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Ultrastructural Characterization of Membrane Rearrangements Induced by Porcine Epidemic Diarrhea Virus Infection

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Abstract

The porcine epidemic diarrhea virus (PEDV) is a coronavirus (CoV) belonging to the alpha-CoV genus and it causes high mortality in infected suckling piglets with substantial losses in the farming industry. CoV trigger a drastic reorganization of host cell membranes to promote their replication and egression, but a detailed description of the intracellular remodeling induced by PEDV is still missing. In this study, we have examined qualitatively and quantitatively, using electron microscopy, the intracellular membrane reorganization induced by PEDV over the course of an infection. With our ultrastructural approach, we reveal that, as most of CoV, PEDV initially forms double-membrane vesicles (DMVs) and convoluted membranes (CMs), which probably serve as replication/transcription platforms. Interestingly, we also found that viral particles start to form almost simultaneously in both the endoplasmic reticulum and the large virion-containing vacuoles (LVCVs), which are compartments originating from the Golgi, confirming that alpha-CoV assemble indistinguishably in two different organelles of the secretory pathway. Moreover, PEDV virons appear to have an immature and a mature form similarly to another alpha-CoV, the transmissible gastroenteritis coronavirus (TGEV). Altogether, our study underlies the similarities and differences between the life cycle of alpha-CoV and that of viruses belonging to other CoV subfamilies.

Keywords: PEDV; alpha-coronavirus; life cycle; electron microscopy; membrane rearrangement
1. Introduction

Coronaviruses (CoV) are enveloped positive single stranded RNA viruses that are characterized by crown-like spikes on the virion surface under the electron microscope (1). Mostly based on phylogenetic clustering, this virus family has been divided into four subgroups: the alpha-, beta-, gamma- and delta-CoV (1, 2). The porcine epidemic diarrhea virus (PEDV) is an alpha-CoV and the causative agent of porcine epidemic diarrhea, which is characterized by severe enteritis, vomiting, watery diarrhea and loss of weight. PEDV infections have a substantial detrimental effect on the swine industry because the morbidity and mortality rates are high, especially in sucking piglets (3-5). Since its first identification in Belgium in 1978 from growing and fattening pigs (6), PEDV has been reported in Europe and Asia, and a remarkable increase of PEDV outbreaks occurred in the pig-producing provinces of China in the late 2010 (7). PEDV also emerged for the first time in the United States in early 2013 (5), and spread to Canada and Mexico (8). These recent outbreaks and the global re-emergence of PEDV have attracted the attention of numerous virologists, as there is the necessity of an urgent attention and deeper understanding of PEDV biology and mechanisms of pathogenesis.

Replication is a fundamental event in the life cycle of viruses, and in the case of positive single strand RNA viruses and some double-stranded DNA viruses, it occurs on cellular compartments that are generated by specialized viral proteins through the modification of one or more host membranes and/or organelles (9-11). CoV-infected cells also undergo a massive remodeling of intracellular membranes (12). Upon CoV entry in the host cell and release of their genomic RNA (gRNA) into the cytoplasm, two large polyproteins known as pp1a and pp1b, are synthesized and their self-processing leads to the generation of 15 to 16 non-structural proteins (NSPs) (13, 14). NSPs trigger the formation of double-membrane vesicles (DMVs) and convoluted membranes (CMs), which provide a platform for the concentration of viral factors to very likely guarantee an efficient replication and transcription of CoV gRNA (12, 15-18). Collectively, the NSPs also form the replication-transcription complex (RTCs), which localize on the DMVs and CMs where they mediate the synthesis of viral RNA (15, 19, 20). Double-stranded RNA (dsRNA), a byproduct of genomic RNA replication, gets concentrated in the lumen of the DMVs through a mechanism that remains totally unknown (15). In contrast, virion assembly takes place at the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and Golgi complex, and involves the inward budding of the limiting membrane of these
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compartments, which is triggered by the interaction between the nucleocapsid (N) protein associated with the gRNA, and the structural spike (S), membrane (M) and envelop (N) proteins (13, 14). Complete virions subsequently reach the extracellular environment following the conventional secretory pathway (21).

The first electron microscopy (EM) analyses of CoV-infected cells were carried out in 1960’s and they mainly characterized the morphology of the viral particles (16-18, 22). Subsequent ultrastructural studies highlighted the DMVs as being a feature of CoV infections, including those of mouse hepatitis virus (MHV), severe acute respiratory syndrome coronavirus (SARS-CoV), human CoV NL63 (HCoV-NL63), Middle East respiratory syndrome coronavirus (MERS-CoV) and infectious bronchitis virus (IBV) (12, 15, 23-26). Over the years, other intracellular structures have been reported to be present in cells infected by CoV in addition to DMVs and virion particles, including CMs, tubular bodies (TBs), vesicle packages (VPs), cubic membrane structures (CMSs) and large viron-containing vacuoles (LVCVs). CMs are reticular inclusions observed in between clusters of DMVs, and are often connected with both DMVs and the ER as reveled by both 2D and 3D ultrastructural studies (15, 17). Like DMVs, MHV- and SARS-CoV-induced CMs are positive for dsRNA and NSPs as well, and this finding has led to postulate that these structures are also involved in viral replication and transcription (12, 15, 27, 28). In later stages of SARS-CoV infection, groups of single-membrane vesicles surrounded by a common outer membrane and called VPs, are arising from the merging of DMVs, viron particles and possibly CMs (15, 27). LVCVs, which are vacuoles filled with viral particles, have been frequently observed in cells infected with CoV and are Golgi cisternae that expand to accommodate the increasing assembly of progeny virions over the course of an infection, as they are positive for Golgi marker proteins (12, 17, 18, 22, 23, 26, 27, 29, 30). Finally, TBs and CMSs, which have been detected in MHV- and SARS-CoV-infected cells, are condensed, highly organized membrane rearrangements connected to the ER. TBs and CMSs appear to be the result of an aggregation of an overproduced protein because they mostly contain a single structural protein, and therefore they are probably a byproduct of a massive infection with no role in virus replication (12, 17, 31, 32).

Despite its veterinary relevance, the intracellular membrane remodeling induced in host cells by PEDV remains largely unknown. The only infections of alpha-CoV characterized at the ultrastructural level so far are the ones of HCoV-NL36 and of transmissible gastroenteritis coronavirus (TGEV) (23, 29, 33). In cells infected with these two alpha-CoV, virions were observed in the ER, Golgi and LVCVs, whereas DMVs were only reported to be present in cells exposed to
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HCoV-NL36. We thus decided to analyze PEDV-infected cells by a quantitative EM and immuno-electron microscopy (IEM) to establish a more comprehensive inventory of the membrane remodelings induced by this virus and in more general by alpha-CoV. We opted for a time-course approach to determine at which stage of the viral life cycle the different membranous structures appear. By combining the ultrastructural data with the measurement of viral RNA synthesis, viral replication, progeny virus release and immunofluorescence analyses, our results show that PEDV infection induces a profound reorganization of the ER and Golgi, which precedes the formation of DMVs, CMs and LVCVs. Consistently with these observations, we also found that ER and Golgi undergo alterations not observed for other CoV, such as ER proliferation and a Golgi vacuolarization, which results in an organelle that we have named irregular vesicle clusters (IVCs). Importantly and similarly to HCoV-NL36 and TGEV, we also found that together with the Golgi, the ER is the major platform of virion assembly. Altogether, our study provides an overall comprehensive picture of the ultrastructural events taking place inside a cell over the course of a PEDV infection.

2. Results

2.1 PEDV induces the formation of multiple membranous structures

As a first ultrastructural analysis, we compared the morphology of uninfected cells that of those infected with PEDV for 72 h by EM in order to make a repertoire of all the membranous rearrangements induced by this virus. We identified six different structures. The most abundant structure was the large double-membrane vesicles, which have a diameter of 230±50 nm, and contained coiled filaments and were often in close proximity to each others (Fig. 1A and B). These are the characteristic DMVs induced by CoV (12, 17, 23-25). Numerous DMVs appeared to have inward invaginations of the limiting membrane that have also previously been observed in specific cell types (Fig. 1B, asterisks) (12, 15). In between and around the DMVs, we frequently observed a network of reticular inclusion, which have already been described in MHV- and SARS-CoV-infected cells as the CMs (Fig. 1B) (12, 15, 17).

PEDV virions were also easily detected and appeared as spherical structures with a diameter range of 90±20 nm (Fig. 1C-F). Based on their morphology, PEDV particles could be grouped in two categories. The first were particles with an annular stained region under the external envelope (Fig. 1C, 1D and 1F, black arrows). The second were smaller virions with a less circular and
denser core, which was more markedly stained (Fig. 1C-E, white arrows). These same phenotypical differences in viral particles have also been observed in TGEV-infected cells, another alpha-CoV, for which it was shown that the particles represent immature and mature virions, respectively (29). PEDV viral particles were mainly present in the lumen of two morphologically distinguishable compartments, where they were assembling through inward budding at the limiting membrane (Fig. 1C and D, arrowheads). One of these two compartments was what appears to be an expanded rough ER with irregular shape, as the surface was decorated with ribosomes and they were often observed connected to an ER with normal morphology (Fig. 1C). These structures have already been reported in cells infected by other alpha-CoV (23, 29). The other compartments were large vacuoles with a diameter of 865±270 nm, limited by a single membrane, which have previously named LVCVs (Fig. 1D) (12, 17, 18, 22, 23, 26, 27, 29, 30). Virions were also detected in large single membrane vacuoles with a diameter of 800±160 nm, which also contained compact membrane whorls and amorphous material (Fig. 1E). Similar compartments, but smaller in size and without virions, were also observed in uninfected cells (data not shown), indicating that they do not represent structures induced by PEDV. These characteristics suggested that these vacuoles could be endolysosomal compartments. This notion was confirmed by immunogold labeling of cryosections obtained from PEDV-infected cells at 72 h p.i. using antibodies against CD63 and the transferrin receptor (TfR). These two marker proteins of the endolysosomal system (44) were present on these virion-containing vacuoles (Fig. S1). Altogether, these characteristics indicate that these vacuoles are expanded endolysosomal compartments. Interestingly, both types of virions could be observed in ER and LVCVs but only the smaller dark mature virions were found in the endolysosomal compartments. Virions were also detected in large single membrane vacuoles with a diameter of 800±160 nm, which also contained compact membrane whorls and amorphous material (Fig. 1E). Similar compartments, but smaller in size and without virions, were also observed in uninfected cells (data not shown), indicating that they do not represent structures induced by PEDV. These characteristics suggested that these vacuoles could be endolysosomal compartments. These two marker proteins of the endolysosomal system (44) were present on these virion-containing vacuoles (Fig. S1). Altogether, these characteristics indicate that these vacuoles are expanded endolysosomal compartments. Interestingly, both types of virions could be observed in ER and LVCVs but only the smaller dark mature virions were found in the endolysosomal compartments.
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Figure 1. PEDV induces the formation of multiple membranous structures. Vero E6 cells were inoculated with PEDV (MOI = 1) and processed for EM at 72 h p.i. as described in Material and Methods. (A, B) Representative electron micrographs of DMVs and CMs are shown. Asterisks indicate invaginations of the DMVs limiting membrane. (C) Expanded ER with luminal virions (asterisks). Arrowheads indicate forming viral particles. Black and white arrows indicate immature and mature virions, respectively. (D) LVCVs are large organelles with a smooth single limiting membrane and virions at their interior. Virions can be seen as invaginations of the limiting membrane (arrowheads). Black and white arrows indicate immature and mature virions, respectively. (E) Large single membrane compartments containing mature virion particles (asterisk), and filled with condensed membrane whorls and amorphous material/structures. White arrows indicate mature virions. (F) Cytoplasmic inclusions composed of condensed tubular structures containing a dense inner core were observed connected with ER, which we named ERBs. From time to time, virus particles were observed in their interior (arrows). ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; PM, plasma membrane. Scale bar, 250 nm.

Finally, we also detected large cytoplasmic inclusions with a length from 910±288 nm, which were characterized by a dense inner core with a geometrical appearance, a limiting membrane and connections with the ER (Fig. 1F). Virions could also be seen to form in those structures (Fig. 1F, arrows), which have already been reported in cells infected by human coronaviral LINDER strain (45). We named this new structure ER bodies (ERBs). We could not detect structures reminiscent to the TBs and CMSs.

2.2 Time-course PEDV infection and measurement of cellular life cycle parameters

To understand the relationship between the different membranous structures induced by PEDV and obtain insights into their role during infection, we subsequently infected cells with PEDV and examined them at the ultrastructural level in a time-course manner, at different time points between 0 h and 72 h p.i. We first measured at each p.i. time point important known parameters that reflect the viral life cycle, i.e. infection efficiency, RNA replication/transcription and secretion of progeny virus, to be able to correlate our EM analyses with the progression of alpha-CoV infection in host cells.

Virus infection was examined at each time point by immunofluorescence using anti-dsRNA antibodies to indirectly monitor the progression of PEDV infection over time. dsRNA is an intermediate in CoV replication and it principally localizes inside the DMVs, and therefore can be used to specifically detect cells in which CoV are replicating (46). As shown in Fig. 2A, the dsRNA could be visualized from 24 h p.i., but the percentage of cells positive for this nucleic acid was still below 10% (Fig. 2A) and increased slightly to 13% at 36 h p.i. A very pronounced PEDV infection was detected at 48 and 72 h p.i., with 80% and almost 100%, respectively, of cells positive for dsRNA to almost 100% (Fig. 2A).
Next, the amounts of both gRNA and the subgenomic RNA encoding for the structural N protein (sgRNA N) were determined at each time point by RT-PCR to assess the RNA replication/transcription of PEDV. Both gRNA and sgRNA N were already detected at 8 h p.i. and their amounts gradually increased until 48 h p.i., when they reach an expression plateau (Fig. 2B).

Finally, the presence of infectious virions in the cell culture supernatants was determined using the TCID\textsubscript{50}/ml titration to monitor the assembly and release of PEDV. Presence of PEDV virions was first detected at 8 h p.i. and also increased until 48 h p.i., when it reached a maximum (Fig. 2C). This observation correlated with the analysis of viral RNA expression because as expected, PEDV assembly and release follow intracellular PEDV replication.

Altogether, these measurements indicated that PEDV life cycle in Vero E6 cells progresses following the established dynamics of CoV, thereby confirming the use of this cell line as a model (40). Moreover, these quantifications showed that the PEDV starts to replicate, assemble and egress in these cells around 8 h p.i., and the infection continuously increases until 48 h p.i. (Fig. 2). At this time point, there is a dramatic augmentation in the number of infected cells, close to 100%, which coincides with an arrest in the augmentation of viral RNA synthesis.

2.3 Quantification of the PEDV-induced structures over the course of an infection

Next, we quantified the number of the PEDV-induced structures that we inventoried at 72 h p.i. (Fig. 1), at each collected p.i. time point by conventional EM. We first morphologically determined the number of cell sections that showed visible signs of infection to see whether the changes observed at the ultrastructural level correlate with the measured infection parameters (Fig. 2). To this end, the number of cell sections displaying at least one of the PEDV-induced structures detected at 72 h p.i. (Fig. 1), was determined. At 24 h p.i., 2% of the cell sections started to show visible signs of infection and this percentage gradually increased over time until reaching 84% at 72 h p.i. (Fig. 3A). Importantly, the percentage of cell sections with visible signs of infection obtained from the EM analysis correlated well with the rest of the measured parameters (Fig. 2), showing that the morphological examination of the cells is a reliable alternative approach to follow PEDV infection.

We next quantitatively analyzed the EM samples prepared at the different p.i. time points to understand the role of the PEDV-induced structures during an infection and to unravel their relationship. In particular, the percentage of cell
Figure 2. Time-course PEDV infection and measurement of cellular life cycle parameters. Vero E6 cells were infected with PEDV (MOI = 1) before to analyze them and the cell culture supernatants at 0, 8, 16, 24, 36, 48, 60 and 72 h p.i. (A) Cells were processed for immunofluorescence using an anti-dsRNA antibody (green) to determine the number of PEDV-positive cells. The DAPI dye (blue) was used to stain the nuclei and determine the total number of cells. Scale bar, 10 μm. Quantification of the percentage of infected cells at each time point is indicated between brackets. (B) The total RNA was isolated from cells and the relative amount of sgRNA N and gRNA mRNAs was quantified by RT-PCR. (C) The production of the virus progeny was assessed by determining the virus titer of the cell culture supernatants by end point dilutions on Vero E6 cells, before calculating the TCID50/ml. Error bars in B and C represent the standard deviation of 3 experiments.
profiles containing a specific structure was determined for each p.i. time point (Fig. 3B and 3C). DMVs were one of the first structures to be detected and they were observed in about 2 % of cell sections at 24 h p.i. The number of cell sections positive for these vesicles gradually increased over time, reaching a maximum of 38 %, at 60 h p.i. (Fig. 3B). Interestingly, the localization and morphology of the DMVs changed over the course of the PEDV infection (Fig. S2). At early time points, i.e. 24 and 36 h p.i., DMVs had always a regular circular shape and they were distributed throughout the cytoplasm in small clusters of less than 5 DMVs. DMVs became organized in larger clusters, with 10 or more vesicles, and mostly were found in the perinuclear region of the cell. The DMVs invaginations became also more pronounced from 48 h p.i. CMs were initially detected at 24 h p.i., but only in 0.5 % of cells (Fig. 3B and S2). They became more apparent at 36 h p.i., reaching a plateau at 60 h p.i. The CMs were always found in between or around the cluster of DMVs. A dramatic change in the percentage of CMs was observed at 60 h p.i., from 6 % to 21 %, when also the DMVs increased markedly. Overall, these data indicated that the CMs are structures that are functionally connected with DMVs as reported (15).

Both LVCVs and virions-positive ER were detectable from 24 h p.i. and the percentage of cell sections with these structures increased over time (Fig. 3C). Interestingly, their morphology changed with the progression of the infection. At earlier time points, from 24 to 48 h p.i., the number of virions per ER and LVCVs section was less than 5. At late time points, i.e. 60 and 72 h p.i., the virion-positive ER domains and LVCVs were larger, had less regular shapes and contained more than 10 viral particles in their interior. Virion-positive endolysosomal compartments started to appear from 36 h p.i., and the number of virions found in their lumen increased over time. These observations suggested that the formation of LVCVs, virion-positive ER and virion-positive endolysosomal compartments are probably induced by a higher production of virions in the cell.

ERBs started to become visible only at 48 h p.i. (Fig. 3C). Initially, positive cell profiles displayed only one ERBs but from 60 h p.i., we occasionally observed more than one ERBs per cell section. We concluded that the ERBs are not required for the early steps of the PEDV life cycle but are rather the result of an advanced infection.
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A

B

C

Post-infection time [h]

Cell sections with signs of infection [%]

Cell sections with the indicated structures [%]

Cell sections with the indicated structures [%]

Post-infection time [h]

DMVs
CMs

ER with virions
Endolysosomal compartments with virions
LVCVs
ERBs
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Figure 3. Quantitative analysis of the PEDV-induced structures over the course of the infection. (A) The EM preparations described in Fig. 1 were used to count the number of cell sections containing at least one of the six PEDV-induced structures (Fig. 1) to assess morphologically the proportion of infected cells. (B) The percentage of cells displaying DMVs and CMs was determined in the experiments shown in Fig. 1. (C) The percentage of structures containing viral particles (i.e. virion-positive ER, virion-positive endolysosomal compartments, LVCVs and ERBs) in the samples shown in Fig. 1 was statistically evaluated. Error bars represent the standard deviation from 4 grids.

2.4 The Golgi complex undergoes reorganization over the course of a PEDV infection

Our ultrastructural quantifications were showing that the LVCVs become very prominent at 60 h p.i. (Fig. 3C). It has previously been shown that LVCVs are expanded Golgi stacks (12, 29). Therefore, we decided to also quantify at the ultrastructural level the number of cell sections displaying the presence of at least one Golgi complex over the course of the PEDV infection (Fig. 4A and 4B). During the first 48 h after the inoculum, the number of cell sections displaying this organelle did not change noticeably (Fig. 4B). The Golgi complex, however, was practically no more detectable with its known morphology (i.e. a series of adjacent stacks) from 60 h p.i., at the exact same time point when the LVCVs became prominent (Fig. 4B). Interestingly, the percentage of cell sections with LVCVs at the late p.i. time points was similar to the one of cell sections with Golgi prior to exposure to PEDV. Altogether, these data indicated that as for MHV and TGEV (12, 29), the LVCVs originate from ERGIC/Golgi, which probably expand as a consequence of a large local production of virions.

During the morphological analysis of the Golgi complex, we also observed another alteration of this compartment, which appeared as large clusters of single membrane vesicles with irregular contours and very variable lengths between 650 nm and 1.9 µm (Fig. 4C). In approximately 20-50 % of the cases, depending on the p.i. time point, these structures also appeared to contain a few of Golgi-like stacks and therefore we speculate that they originate from the Golgi complex (Fig. S3A and S3B). Quantification of these structures revealed that they are not abundant and the number of cell sections displaying them peaked at 24 h p.i. before to decrease (Fig. 4D). These structures have not been detected in other CoV infections and therefore they could be specifically induced by PEDV. Alternatively, they could represent Vero E6 cell-specific Golgi complex alterations that take place when those cells are exposed to PEDV. We named those structures irregular vesicle clusters (IVCs).

Subsequently, we also examined whether the Golgi organization and subcellular distribution changes during the course of a PEDV infection by
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We inoculated cells with PEDV (MOI = 1) before analyzing them by immunofluorescence at 0, 24 and 72 h p.i. using antibodies against dsRNA and GM130, a Golgi marker protein. In non-infected cells, GM130-labelled Golgi localized perinuclearly (Fig. 4G) (47). GM130 lost its compact organization in infected cells, recognized by dsRNA staining, and became more scattered throughout the cytoplasm in numerous puncta at 24 h p.i. (Fig. 4G). At 72 h p.i., the distribution of GM130 signal changed again forming cytoplasmic clusters, but those had a less compact shape compared to the ones observed in uninfected cells. We did not observe at any time point a colocalization between dsRNA and GM130 indicating that the Golgi membranes very likely do not contribute to the establishment of the PEDV replication sites (Fig. 4G). When the same samples were labeled with another Golgi protein marker, GIANTIN (48), and one for the trans-Golgi network, TGN46 (48), and analyzed by immunofluorescence, we detected the same reorganization of the Golgi in infected cells as the one observed for GM130 (Supplementary Fig. S3C and S3D).

To demonstrate that LVCVs indeed originate from Golgi complexes, Vero E6 cells were infected with PEDV for 72 h before to be processed for immun-EM as described in Material and Methods. Cryo-sections were subsequently immunogold labelled with anti-GM130 and anti-TGN46 antibodies. In uninfected cells, GM130 and TGN46 was exclusively localized to the Golgi complex (Fig. S3E and not shown), where they was present in the stacks and trans-Golgi network, respectively, as expected. In contrast, GM130 and TGN46 labelings were mainly found on LVCVs at 72 h p.i. confirming that these compartments have a Golgi origin (Fig. 4E and 4F).

Altogether, the immunofluorescence results corroborate the ones obtained in EM analyses and revealing that the Golgi complex undergo a massive reorganization during PEDV infection.

2.5 PEDV infections involve ER membrane rearrangements in Vero cells

The presence of virions in the ER and the formation of ERBs strongly suggested that there could also be a reorganization of the ER over the course of the PEDV infection. During the time-course EM analysis, we indeed observed that the ER proliferated over time, as numerous cross-sections of this organelle, often appearing as 5-10 adjacent ER tubules, where observed in the cytoplasm of infected cells (Fig. 5A and 5B). The ER proliferation first appeared at 8 h and reached a peak at 24 h p.i., before becoming less prominent during the following
Figure 4. The Golgi complex undergoes reorganization over the course of a PEDV infection. Vero E6 cells infected with PEDV were collected at different p.i. time points as described in Material and Methods before being processed for conventional EM (A, B, C, D), immuno-EM (E, F) and immunofluorescence (G). (A) A Golgi complex observed at 0 h p.i., which is typically composed by few long tubular stacks, with vesicles in their proximity. (B) The percentage of cell sections in the experiment shown in Fig. 1 that displays a Golgi complex and/or a LVCVs. (C) Typical morphology of an IVCs, which are closely packed, irregular shaped, single-membrane vesicles with a light content. (D) The percentage of cell sections in the experiment shown in Fig. 1, which are positive for IVCs. (E, F) PEDV induces the formation of LVCVs, which are positive for the Golgi stack and trans-Golgi protein markers GM130 (E) and TGN46 (F), respectively. Asterisks mark the LVCVs. (G) The subcellular distribution of GM130 was examined by immunofluorescence over the course of a PEDV infection, at 0, 24, 48 (not shown) and 72 h p.i. The anti-dsRNA staining was employed to identify infected cells and nuclei were stained with the DAPI dye (blue). Representative immunofluorescence images were from 3 independent experiments. Error bars represent the standard deviation from 3 grids. G, Golgi; M, mitochondrion; N, nucleus; PM, plasma membrane. Black scale bar, 500 nm; white scale bar, 10 μm.
hours of infection (Fig. 5C). These changes in ER area were confirmed by estimating the ER surface area using the point-hit method (Fig. 5D). Interestingly, the ER proliferation alleviation starting from 24 h p.i. coincided with when ER-derived structures such as DMVs, CMs and virion-positive ER start to emerge in the infected cells (Fig. 5C). Next we confirmed that the ERBs as well as the virion-containing compartments seen in Fig. 1C, originate from ER by immuno-EM analysis using an anti-KDEL antibody (Fig. 5E, 5F and S4). Since the ERBs only appeared from 48 h p.i. and increased gradually afterwards (Fig. 5C), this observation suggests that these structures are probably not functionally connected with the ER proliferation.

To sustain the changes in the ER organization, we examined the ER organization during the course of PEDV infection by immunofluorescence. We inoculated cells with or without PEDV before analyzing them at 24 h and 72 h p.i. using antibodies against PDI, an ER resident protein (49). In uninfected cells and at all time points, PDI localized in a large tubular network extending throughout the cytoplasm as expected (Fig. 5G) (50). In infected cells, in contrast, PDI lost its homogenous distribution and concentrated perinuclearly at 24 h and 72 h p.i. This observation supports the EM analyses showing that ER undergoes a massive reorganization in PEDV-infected cells.

3. Discussion

The description of the membrane remodeling induced by PEDV in host cells remains poorly described despite the increasing veterinary importance of this virus (40, 51). This type of information is also scarce for alpha-CoV in general, as the infection of only two other viruses belonging to this genus, i.e. HCoV-NL36 and TGEV, has been characterized at the ultrastructural level so far (23, 29, 33). HCoV-NL36 and TGEV virions have been observed in ER, Golgi and LVCVs, while DMVs were solely detected in cells exposed to HCoV-NL36. Thus, although those studies have revealed some similarities between the host membrane rearrangements caused by alpha-CoV and members of the other CoV genera, a temporal and comprehensive description of all morphological alterations induced in host cells by alpha-CoV, is still lacking. The limitation in the ultrastructural studies on HCoV-NL36 and TGEV has been the analysis of a single infection time point, which makes it difficult to obtain a complete repertoire of induced intracellular structures because their frequency and morphology change over the course of an infection as shown for MHV (12). Therefore, we decided to take a time-course approach to examine PEDV infection in Vero E6 cells by qualitative and quantitative EM. In addition to
structures like DMVs and LVCVs already shown to be induced by alpha-CoV, this strategy allowed us to detect structures such as CMs reported for other CoV and novel ones, the IVCs and ERBs. It has recently been reported that PEDV triggers autophagy (52), but we did not have evidences in our samples indicating an induction of this pathway. In particular, we have not detected autophagosomes, which are morphologically different than the observed DMVs. These latter are much smaller (180-280 versus 500-1500 nm (53)) and they do present cytoplasmic component in their interior.

Here, we report for the first time the presence of CMs in cells infected with an alpha-CoV (Fig. 1B), which are morphologically similar to the ones generated in cells exposed to other CoV (15, 17, 24). In contrast to what is observed for MHV and SARS-CoV (12, 15), the CMs appear at the same time as DMVs but with lower frequency (Fig. 3B). We favor the idea that CMs could originate from the DMVs and could have a similar function in replication and transcription like these structures. In contrast to MHV- and SARS-CoV-infected cells (15, 17), we never observed continuity between these two structures using our EM approach.

It is known that ER is capable of increasing in size upon treatment with specific drugs or protein overexpression (54, 55). Interestingly, we saw a proliferation of the ER at the early stage of PEDV infection, from 8 h p.i., which peaked at 24 h p.i. just before the appearance of the DMVs and the virion-positive ER (Fig. 5C). Considering the functional interrelationship between DMVs and ER (15, 45, 50), one hypothesis is that the ER is proliferating to accommodate the increasing amassing of viral proteins that will form the DMVs and luminal virions. Another hypothesis is that one or more PEDV proteins present some structural and/or functional characteristics that lead to a more pronounced ER stress response.

At the late stages of PEDV infection, we observed at low frequency a novel structure similar to the one described in cells infected by human coronaviral LINDER strain (45), that we named ERBs (Fig. 1F). ERBs have a geometrical organization and they are connected with the ER (Fig. 1F). Their ER origin is also demonstrated by the fact that they are positive for the ER marker peptide KDEL (Fig. 5D and E). These observations make the ERBs reminiscent with the TBs and CMSs induced by MHV and SARS-CoV (12, 16, 17, 31). TBs and CMSs also appear at the late stage of an infection and they contain a single viral protein, which self-aggregates when massively overproduced. As a result, it is tempting to speculate that ERBs also represent aggregates formed by one or more PEDV proteins that are present in the ER in elevated amounts. The
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A, B, E: Electron micrographs showing the ultrastructure of cells infected with Alpha-CoV at different time points post-infection (p.i.).

C, D: Bar graphs representing the percentage changes in ER proliferation and relative ER area over time post-infection.

G: Immunofluorescence images showing the expression of PDI and DAPI in mock and PEDV-infected cells at 24 and 72 hours post-infection.
Figure 5. PEDV infection causes different ER membrane rearrangements. Vero E6 cells infected with PEDV were collected at different p.i. time points as described in Material and Methods, before being processed for conventional EM (A, B, C), immuno-EM (D, E) and immunofluorescence (F). (A) ER observed at 0 h p.i., which typically appears as long dispersed tubular structures, with many ribosomes sitting on the limiting membrane. (B) Representative morphology of proliferating ER in PEDV-infected cells, showing an arrangement of 5-10 layers of tubular rough ER. (C) The percentage of cell sections in the experiment shown in Fig. 1 that displays a ER proliferation, DMVs, CMs, virion-positive ER and ERBs. (D) The ER surface area at each p.i. time point was determined using the point-hit method. The data are presented relative to the 0 h p.i. control. (E) Morphology of the ER in non-infected cells labeled with an anti-KDEL antibody. (F) Representative images of ERBs labeled with the anti-KDEL antibody. (G) The subcellular distribution of PDI, an ER protein marker, was examined by immunofluorescence with or without PEDV infection at 24 and 72 h p.i. Representative immunofluorescence images were from 3 independent experiments. Error bars represent the standard deviation from 3 grids. M, mitochondrion; N, nucleus; PM, plasma membrane; ER, endoplasmic reticulum. Black scale bar, 500 nm; white scale bar, 10 μm.

morphological differences between ERBs, TBs and CMSs are probably due to a difference in the structural and self-aggregating properties of the proteins that lead to their formation.

We also identified a novel structure that we called IVCs, which we speculate that probably originate from the Golgi because some of the IVCs appear to contain stacks of this organelle (Fig. S3A and S3B). Like the ER proliferation, IVCs also became apparent at the early stage of PEDV infection, at 8 h p.i., and became the most frequent at 24 h p.i. (Fig. 4D). As a result, a hypothesis analogous to the one drawn for the ER proliferation, would be that IVCs are Golgi complexes that are expanding and/or getting altered because of the increasing amounts of PEDV proteins. One could also imagine that IVCs are the precursors of LVCVs.

In our study, we have observed virions in several compartments, i.e. ER, LVCVs, endolysosomal organelles and very rarely in the ERBs (Fig. 1C-F). However, we first detected them forming in both ER and LVCVs at the same time point (i.e. 24 h p.i.). This is in contrast with what reported for CoV belonging to other subfamilies, i.e. their viral particles assemble in the ERGIC/Golgi compartments (12, 56), but also TGEV. The virions of this alpha-CoV start to form in the ERGIC/Golgi compartment and only at later time point in the ER (29). The reason behind these differences might be due to differences between genera, viruses and/or the infected cell types. Nonetheless, our findings suggest that PEDV particles assemble in both the ER and Golgi complex. PEDV virions were also detected in what appear to be endolysosomal-like compartments from 36 h p.i., but intriguingly we never noted them assembling on the limiting membrane of these organelles. Altogether, these observations lead us to postulate that the endolysosomal compartments are not the site of viral particle assembling, but rather a location where excess virions are nonspecifically delivered or
eventually could represent a transport intermediates involved in the secretion of viral particles. Alternatively, those could be secreted virions that are re-infecting cells through endocytosis. Very interestingly, we observed two types of virions appearing simultaneously (Fig. 1C and 1D), which have been so far described exclusively in cells exposed to another alpha-CoV, TGEV, suggesting that this could be a unique characteristic of this CoV genus (29). In the morphological study of TGEV-infected cells (29), the smaller viral particles with a denser core were identified as mature virions while the larger annular ones as the immature precursors. Our observations suggest that PEDV virion maturation does not occur in a specific organelle as both types of viral particles are present in the ER and LVCVs (Fig. 1C and D), indicating the structural maturation of PEDV virion particles could happen in both of ER and LVCVs. This result is in part dissimilar from the one obtained with TGEV, where it was shown that virion maturation mainly take place in the Golgi (29). Again, this difference could be specific to the virus or the infected cell type. Another possibility is that some mature virions are retro-transported to ER after maturation in Golgi due to their local massive production. Interestingly, we also found that only mature virions are present in the compartments of the endolysosomal system (Fig. 1E), further supporting one of our hypotheses made above.

In conclusion, our study has characterized and temporarily ordered the different membranous rearrangements induced by PEDV. This information paves the way for future investigations about the function of these structures, which is crucial to understand PEDV and more in general alpha-CoV cellular life cycle, and might eventually help to develop novel therapies against them.

4. Material and Methods

4.1 Cell culture, virus propagation and time-course analysis of PEDV infection

Vero E6 cells, a kindly gift from Jolanda Smit (34, 35), were maintained with Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY) containing 5% fetal calf serum (FCS, Gibco). The used PEDV strain was CV777 and it was grown as previously described in Vero E6 cells (36).

Vero E6 cells were also employed for the time-course infection (36, 37). Vero E6 cells at 60% confluence were inoculated with 1 multiplicity of infection (MOI) of PEDV and 25 µg/ml of trypsin (Sigma-Aldrich, St. Louis, MO). After 1 h of incubation, cells were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) to remove the excess virus and synchronized the PEDV life cycle. Infected cells were
maintained in complete DMEM without FCS, and aliquots of cells and culture supernatants were collected for analysis at 0, 8, 16, 24, 36, 48, 60 and 72 h post-infection (p.i.).

4.2 Extracellular virus titration

The amount of infectious PEDV particles released into culture medium was determined by calculating the 50% tissue culture infectious (TCID$_{50}$) values in Vero E6 cells. Briefly, monolayers of Vero E6 cells were inoculated with serial dilutions in DMEM of supernatants collected at 0, 8, 16, 24, 36, 48, 60 and 72 h p.i. Cytopathic effects, i.e. cell death, were morphologically assessed under a light microscope after incubation at 37°C for 4 days. The TCID$_{50}$ value was calculated using the Spearman/Kaerber formula (38, 39).

4.3 Isolation of the total RNA and Real-Time PCR (RT-PCR)

Quantification of viral replication was determined by RT-PCR. Briefly, the total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized by using the Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) (both reagents were from Invitrogen, Carlsbad, CA). RT-PCR was then performed using primers to detect the mRNA levels of the ORF3 (GAGACTCGAGCGATTGACACAGTTG and GAGAGGTACC GCCTCAAAGACGC) and the N protein (GAGACTCGAGCTGCAACGACACGGCACCAATC and GAGAGGTACC CGTCTGAAAAGCCAATC) in a CFX Connect™ Real-Time PCR detection system (Bio-Rad, Hercules, CA) using the following conditions: 10 s at 95°C, then 40 cycles of 5 s at 95°C and finally 34 s at 60°C.

4.4 Immunofluorescence analyses

Immunofluorescence analyses were carried out as previously described (12, 40). Preparations were first incubated with the primary antibodies in PBS containing 0.1% bovine serum albumin (BSA) at room temperature for 1h and after extensive washing with PBS, they were incubated with the secondary antibodies also in PBS containing 1% BSA at room temperature for 45 min. Preparations were finally mounted in DAPI-containing ProLongTM Gold antifade (Invitrogen). Fluorescence signals were captures with either a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) or a DeltaVision Elite system (GE Healthcare Life Sciences, IL).
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Primary antibodies used in this study were monoclonal antibodies against dsRNA (English and Scientific Consulting Kft, Szirák, Hungary), TGN46 (Sigma, St. Louis, MO) and PDI (Enzo, Farmingdale, NY), and polyclonal antisera against GIANTIN (Abcam, Cambridge, United Kingdom) and GM130 (Abcam, Cambridge, United Kingdom). Secondary antibodies were Alexa488- or Alexa568-conjugated goat anti-rabbit or anti-mouse antibodies (Life Technologies).

4.5 Transmission electron microscopy

Cells were fixed with Karnovsky (2% para-formaldehyde (PFA), 2.5% glutaraldehyde (GA) in 0.1 M sodium cacodylate pH 7.4) for 140 min at room temperature and then post-fixed with 1% OsO4, 1% KCNFe in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h on ice. Samples were subsequently dehydrated stepwise with increasing concentrations of ethanol before rinsing them with 1,2-propylene oxide (Merck, Darmstadt, Germany) at room temperature and embedding in Epon resin (41). After resin polymerization for 4 days at 60°C, 65–70 nm sections were cut using an UC7 ultra-microtome (Leica Microsystems) and contrasted with uranyl acetate and lead citrate (41), before being analyzed in a CM100bio TEM (FEI, Eindhoven, The Netherlands).

For the quantitative analyses, at least 200 cell profiles were randomly selected at each p.i. time point and three different quantifications were performed on 4 grids. First, the number of cell sections containing at least one of the PEDV-induced structures was counted to determine morphologically the proportion of infected cells. Second, we calculated the percentage of cell sections positive for each PEDV-induced structure. Third, we determined the average number of each structure per cell section and calculated the standard deviation. The average diameter of length of each structure was determined by measuring 10–40 profiles using the ImageJ software (http://rsb.info.nih.gov/ij/), which also calculated the standard deviations. At each p.i. time point, the ER surface area per cell was calculated using the point-hit method (42) on 50 randomly selected electron micrographs from 3 different grids. The results are presented relative to the 0 h p.i. control.

4.6. Immuno-electron microscopy

Cells were fixed in 4 % PFA in 0.1 M phosphate buffer (19 mM NaH2PO4, 81 mM Na2HPO4, pH 7.4) overnight at 4°C. Before scraping them from the petri dish in PBS containing 1% gelatin, cells were washed 3 times in PBS and 1 time
in PBS containing 50 mM glycine. Cells were successively embedded in 12% gelatin, cryo-sectioned and immunogold labelled as previously described (43). Primary antibodies were mouse anti-KDEL (Calbiochem, San Diego, CA), sheep anti-TGN-46 (Serotec, Oxford, United Kingdom) mouse anti-GM130 (BD Biosciences, Franklin Lakes, NJ), mouse anti-CD63 (Developmental Studies Hybridoma Bank, Iowa City, IA) and mouse anti-transferrin receptor (TfR, ThermoFisher Scientific, Waltham, MA), and they were detected after bridging with a rabbit anti-mouse IgG (Rockland, Limerick, PA) or an rabbit anti-sheep IgG antibody (Nordic, Tady, Sweden) respectively, with 10 nm gold particles conjugated to protein A (CMC, Utrecht, the Netherlands). Labelled cryosections were contrasted with a 2% uranyl oxalate (pH 7) and methyl cellulose-uranyl acetate (pH 4) solution on ice, and imaged as described for the conventional EM.

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Author Contributions

F.R., X.Z., M.M. and D.S. conceived and designed the experiments; X.Z., Y.C. and T.V. performed the experiments; X.Z., M.M. and F.R. analyzed the data; J.K. contributed reagents and materials; X.Z. and F.R. wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest. The founding bodies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.
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Supplementary figure

Figure S1. PEDV viral particles can be detected in compartments of the endolysosomal system. Immunogold labelling of PEDV-infected Vero cells at 72 h p.i. with anti-CD63 (A) and anti-TfR (B) antibodies. Asterisks mark endolysosomal compartments. M, mitochondrion; N, nucleus; PM, plasma membrane. Scale bar, 500 nm.
Figure S2. DMVs morphology and subcellular distribution change over the course of the PEDV infection. Representative micrographs of cell sections with DMVs observed at 24, 48 and 72 h p.i.. Asterisks mark DMVs while arrowheads indicate invaginations at the limiting membrane of DMVs. LVCVs, large virion-containing vesicle. M, mitochondrion; PM, plasma membrane. Scale bar, 250 nm.
**Figure S3. PEDV infection induces a reorganization of the Golgi complex.** Vero E6 cells infected with PEDV were collected at different p.i. time points as described in Material and Methods before being processed for conventional EM (A, B) and immunofluorescence (C, D). (A) IVCs observed at 16 h p.i. containing few Golgi stacks (arrow). (B) Quantification of the percentage of IVCs with or without Golgi stacks in the experiment shown in Fig. 1. Error bars represent standard deviations from 3 grids. (C) The localization of TGN46 was analyzed by immunofluorescence at the indicated p.i. time points. Nuclei were stained with DAPI and representative images were from 3 independent experiments. (D) The subcellular distribution of GIANTIN was examined by immunofluorescence at the indicated p.i. time points. Infected cells were identified by dsRNA labeling and nuclei were stained with the DAPI dye (blue). Representative immunofluorescence images were from 3 independent experiments. (E) A representative Golgi in non-infected cells where the trans-Golgi network is labeled with an anti-TGN46 antibody. M, mitochondrion; N, nucleus; PM, plasma membrane; G, Golgi. Black scale bar, 500 nm; white scale bar, 10 μm.
Figure S4. PEDV viral particles assemble in the ER and lead to its expansion. (A) Immunogold labelling of PEDV-infected Vero cells at 72 h p.i. with anti-KDEL antibodies. (B) Higher magnification of the inset in panel A. Arrowheads highlight virions forming with inward budding at the limiting membrane of the ER. ER, endoplasmic reticulum; M, mitochondrion; PM, plasma membrane. Scale bar, 500 nm.