Three Main Inducers of Alphacoronavirus Infection of Enterocytes: Sialic Acid, Proteases, and Low pH

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Abstract

Transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) are similar coronaviruses, causing diseases characterized by vomiting, diarrhea, and death from severe dehydration in piglets. Thus, they have caused huge losses to the swine-breeding industry worldwide. Nowadays, they are easily transmitted among the continents via vehicles, equipment, and cargo. Both viruses establish an infection in porcine enterocytes in the small intestine, and their spike (S) proteins play a key role in the virus-cell binding process under unfavorable conditions when the intestine with a low pH is filled with a thick layer of mucus and proteases. Sialic acid, proteases, and low pH are three main inducers of coronavirus infection. However, the details of how sialic acid and low pH affect virus binding to the host cell are not determined, and the functions of the proteases are unknown. This review emphasizes the role of three factors in the invasion of TGEV and PEDV into porcine enterocytes and offers more insights into Alphacoronavirus infection in the intestinal environment.

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Introduction

Two porcine coronaviruses, transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV), are clustered as different species into the Alphacoronavirus genus. They both are important viral pathogens in piglets, causing similar pathological characteristics with acute diarrhea and dehydration [1, 2], leading to massive losses in the modern swine-breeding industry worldwide. Although their transmission route is limited to the fecal-oral route, as economic globalization increases rapidly and transportation develops remarkably, vehicles, equipment, and cargo have been convenient for these viruses to spread to all continents.

TGEV and PEDV replicate in enterocytes of the small intestine and are the causative agent of a fatal diarrhea in newborn piglets. Doyle and Hutchings [3] described the

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Table 1. Representative prototypes of coronaviruses with their own receptor or coreceptor

Genus	Representative prototypes	Receptor, coreceptor
Alphacoronavirus	TGEV	pAPN, Neu5Ac, Neu5Gc
	HCoV-229E	hAPN
	HCoV-NL63	ACE2
	PEDV	pAPN, Neu5Ac
Betacoronavirus	MERS-CoV	DPP4
	SARS-CoV	ACE2
	BCoV	Neu5, 9Ac2
	MHV	CEACAM1
	HCoV-OC43	Neu5, 9Ac2
Gammacoronavirus	IBV	Neu5Ac
Deltacoronavirus	Bulbul coronavirus HKU11	Unidentified
	Thrush coronavirus HKU12	
	Munia coronavirus HKU13	

In the table, each color/line corresponding to one species is listed in the order as presented in the phylogenetic tree. The full names of the species in the table have been mentioned except for bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43). The full names of the protein receptors and sugar receptors are shown in the table.

disease caused by TGEV in America for the first time. TGEV was then spreading to various continents: North America (Canada, 1989), Europe (England, 1957), and Asia (Japan, 1956, Korea, 2000, and Thailand, 2000). In China, TGEV was reported in the 1960s, and widespread outbreaks have occurred since 2010 [4]. In the 1970s, PEDV was first reported in Europe [5, 6]. Subsequently, the virus affected Asian countries extensively, and PEDV infection was usually mild before 2010. The current virulent PEDV strains appeared in 2010 in China. Then, an outbreak occurred in Ohio in the USA in 2013, which spread throughout the USA. Since then, PEDV has resulted in significant economic damage worldwide and is thus receiving increased attention around the world [7].

TGEV and PEDV cluster in the genus Alphacoronavirus, which belongs to the Coronavirinae subfamily (Fig. 1) (order Nidovirales, family Coronaviridae). Coronaviruses (CoVs) are enveloped viruses with a single-stranded positive-sense RNA genome of up to approximately 30 kb with a 5′ cap and a 3′ polyadenylated tail [8]. They are capable of cross-species transmission and may gain new features. CoVs are significant infectious agents involved in gastroenteric, respiratory, hepatic, and neuronal diseases in animals and humans, causing cough and diarrhea, as well as high mortality rates, and bringing huge damage to human health and society [9]. α- and β-CoVs infect mammals such as humans and swine. By contrast, γ- and δ-CoVs are mostly detected in birds, such as Bulbul coronavirus HKU11, Thrush coronavirus

HKU12, and Munia coronavirus HKU13. Both γ- and δ-CoVs have also been found in mammals [10]. α-, β-, γ-, and δ-CoVs are 4 genera of Coronaviridae that are clustered together based on numerous studies and serological and genotypic criteria (Table 1). Each genus has its own representative CoV species. TGEV, PEDV, and human coronaviruses (HCoV-229E and HCoV-NL63) are typical viruses of the genus α-CoV. The representative species of β-CoV are mouse hepatitis virus and severe acute respiratory syndrome coronavirus (SARS-CoV). Infectious bronchitis virus is currently the most studied virus in the γ-CoV genus [11]. Little is known about δ-CoV.

This review emphasizes the role of three factors (sialic acid, proteases, and low pH) in the invasion of TGEV and PEDV into porcine small intestine epithelial cells and provides information with respect to α-CoV infection that brings new insights into virus research.

The Role of the Spike Protein of TGEV and PEDV

The spike (S) protein of CoVs is essential for the interaction with receptors and the fusion of the viral particles and for cellular membranes. It also plays a crucial role in the interspecies transmission of CoVs. The interactions of CoV S glycoproteins with receptors on the cell surface determine the host range and tissue tropism of CoVs [12, 13]. Virus infection begins with the interplay between the
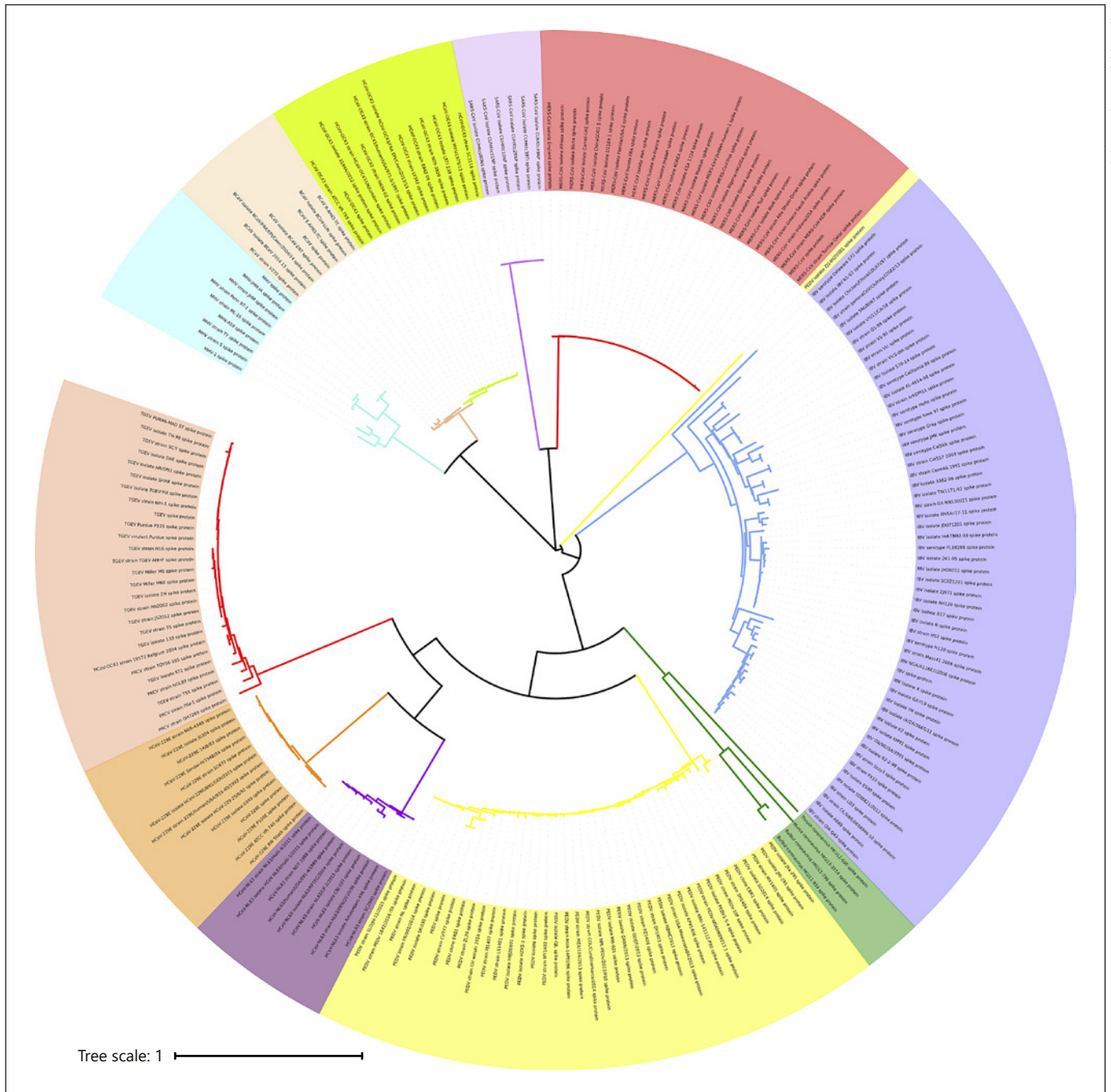
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Fig. 1. Phylogenetic tree of the Coronavirinae subfamily. The phylogenetic tree was built on the basis of the nucleotide sequences of complete spike genes from 213 coronaviruses. The nucleotide sequence alignment and the construction of the phylogenetic tree were completed using the MEGA5.0 program with a proper substitution model: d = transitions + transversions, and all other settings were maintained as default. The final depiction of the phylogenetic tree was completed using iTOL on the Internet. As the map shows, the coronavirus family is divided into 4 main groups, and 11 coronaviruses are regarded as representatives, which are shown in various colors. Interestingly, the spike gene of PEDV isolate strain ZJ14HZ030301 is separated into a single group in the phylogenetic tree.

Fig. 2. Simplified graphic of the structural domains of the main coronaviruses' spike proteins. The spike protein structure can be divided into the S1 and S2 domains, and the structural domains in the spike protein are located in the order (from C to the N terminus) as: transmembrane (TM), heptad repeats (HRs) in the S2 domain, C-terminal domain (CTD), and N-terminal domain (NTD) in the S1 domain as well as the signal peptide (SP). In the graphic, compared with TGEV, PRCoV lacks an NTD, which is associated with enteric tropism. The RBD in the SARS spike protein is shown as discontinuous in the graphic.
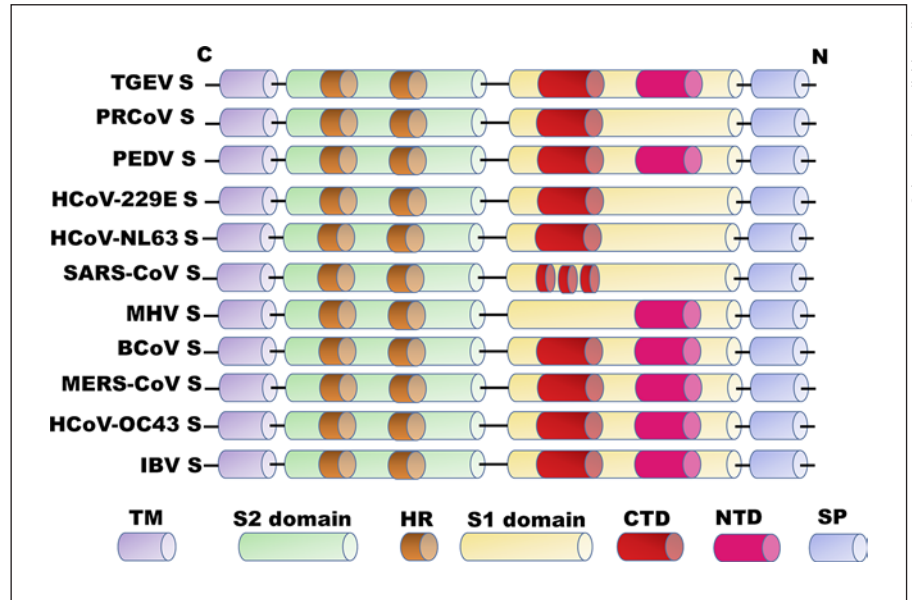
S protein and its specific receptors, followed by penetration into the cells by a fusion event [14, 15].

The S protein, a class I fusion protein, is a membrane protein. It is the largest glycoprotein of CoVs [16], projecting out from the surface of CoV particles and forming a homotrimer structure called a peplomer. The S protein is responsible for the corona-like appearance of the surface projections in the electron microscope. The peplomer includes a globular portion and a protein stalk. By adopting the helical structure that is characteristic of class I virus fusion proteins, the protein stalk connects the globular portion to the transmembrane domain [17]. The N-terminal S1 domain constitutes the globular region, and the stalk is made up of the membrane-proximal S2 domain. The N-terminal S1 domain and C-terminal S2 domain of the S protein play a similar role in all CoVs, the S1 region is related to receptor binding, and the S2 domain plays a role in the membrane fusion process. In addition, the S1 domain contains two subdomains, an N-terminal domain (NTD) and a C-terminal domain (CTD) (Fig. 2). The two subdomains, called RBDs (receptor binding domains), bind with specific cell receptors, including a series of proteins and sugars. Thus, determinants in the S1 domain are not only crucial for initiating virus entry into cells, they also determine the cell and host tropism of CoVs [12].

TGEV and PEDV S proteins have many similarities in their secondary structure. The TGEV S protein is produced as a 1,447-amino acid precursor polypeptide with a 16-residue signal peptide. According to analysis of the S protein sequences of CoVs with different biological phenotypes, there are 4 antigenic sites (C, B, D, and A in that order) in the N-terminal half of the S protein, among which the A site can induce neutralizing antibodies [4] and is highly conserved in TGEV and porcine respiratory coronavirus (PRCoV), a respiratory variant of TGEV. The S protein encoded by PRCoV lacks about 200 amino acids in the N-terminal region that contain determinants related to the enteropathogenicity of TGEV [18]. PEDV has a 150- to 220-kDa spike glycoprotein with a homotrimeric structure. The PEDV S protein is also a type I transmembrane glycoprotein that contains 5 regions, a signal peptide (residues 1–20), an S1 region (residues 21–793), an S2 region (residues 794–1385), a transmembrane domain (residues 1335–1358), and a cytoplasmic tail (residues 1359–1385) [19]. The S1 region is responsible for virus particle binding to the cellular receptors, whereas the S2 region participates in membrane fusion of the virus and host cells. Like other CoVs, the S1 region possesses two subdomains comprising an NTD (residues 21–324) and a CTD (residues 253–638). The CTD of the S1 domain binds to a functional cellular receptor for PEDV infection. Sequence analyses of PEDV prototype and variant strains reveal that the N terminus of the S protein changes more easily than the C terminus. In addition, a previous report suggested an interaction of NTD of the S1 domain with a coreceptor [20, 21].

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The Conformation of the RBD in TGEV and PEDV

The α-CoV RBD comprises about 150 residues that adjoin the CTD in the S1 region (Fig. 2). Previous studies on the CTD concluded that the structure had the characteristic of independent expression of the S protein and preservation of its native structure maintained the binding specificity [4]. Several structural studies indicate that the α-CoV RBD adopts a β-barrel fold with 2 highly twisted β-sheets, in which 3 β-strands (β1, β3, and β7) run parallel and 3 disulfide bonds exist in the 3 β-structures [22]. The RBD of TGEV is located within the CTD of the S1 domain [23]. In the TGEV RBD crystal structure, the bent-strand β5 crosses both β-sheets. N-linked glycans are concentrated at one side of the β-barrel; the opposite side is not glycosylated and might be closer to other S protein domains. The N- and C-terminal ends of the RBD are located on the same side of the domain (terminal side); at the opposite side, 2 β-turns form the tip of the barrel in the TGEV RBD [12].

Like TGEV, the crystal structure of a single domain unit in the PRCoV RBD adopts a β-barrel fold with 2 highly twisted β-sheets located in the CTD of the S1 domain and engages in binding to the host cell surface receptor. Compared with the TGEV S domain, the related PRCoV lacks the NTD, which is related to enteric tropism. The TGEV or PRCoV RBD tips consist of 2 protruding β-turns (β1–β2 and β3–β4), each having a solvent-exposed aromatic residue (tyrosine or tryptophan) [20]. In the tertiary structure of the PRCoV RBD, 3 loops (β1–β2, β3–β4, and β5–β6) at the tips of the β-barrel domains are responsible for receptor binding. Some researchers found that single amino acid mutations in the 3 loops completely or significantly reduced the ability of PRCoV RBD to bind to host receptors, and mutations outside the RBD had no effect on receptor recognition [24].

To date, there have been few reports on the RBD structure of PEDV. It is interesting that 3 receptor binding mutant proteins, RBM1-1, RBM2-1, and RBM3-2 proteins, did not significantly reduce PEDV pAPN-binding activities in virus infection [4], suggesting the PEDV S protein uses a receptor-binding mechanism different from TGEV and PRCoV. PEDV is further confirmed to have a broader receptor range than other α-CoVs [21].

Cell Entry Receptors of TGEV and PEDV

During the progress of evolution and adaptation to diverse hosts, CoVs have evolved to use various receptors to enter host cells. Different hosts or virus strains produce evolutionary diversity in the same virus family, and the binding of CoVs to susceptible cells seems to show variation in the receptors used that correspond to viral groups and species. These CoVs recognize distinct cellular receptors and coreceptors, such as proteins and sugars, to facilitate their penetration into cells [8]. Currently, there are 4 main protein receptors: APN, angiotensin-converting enzyme 2 (ACE2), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and dipeptidyl peptidase 4 (DPP4). Most members of α-CoV use APN as the receptor for infecting host cells, such as TGEV, PEDV, and HCoV-229E. APN, also known as CD13, is a 150-kDa type II transmembrane protein that belongs to a membrane-bound metalloprotease family [24]. Interestingly, HCoV-NL63, as an α-CoV, shares the same cell entry receptor, identified as ACE2, with SARS-CoV, which is a β-CoV. ACE2 is a type I integral membrane glycoprotein with an N-terminal extracellular domain comprising 2 α-helical lobes, between both of which there is a catalytic site with a coordinated zinc ion [25]. By contrast, the β-CoV mouse hepatitis virus utilizes CEACAM1 as a cell surface receptor for the S protein. CEACAM1, the first identified CoV receptor [26], is a type I transmembrane multifunctional protein and a member of the immunoglobulin superfamily termed IgSF. Middle East respiratory syndrome-related CoV, belonging to β-CoV, has been shown to use DPP4 as a cell entry receptor. DPP4 (also known as CD26), a type II membrane protein, is a multifunctional membrane-bound serine protease that forms homodimers on the surface of host cells. The DPP4 ectodomain comprises about 730 amino acids and has 2 domains, an α/β-hydrolase domain and an 8-bladed propeller [27]. Among these cellular receptors and coreceptors, APN is a major cell entry receptor for CoVs. APN exists on the epithelial cell surface of different tissues. In particular, it is expressed abundantly in the brush border membrane of the small intestine, the kidney, and the respiratory tract [28]. Most α-CoVs use APN as cell entry receptor. For example, previous studies showed that TGEV uses porcine (p) APN as the receptor in the entry process [24], whereas human (h) APN is a receptor for the infection by HCoV-229E [29].

The reason why TGEV uses APN as an entry receptor has not been clarified. It might be linked to its abundance on the surface of epithelial cells rather than its biological function, which seems to be dispensable for CoV binding capacity [30, 31]. In the small intestine mucosa, APN occupies about 8% of the total protein content of the differentiated enterocytes [32]. For PEDV, although there are many articles in which pAPN was proposed as the recep-

tor in PEDV infection, this view has been questioned due to the lack of robust direct evidence. The characteristic structure of APN is a large glycosylated ectodomain with a zinc metal ion at the active site, which functions as a zinc-dependent protease responsible for cleavage of the N-terminal amino acids, mediated by the HELAH motif [33]. The enzymatic function of the pAPN catalyzes the removal of amino acid residues from the N termini of oligopeptides, and APN has been termed the "moonlighting enzyme" because of its many cell functions. APN can be cleaved into N-terminal (95 kDa) and C-terminal (50 kDa) subunits by trypsin digestion and comprises 4 domains (DI–DIV) [29, 32]. It is heavily glycosylated and forms dimers through extensive DIV–DIV interactions. Sequence conservation in the RBD tip of α-CoVs exerts a crucial function in which the APN recognition mode is highly conserved [29]. Moreover, the specificity of APN with the recognition structure in the S protein is linked to the structure of the APN N-linked glycan and fusion with the RBD β1–β2 turn. In addition, the CoVs tyrosine and tryptophan residues are critical in forming the TGEV RBD-APN structure. HCoV-229E does not have a tyrosine in its RBD β1–β2 turn, hence it recognizes the human APN that lacks this form of glycosylation [20, 34], meaning that HCoV-229E recognition of APN must be unique. There have been some studies that determined the structure of a protruding tip for binding to small APN cavities in this human α-CoV. The S proteins of PEDV and TGEV share high homology, but they have different host preferences. In addition, PEDV has been verified to use a different receptor recognition model compared with TGEV, PRCoV, and HCoV-NL63. The N-terminal region in the PEDV S1 domain binds to sugars, which are regarded as its coreceptor [20].

Sialic Acid Promotes TGEV and PEDV Binding to Host Cell Receptors

In addition to binding to defined protein receptors, some CoVs show a sialic acid-binding activity. At present CoVs in α-, β-, and γ-CoV have developed variant sialic acid binding activities [35]. According to current research, 3 types of sugars have been characterized as receptors or coreceptors for CoV entry into host cells: 5-N-acetylneuraminic acid (Neu5Ac), 5-N-glycolylneuraminic acid (Neu5Gc), and 5-N-acetyl-9-O-acetyl neuraminic acid (Neu5, 9Ac2) [36]. Recognition of sugars as co-receptors of TGEV and PEDV seems to be a strategy by which these viruses adapt to the living organism, meaning that TGEV and PEDV bind to sialic acid to survive under unfavorable intestinal tract conditions.

TGEV was first described to have a sialic acid binding activity in 1996 [37]. The sialic acid binding activity resides in the N-terminal portion of the S1 subunit that has been linked to the enteropathogenicity of TGEV and that is absent from the S protein of PRCoV. The sialic acid preferentially recognized by TGEV is N-glycolylneuraminic acid (Neu5Ac) [37]. The spike protein has a trimeric structure and retains its sialic acid binding activity in soluble forms of the protein [38, 39]. TGEV recognizes and binds the sugar moieties of glycoconjugates that are highly O-glycosylated which promotes binding but is not sufficient for initiation of infection. It is believed that abundant sialic acid in mucins aids TGEV to penetrate the mucus layer and then to get access to pAPN on the surface of the intestinal epithelial cells. Thus, the efficiency of infection can be enhanced under unfavorable conditions. Binding to pAPN and sialic acid are two independent processes. Interestingly, binding to pAPN is more efficient in the absence of sialic acid [40].

There are few studies on the binding of PEDV to sialic acids. PEDV has been proven to have the ability to bind sialic acids. Neu5Ac presented the highest binding affinity with PEDV S1 in experiments using a glycan array screening. Moreover, the sialic acid binding region of PEDV was confirmed as being in the NTD of the S protein (residues 1–320), similar to other CoVs [21]. However, it is unknown how the binding of sialic acid to the S protein can affect PEDV entry into cells.

The Function of Proteases during TGEV and PEDV Infection of Host Cells

Increasing numbers of proteases have been demonstrated to participate in PEDV and TGEV infection of host cells in mechanisms where they do not act as receptors. These proteases are reportedly involved not only in adaptation of virus to innate immune response, but also in proteolytic processing of the S protein.

CoVs always produce two types of cysteine proteases, a chymotrypsin-like main protease and papain-like proteases (PL1pro and PL2pro). In general, they are essential for replicase polyprotein processing and viral replication-transcription. PL1pro, a nonstructural protein of TGEV, resides in the nonstructural protein nsp3 subunits of TGEV [41]. The structure of TGEV PL1pro resembles a right hand with 3 distinct regions, the palm, thumb, and fingers (Fig. 3). It contains a zinc-binding domain, with 4

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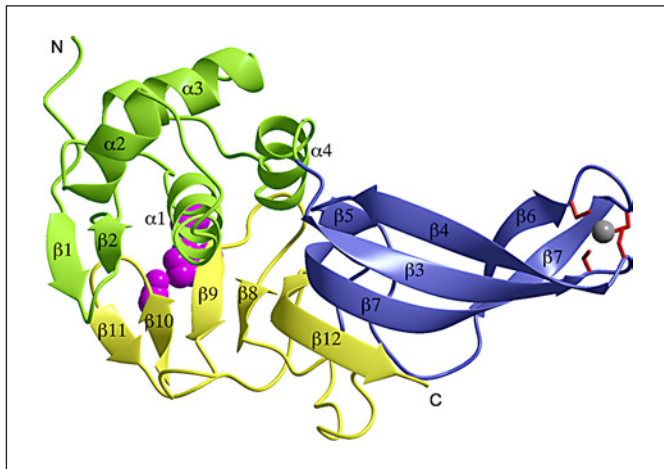
Fig. 3. Ribbon drawing of the TGEV PL1pro [40]. The entire structure resembles a hand, with the palm, thumb, and fingers represented in yellow, green, and blue, respectively. The catalytic triad is presented as magenta spheres, and the zinc ion is a gray sphere.

cysteine residues binding the zinc ion, and a catalytic triad formed by residues Cys32, His183, and Asp196 [42–44]. The TGEV PL1pro protein, which contains a so-called USP-like binding site, exhibits deubiquitinating enzyme activity in vitro [45]. Deubiquitinating enzymes play essential roles in the innate immune response with interferon (IFN) secretion into the intestine mucosa.

In the intestine, the TGEV entry is restricted by IFNs in epithelial mucosa. The pathogen-associated molecular patterns of TGEV, virus RNAs, are sensed by pattern recognition receptors in host cells [46], triggering the IFN expression. TGEV arouses a early IFN-α production in intestinal secretions [47], IFN-α can improve B-cell response to provide protection against reinfection [48]. IFN-λ is the main mucosal antiviral cytokine responsible for resisting virus invasion in the gut, such as TGEV and PEDV [49], together with interleukin-22, which forms the tissue barrier in intestinal epithelial cells to reduce viral infection [50].

TGEV PL1 protease possesses deubiquitinating activity and hydrolyzes the peptide that binds both Lys48- and Lys63-linked polyubiquitin chains [41]. Regulation of signaling molecules by ubiquitin has a significant function in the activation of the IFN response. TGEV PL1 binds and deubiquitinates retinoic acid-induced gene RIG1 and stimulator of interferon gene STING, which are regulators in the IFN signaling pathway, and then the levels of phosphorylated IFN regulatory factor 3 exhibit reduced activity [51], which counteracts IFN regulatory

factor 3 translocating into the nucleus to activate the transcript of IFNs, such as IFN-α/β and IFN-λ. TGEV adopts the strategy to interfere and inhibit IFN secretion into the intestine gut to improve the efficiency of virus invasion.

Previous reports confirm that proteolytic processing of the S protein contributes to the fusion of the viral membrane with cellular membranes and is necessary for virus entry. Proteases in virus entry are capable of cleaving the S protein. These proteases in the pig small intestine potentially facilitate PEDV infection of host cells [52].

PEDV successfully infects African green monkey kidney (Vero) cells in vitro with extracellular trypsin [53]. The use of trypsin improves the possibility of PEDV entry, because trypsin facilitates S protein-mediated fusion with the plasma membrane to deliver viral genomes into host cells. It has been shown that trypsin is helpful for syncytium formation in PEDV infection of MDCK cells [54] Interestingly, PEDV can also propagate without trypsin, suggesting that trypsin might be relevant for cell-cell fusion rather than viral envelope-cell membrane fusion [55]. For PEDV entry into Vero cells under the trypsin-free conditions, endogenous proteases in endosome may adopt trypsin-like function, prompting PEDV S-mediated fusion.

TTSP is a type of trypsin-like serine protease termed type II transmembrane serine protease. TTSP is confirmed to cleave and activate proteins on the surface of influenza viruses and CoVs, allowing multicycle replication in the absence of trypsin. TTSPs are reportedly involved in the release of PEDV virions [56]. TMPRSS2 and MSPL are members of TTSPs. TMPRSS2 and MSPL exhibit trypsin-like features in the amplification of PEDV in vitro in the absence of trypsin and play a vital role in cell-cell fusion and virus-cell fusion. It is found that PEDV S protein is colocalized with TMPRSS2 and MSPL extensively and cleaved by coexpression with TMPRSS2 or MSPL. TMPRSS2 and MSPL could cleave PEDV S protein into two fragments of the same size. The two TTSPs interact with the PEDV S protein to promote viral entry into cells by promoting cell-cell fusion and virus-cell fusion. Moreover, MSPL exhibited the strongest effect in the replication of PEDV compared to TMPRSS2. Interestingly, the adaptive capacity and the growth of PEDV in Vero cells expressing TMPRSS2 and MSPL are higher than those in cells treated with trypsin [57].

It is comfirmed that PEDV requires serine and serine-like proteases (Fig. 4) for its entry through endocytosis in the early stages of infection. For cell-cell fusion, serine proteases are involved in PEDV entry in an acidic pH-independent manner [58]. Cellular serine proteases pos-
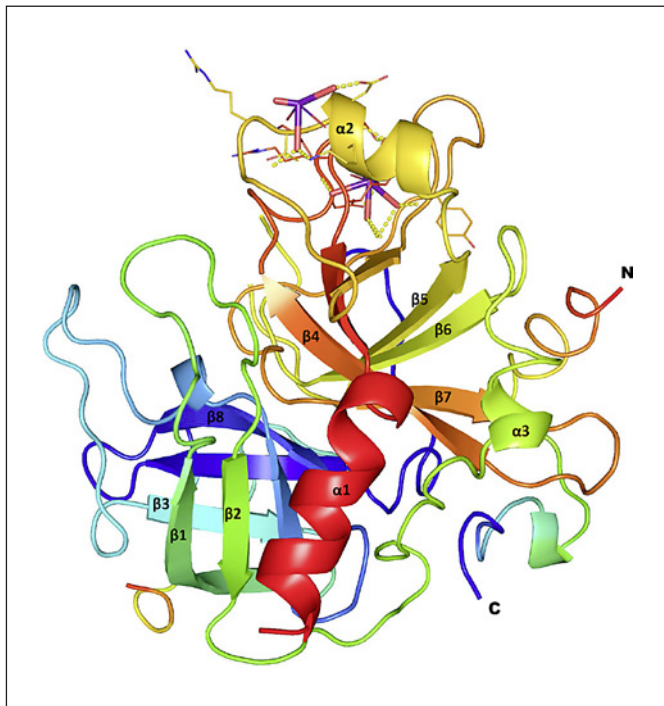
Fig. 4. Ribbon drawing of the serine protease (PBD ID 1hxe). The X-ray diffraction method was used to test the structure. The structure comprises 4 α-helixes (α1–α4) and 8 β-sheets (β1–β8) presented in diverse colors in the light of different domains. There is an Na$^+$ site close to β2 (yellow), shown in stick form.

sess a catalytic triad of amino acids comprising His, Asp, and Ser, which are located in similar positions at the 3-dimensional structure. The nucleophilic Ser is responsible for cleavage and is often replaced by a functionally and spatially equivalent Cys in viral trypsin-like proteases [42, 44]. The Asp of the active-site residues can be substituted by an equivalent Glu. Serine or serine-like proteases in the cytoplasm are required for the fusion between the PEDV envelope and the host endosomal membrane. Serine proteases activate PEDV entry in a low pH-independent manner.

New evidence has emerged that pAPN in the small intestine acts as a protease for PEDV, which contrasts with the idea that pAPN plays role as a cell surface receptor. The structure of APN is shown in Figure 5. With regard to the similarities between PEDV and TGEV and the accordance of the pathology of PEDV infection with the tissue distribution of pAPN, pAPN used to be regarded as a receptor for PEDV, which was supported by some indirect evidence [24, 59]. However, PEDV can also infect

and propagate in pAPN-negative Vero cells [53, 58], indicating that there is no direct evidence to support the view that pAPN is a receptor for PEDV.

In current research, by employing CPK cells to express porcine homologs of pAPN, ACE2, CEACAM1, and DPP4, results were obtained showing that CPK cells were susceptible to TGEV, but not to PEDV. In addition, PEDV infection was not affected by soluble pAPNs, suggesting that PEDV utilizes different receptors compared with other CoVs. By contrast, another study showed that when pAPN was overexpressed in porcine CPK cells, pAPN enabled PEDV multiplication [59, 60]. Another study showed that nonpermissive ST cells expressing a pAPN gene supported productive infection of PEDV of the host cells, showing that constitutive overexpression of pAPN directly promotes PEDV multiplication [29]. In summary, pAPN more likely functions as a protease in PEDV infection rather than as a receptor, which is supported by research evidence; however, the mechanism by which pAPN promotes PEDV infection remains unknown.

Structural Changes and Activation of TGEV and PEDV Proteins at Low pH

It is unquestionable that pH is important for the process of CoV entry into cells. Low pH is necessary for conformational changes of viral glycoproteins and proteolytic activation of viral glycoproteins by endosomal proteases [61]. It is reported that pH-dependent conformational changes occur in the CoV peplomer [62], and the S1 domain is released from peplomers on the surface of the virus. Inside the endosome, endogenous proteases participating in membrane fusion are active in a low-pH environment. TGEV and PEDV tend to multiply at a low pH. The amount of TGEV in the medium at pH 6.5 was 10-fold greater than that at pH 7.2, and the yield was almost 100-fold higher than that at pH 8.0 [63].

TGEV binds to APN and enters the host cell via endocytosis. Upon attaching to the porcine APN receptor, TGEV is incorporated into the cell membrane and enters by way of caveola-dependent endocytosis [64]. Subsequent membrane fusion is promoted by active cellular proteases in the endosome in a low-pH environment [65, 66]. Using MDCK cells overexpressing porcine APN, it has been demonstrated that acidification of the intracellular compartment promotes membrane fusion inside the endosome and cellular proteases are activated in a pH-dependent manner to facilitate membrane fusion [59, 65].
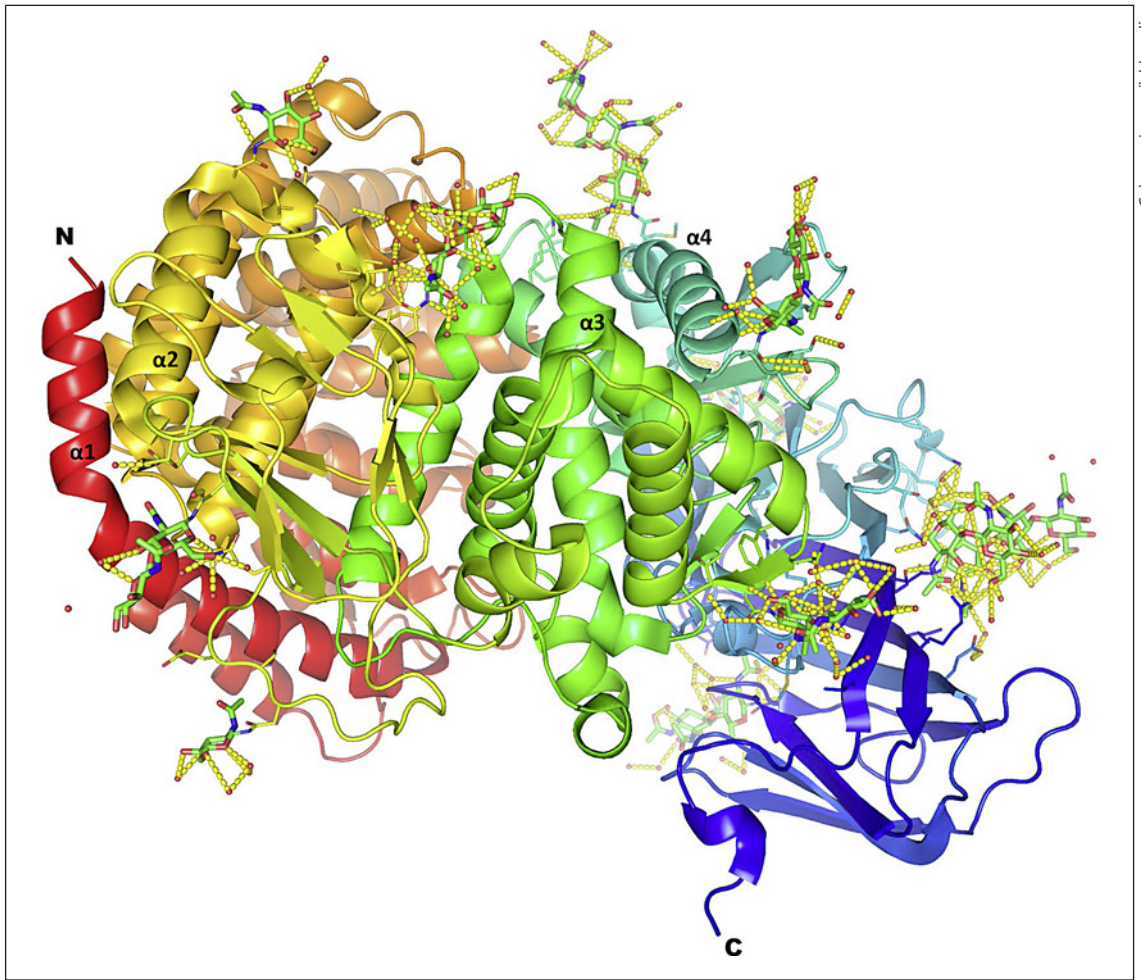
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Fig. 5. Ribbon drawing of the pAPN structure (PBD ID 4FKE). The structure was well characterized using X-ray diffraction. The pAPN structure contains 4 main domains in the order: N-terminal DI (red), DII (yellow), DIII (green), and C-terminal DIV (blue).

Different from the caveola-mediated uptake of TGEV, PEDV enters cells using endocytosis via clathrin-coated pits. After the first step of interacting with receptors on the cell surface, PEDV penetration is facilitated by viral envelope fusion with the host cell plasma membrane, which happens inside a pH-dependent endosomal compartment. The endosomal cellular elements are very important for the activation of low pH-dependent proteases, rather than the virus needing an acidic environment to trigger its entry [41, 67]. The acidic conditions do not affect PEDV itself, suggesting that acidic pH is not the only factor that affects viral infection of host cells.

Conclusions

Interaction between the S protein and the host cells is an indispensable step for viral entry into host cells. In the process of TGEV and PEDV entry into cells, the environmental conditions in the intestinal gut are complex and unfavorable. A thicker layer of mucus containing sialic acid covers the intestinal epithelium, and the small intestine generally has a low pH. In addition, the intestine is filled with proteases from the stomach and intestinal wall. These conditions make it hard for viruses to infect intestinal cells compared with infection of cultured cells. The selection of TGEV and PEDV RBDs interacting with cell surface receptors is the result of pressure from immune

surveillance. Sialic acid usage by TGEV and PEDV promotes the efficient invasion into host cells. PEDV and TGEV invade enterocytes in the intestinal epithelium by exploiting proteases in the small intestine. TGEV induces PL1pro to hijack cellular canonical pathways to prevent viral protein degradation, facilitating later virus entry into targeted cells. TMPRSS2 and MSPL interact with the PEDV S protein to promote viral entry into cells by promoting cell-cell fusion and virus-cell fusion. Recently, porcine APN has been observed to function as a protease for PEDV, not as a receptor, to promote viral multiplication during PEDV entry into the cell, the mechanism of which requires further study. Low pH in the initial stage of PEDV entry into intestinal epithelial cells allows structural changes and protease activation during binding to surface receptors.

Disclosure Statement

No potential conflict of interest is reported by the authors.

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