Identification and subcellular localization of porcine deltacoronavirus accessory protein NS6

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Abstract

Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus. Accessory proteins are genus-specific for coronavirus, and two putative accessory proteins, NS6 and NS7, are predicted to be encoded by PDCoV; however, this remains to be confirmed experimentally. Here, we identified the leader-body junction sites of NS6 subgenomic RNA (sgRNA) and found that the actual transcription regulatory sequence (TRS) utilized by NS6 is non-canonical and is located upstream of the predicted TRS. Using the purified NS6 from an Escherichia coli expression system, we obtained two anti-NS6 monoclonal antibodies that could detect the predicted NS6 in cells infected with PDCoV or transfected with NS6-expressing plasmids. Further studies revealed that NS6 is always localized in the cytoplasm of PDCoV-infected cells, mainly co-localizing with the endoplasmic reticulum (ER) and ER-Golgi intermediate compartments, as well as partially with the Golgi apparatus. Together, our results identify the NS6 sgRNA and demonstrate its expression in PDCoV-infected cells.

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1. Introduction

Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus that causes diarrhea in nursing piglets (Chen et al., 2015b; Hu et al., 2015; Jung et al., 2015; Ma et al., 2015). It was first detected in Hong Kong in 2012 (Woo et al., 2012). In early 2014, outbreaks of PDCoV were announced in swine populations in Ohio, Illinois, and Iowa, and it rapidly spread to multiple states in the United States (Li et al., 2014; Marthaler et al., 2014a; Marthaler et al., 2014b; Wang et al., 2014a, 2014b). Thereafter, PDCoVs were detected or caused outbreaks in Korea (Lee et al., 2016; Lee and Lee, 2014), China (Dong et al., 2015; Song et al., 2015; Wang et al., 2015) and Thailand (Janetanakit et al., 2016; Madapong et al., 2016), posing significant economic concerns and gaining considerable attention (Jung et al., 2016; Lorsirigool et al., 2016; Zhang, 2016).

PDCoV is an enveloped, single-stranded, positive-sense RNA virus belonging to the newly identified genus Deltacoronavirus within the family Coronaviridae. Its genome is approximately 25.4 kb in length, making it the smallest genome among the known coronaviruses (CoVs). The genome arrangement of PDCoV is similar to those of other CoVs with the typical gene order: 5’UTR-ORF1a-ORF1b-S-E-M-NS6-N-NS7-3’UTR (Song et al., 2015; Woo et al., 2012). Although the biological functions of PDCoV-encoded proteins have not been studied in detail, based on studies of other known CoVs, PDCoV ORF1a and ORF1b probably encode two viral replicase polyproteins, pp1a and pp1ab, which are predicted to be proteolytically cleaved into 15 mature nonstructural proteins responsible for viral replication and transcription; ORFs S, E, M, and N encode viral structural proteins, Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N), respectively (Chen et al., 2015a; Lee and Lee, 2014; Li et al., 2014; Woo et al., 2010). Additionally, NS6 and NS7 each encode a putative accessory protein (Fig. 1A).

Accessory proteins are genus-specific for coronavirus (Tan et al., 2006); however, different CoVs contain different numbers of accessory genes and proteins. For example, alphacoronavirus feline infectious peritonitis virus encodes five accessory proteins (p3a, p3b, p3c, p7a, and p7b), while only one accessory protein is encoded by porcine epidemic diarrhea virus (PEDV); betacoronavirus severe acute respiratory syndrome coronavirus (SARS-CoV) encodes a total of eight accessory proteins; and the most studied gammacoronavirus, infectious bronchitis virus (IBV), encodes four accessory proteins (Liu et al., 2014). Although coronavirus accessory proteins have generally been considered to be dispensable for viral replication in vitro (Hajjara et al., 2004; Yount et al., 2005), extensive functional studies have shown that many accessory...
proteins are involved in immune modulation (Kopecky-Bromberg et al., 2007) and viral pathogenesis in vivo (De Haan et al., 2002). The field of coronavirus accessory proteins has gained considerable attention in recent years.

In the PDCoV genome, there are two putative accessory genes, NS6 and NS7. NS6 is predicted to be located in the genome between M and N and to encode a 94-amino acid peptide, while NS7 is predicted to be located within the N gene (Lee and Lee, 2015; Woo et al., 2012). To date, there are no reports regarding the expression of PDCoV accessory genes or the identification of an associated transcription regulatory sequence (TRS) for production of these subgenomic RNAs (sgRNAs) in virus-infected cells.

Here, we identified the leader-body fusion site and TRS of NS6 sgRNA. By using monoclonal antibodies (MAbs) that recognize the putative NS6 protein, we demonstrated that the predicted NS6 could be expressed and localized to the cytoplasm in PDCoV-infected cells, providing the first biochemical evidence for the existence of PDCoV NS6.

Fig. 1. Analysis of the leader-body TRS junction of putative PDCoV NS6 sgRNA. (A) The primer design for the leader-body junction RT-PCR analysis is shown in a schematic diagram of the PDCoV full-length genome. (B) A representative gel from agarose gel electrophoresis of RT-PCR products amplified from PDCoV mRNA is shown. M, molecular size ladder. (C) Analysis of the sgRNA NS6 sequence. The primers and the leader sequences are displayed as underlined and as italicized, respectively. The positions of the nucleotides in the genome sequences are indicated by black arrowheads. The start codon ATG in NS6 sgRNA is marked in bold. Boxed regions represent the authentic TRS and putative TRS used for sgRNA synthesis. The N100 indicates that the 100 nucleotides at that region are not shown.
2. Results

2.1. Identification of PDCoV NS6 sgRNA

A vital feature of coronavirus transcription is the set of sgRNAs produced by discontinuous transcription. Each sgRNA contains a common 5′ leader sequence derived from the 5′-end of the viral genome and a so-called “body” sequence, which represents sequences from the 3′-poly(A) stretch to a position that is upstream of each genomic ORF encoding a structural or accessory protein (Sawicki et al., 2001, 2007). The fusion of leader and body sequences is processed, at least in part, by cis-acting elements termed transcription regulatory sequences (TRSs) (Hussain et al., 2005; Sawicki et al., 2007). Thus, determining the junction region of the leader and body sequences can verify the existence of the corresponding sgRNAs. To identify the possible NS6 sgRNA, total intracellular RNA was extracted from LLC-PK cells infected with PDCoV, and sgRNAs were amplified by leader-body junction RT-PCR with the primers Leader-F and NS6r (Fig. 1A) as reported previously (Dye and Siddell, 2005; Hussain et al., 2005). As shown in Fig. 1B, two specific RT-PCR products of approximately 1.0 kb and 500 bp were obtained and then isolated from the agarose gel, followed by cloning and sequencing. At least 10 independent clones were sequenced. The results reveal that the two specific RT-PCR products are sgRNAs M and NS6 (Fig. 1B). Our sequence analysis of these RT-PCR products indicates that sgRNA M contains a leader sequence followed by the typical PDCoV TRS (ACACCA) (Woo et al., 2009, 2012), as expected for the predicted M sgRNA transcript. However, to our surprise, the difference (underlined) in the TRS sequence (ACACC) typical PDCoV TRS (ACACCA) (Woo et al., 2009, 2012), as expected indicates that sgRNA M contains a leader sequence followed by the NS6 (Fig. 1B). Our sequence analysis of these RT-PCR products in-sults reveal that the two specific RT-PCR results of western blotting assays also show that approximately 11-kDa lysates of cells transfected with pCAGGS-NS6.

To further investigate the existence of NS6 at the protein level, we needed to prepare an NS6-specific antibody. To this end, a 285-bp cDNA fragment of the NS6 gene was amplified by RT-PCR and cloned into a prokaryotic expression vector, pET28a (+), resulting in pET28a-NS6. This plasmid was then transformed into Escherichia coli Rosette (DE3), and gene expression was induced with 0.8 mm isopropylthiogalactopyranoside (IPTG). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the fusion protein His-NS6 was efficiently expressed in the form of inclusion bodies in E. coli, the size of which was consistent with the predicted molecular weight of the recombinant protein (Fig. 2A). The results of western blotting assays demonstrated that the recombinant NS6 protein was specifically recognized by anti-His MAb (Fig. 2B). Subsequently, the protein was purified (Fig. 2C), and the concentration of the purified NS6 protein was 0.46 mg/ml, as measured by using a Trace ultraviolet spectrophotometer.

Using the purified NS6 protein as an immunization antigen and the hybridoma technique, two hybridoma cell lines, named 4B9 and 2G3, were acquired through screening and subcloning. To further confirm the specificity of these two MAbs, a eukaryotic expression plasmid encoding NS6 was constructed by cloning the cDNA of the NS6 gene into pCAGGS with the primers NS6-F and NS6-R (Table 1), which was then transfected into LLC-PK cells; indirect immunofluorescence assays (IFAs) and western blotting assays were then performed with the obtained MAbs. As shown in Fig. 3A, specific fluorescence could be observed in cells transfected with pCAGGS-NS6, while neither MAb recognized cells transfected with the empty vector, pCAGGS. The results of western blotting assays also show that approximately 11-kDa specific protein bands, the size of which is identical to the size of the predicted NS6 protein (Fig. 3B), could be detected by both MAbs in the lysates of cells transfected with pCAGGS-NS6.

Table 1. Sequence of the primers used for identification of sgRNA and construction of plasmids.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5′–3′)</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leader-F</td>
<td>AATTTTATCCTCTCTAGCTTCG</td>
<td>40–60</td>
</tr>
<tr>
<td>NS6r</td>
<td>AAGAATGACTTGGAGCGATGGTCGA</td>
<td>23936–23960</td>
</tr>
<tr>
<td>NS6-F</td>
<td>ACTGAAATCAGTGGCAACTGCCTCACCCGG</td>
<td>23984–2374</td>
</tr>
<tr>
<td>NS6-R</td>
<td>GCCCTGCAGATTTAATTCATCTCTCAGAATG</td>
<td>23954–23975</td>
</tr>
</tbody>
</table>

* Restriction enzyme recognition sites are shown in bolded and underlined type.
* The number represents nucleotide position corresponding to the nucleotide sequence of the PDCoV CHN-HN-2014 (GenBank accession no. KT316560).
2.3. Expression and subcellular localization of NS6 protein in PDCoV-infected cells

To test if NS6 is indeed expressed during PDCoV infection, LLC-PK cells were infected with PDCoV at a multiplicity of infection (MOI) of 5.0. The cells were fixed and either subjected to IFA or collected for western blotting at 12 h post-infection (hpi). For IFA, obvious immunofluorescence was observed in the cytoplasm in PDCoV-infected LLC-PK cells, while no fluorescence signals were observed in mock-infected cells (Fig. 4A). Also, specific protein bands similar to the size observed in pCAGGS-NS6-transfected cells could be detected by both MAbs in lysates of PDCoV-infected LLC-PK cells (Fig. 4B).

Fig. 3. Expression of NS6 in cells transfected with pCAGGS-NS6. LLC-PK cells were plated onto 24-well plates following transfection with pCAGGS-NS6 or empty vector. At 28 h post-transfection, cells were fixed for use in an IFA (A) with MAbs 4B9 or 2G3 against NS6 and Alexa Fluor 594-conjugated donkey anti-mouse IgG (red). DAPI (blue) indicates the locations of the cell nuclei. Fluorescent images were acquired with a confocal laser scanning microscope (Fluoview ver. 3.1; Olympus, Japan). The transfected cells were also collected for use in western blotting (B) analyses performed with MAbs 4B9 or 2G3.

Fig. 4. Expression of NS6 in PDCoV-infected LLC-PK cells. LLC-PK cells were plated onto 24-well plates and then mock-infected or infected with PDCoV at a MOI of 5.0. At 12 h post-infection, IFAs (A) and western blotting assays (B) were performed with MAbs 4B9 or 2G3, as described in Fig. 3.

To further investigate the expression kinetics of NS6 protein in detail during PDCoV infection, LLC-PK cells seeded onto microscope coverslips in 24-well plates were mock-infected or infected with PDCoV at a MOI of 10. At 3, 6, 9, 12, and 15 hpi, cells were fixed for IFA with MAb 4B9. As shown in Fig. 5A, specific fluorescence could be observed at 6 hpi, and increasingly more fluorescence was readily detected as the infection progressed. Up to 15 hpi, obvious cytopathic effects characterized by cell shrinkage and detachment were observed.
Fig. 5. Expression kinetics and subcellular localization of NS6 protein in PDCoV-infected LLC-PK cells. LLC-PK cells were plated onto coverslips and then mock-infected or infected with PDCoV at a MOI of 10. (A) At the indicated times post-infection, cells were fixed for IFA with MAb 4B9 and Alexa Fluor 488-conjugated donkey anti-mouse IgG (green). (B) At 12 hpi, cells were fixed and stained with NS6-specific MAb (4B9) and rabbit anti-GRP94 (ER marker), rabbit anti-GS28 (Golgi marker), or rabbit anti-SEC31 (ER-Golgi intermediate compartment), followed by staining with secondary antibodies Alexa Fluor 488-conjugated donkey anti-rabbit IgG (green) or Alexa Fluor 594-conjugated donkey anti-mouse IgG (red). Nuclei were counterstained with DAPI (blue). Fluorescent images were acquired with a confocal laser scanning microscope (Fluoview ver. 3.1; Olympus, Japan).
The NS6 protein was localized in the cytoplasm during the entire experimental period (Fig. 5A).

To examine the precise subcellular localization of the NS6 protein in PDCoV-infected LLC-PK1 cells, the cells were fixed at 12 hpi and stained with both mouse anti-NS6 monoclonal antibody (MAb 4B9) and rabbit polyclonal antibodies against either the endoplasmic reticulum marker GRP94, the Golgi apparatus marker GS28, or the ER-Golgi intermediate compartment marker SEC31. NS6 protein was observed to predominantly colocalize with both the ER compartment and the ER-Golgi intermediate compartment, as well as to partially colocalize with the Golgi complex (Fig. 5B).

3. Discussion

Although coronavirus accessory proteins are not essential for virus replication in vitro (Casais et al., 2005; Yount et al., 2005), previous reports have made it clear that these accessory proteins are not redundant, but rather possess many functions, including modulating viral pathogenicity (De Haan et al., 2002) and acting as cell death inducers (Law et al., 2005) or interferon antagonists (Niemeyer et al., 2013; Siu et al., 2014). Furthermore, recent studies have reported the discovery of novel accessory proteins in some coronaviruses, such as the newly identified accessory protein ORFk of bat severe acute respiratory syndrome-like coronavirus (Zeng et al., 2016). For deltacoronavirus, no information on the expression, intracellular localization, function of accessory proteins or on their unique sgRNAs has been reported. Indeed, given the absence of a TRS preceding NS6 and the relatively high (0.500) Ka/Ks ratio of NS6 in Bulbul Coronavirus (BuCoV), another deltacoronavirus, it seems likely that BuCoV does not express this accessory protein (Woo et al., 2009). In contrast, a typical putative TRS preceding NS6 is present in other deltacoronaviruses, such as PDCoV, White-eye Coronavirus, and Sparrow Coronavirus (Woo et al., 2012). However, these deltacoronavirus accessory proteins have been predicted in the absence of any experimental evidence to confirm their expression during virus infection. Here, we identify the sgRNA of the PDCoV NS6 gene for the first time and provide experimental evidence to confirm its expression in PDCoV-infected cells.

To analyze the PDCoV sgRNAs, we initially used a non-radioactive labeling Northern blot. Unfortunately, NS6 sgRNA could not be detected, even though other sgRNAs including S, M, and N, were easily identified (data not shown). This result may be due to a lower sensitivity of the non-radioactive labeling technique or a lower abundance of NS6 sgRNA. Thus, we performed leader-body junction RT-PCR, which had been extensively used in previous studies (Hussain et al., 2005; Zhang et al., 2007), to examine the existence of sgRNA NS6. The advantages of this method include higher sensitivity and simplicity of operation. Moreover, it can determine the leader-body junction site of each sgRNA by DNA sequencing. To avoid interference from other high abundance sgRNAs, such as sgRNAs S and N, the reverse primer was designed to target the end of NS6 and the extension time in each PCR cycle was set as 20 s. As expected, only M and NS6 sgRNAs were amplified in our experimental conditions (Fig. 1B).

Unexpectedly, the location and sequence of the leader-body junction site of NS6 sgRNA did not match previous projections. The predicted leader-body fusion site for NS6 sgRNA is 46 nucleotides upstream of the NS6 gene AUG start codon; however, sequence analysis of the RT-PCR product shows that the actual site for NS6 sgRNA is located at 148 nucleotides upstream of the AUG start codon of NS6 gene. Additionally, each sgRNA contains a TRS characterized by an AU-rich section of about 10 nucleotides (the core is a hexanucleotide motif) (Bentley et al., 2013). The TRS is the fusion site of leader and body sequences of coronavirus sgRNAs (Dye and Siddell, 2005). Although the molecular mechanisms of the leader-body fusion in coronavirus sgRNAs have not been elucidated fully, accumulating evidence suggests that most TRSs are derived from the leader TRS (TRS-L), which are considered canonical TRSs, while some non-canonical TRSs are derived from the body TRS (TRS-B) (Van Marle et al., 1999; Zuniga et al., 2004). The conserved hexanucleotide TRS used by PDCoV is ACACCA, as determined for M sgRNA by our study, while the TRS of NS6 sgRNA is ACACCT, which is identical to the core sequence of TRS-B, suggesting that NS6 uses a non-canonical TRS. Similar phenomena have also been found in sgRNAs 2-1 and 3-1 of SARS-CoV (Hussain et al., 2005). The differences in TRS sequence for different sgRNAs could play a regulatory role in controlling the abundances of different mRNAs (Bentley et al., 2013; Hussain et al., 2005; Zuniga et al., 2004). It is possible that the lower abundance of NS6 sgRNA is associated with its non-canonical TRS.

Although many sgRNA of different coronaviruses have been identified, their natural expression products in virus-infected cells are yet to be confirmed due to lack of specific antibodies. Here, we generated two MAbs against PDCoV NS6 and demonstrated that the NS6 protein could be detected in both PDCoV-infected cells and cells transfected with a eukaryotic expression construct of the NS6 gene. We also found that a similar intracellular localization distribution could be observed in both cells infected with PDCoV and those overexpressing NS6. Furthermore, the expression of NS6 could be detected at the early phase (6 hpi) of PDCoV infection. Subcellular localization analyses showed that NS6 colocalized partially with the Golgi, and predominantly with the ER complex and ER-Golgi intermediate compartment. Similar subcellular localization has also been reported for SARS-CoV ORF7a (also known as U122), an accessory protein incorporated into purified SARS-CoV particles (Fielding et al., 2004; Huang et al., 2006). It is well known that the ER-Golgi compartment is the site of coronavirus assembly and packaging (Klumpperman et al., 1994; McBride and Fielding, 2012). The presence of NS6 in the ER-Golgi intermediate compartment suggests the possibility that NS6 plays a role in viral assembly and budding events. Indeed, our preliminary study found that NS6 could be detected in the purified virion by western blotting (data not shown); however, additional experimental evidence, especially confirmation with immunogold electron microscopy is required to support this idea.

Previous studies suggested that coronavirus accessory proteins are often dispensable for virus replication in vitro, but are required for optional replication and virulence in the natural host (McBride and Fielding, 2012; Zhao et al., 2009). PDCoV infection suppresses RIG-I-mediated interferon-β production (Luo et al., 2016), and previous studies suggested that some accessory proteins of coronavirus, such as SARS-CoV ORF3b (Freundt et al., 2009) and MERS-CoV ORF4b (Thornbrough et al., 2016), are interferon antagonists. Whether PDCoV NS6 possesses this property requires further study. In addition, BLAST search revealed that NS6 proteins share no amino acid similarities with other CoV accessory proteins or known host proteins. No putative transmembrane domain and functional domain were identified by TMHMM (www.cbs.dtu.dk/services/TMHMM) and InterProScan (www.ebi.ac.uk/interpro) analyses, respectively. Precise functions of NS6 protein need to be yet further explored. At present, we are making efforts to construct an infectious cDNA clone of PDCoV and hope to use a reverse genetics system to study the NS6 function in the PDCoV viral life cycle and pathogenesis.

In summary, we confirmed the existence of a separate NS6 sgRNA with a non-canonical leader-body fusion site. We also prepared two MAbs against PDCoV NS6 and demonstrated that NS6 proteins are indeed expressed in PDCoV-infected cells. The expression of NS6 could be detected as early as 6 hpi in the cytoplasm of PDCoV-infected cells. The expressed NS6 proteins in PDCoV-infected cells predominantly localize to the ER complex and ER-Golgi intermediate compartment. The identification of sgRNA NS6 and the expression and subcellular localization of NS6 protein lay the foundation for elucidation of the structure and function of NS6 in the PDCoV viral replication cycle.
4. Materials and methods

4.1. Cells, viruses, and reagents

LLC-PK and SP2/0 myeloma cells were respectively grown in Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen) and RPMI 1640 Medium, each supplemented with 10% heat-inactivated fetal bovine serum, at 37 °C in a humidified 5% CO₂ incubator. PDCoV isolate CHN-HN-2014 (GenBank accession no. KT336560), which was isolated from a suckling piglet with acute diarrhea in China in 2014, was propagated in LLC-PK cells in DMEM with 10 μg/mL of trypsin. The titer of the supernatant from infected cells was up to 10^{8.5} TCID₅₀ (median tissue culture infectious dose) per milliliter. Horseradish peroxidase-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated donkey anti-mouse (rabbit) IgG, and Alexa Fluor 488-conjugated donkey anti-mouse (rabbit) IgG were purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal antibodies against hemagglutinin were purchased from Medical and Biological Laboratories (Japan).

4.2. Leader-body junction analysis

Total intracellular RNA was extracted from PDCoV-infected LLC-PK cell lysates using Trizol reagent (Invitrogen) and then reverse-transcribed using a Transcription First Strand cDNA Synthesis kit (Roche) according to the manufacturer’s instructions with specific primer NS6r, which binds to a location within the C terminal of the NS6 gene. The obtained cDNAs were amplified by PCR with NS6f and a forward primer Leader-F, which binds to a location in the 5’ leader sequence (Table 1). PCR reaction conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 20 s, and a final extension of 72 °C for 5 min. PCR products were analyzed on a 1.2% agarose gel and then cloned into a pMD18-T cloning vector (Takara) for sequencing.

4.3. Cloning of the NS6 gene

Viral genomic RNA was extracted from the supernatant of PDCoV-infected LLC-PK cells using Trizol reagent (Invitrogen) and used immediately for cDNA synthesis according to the manufacturer’s instructions. NS6 gene-specific primers were designed using Primer 3.0 software based on the published CHN-HN-2014 sequence (Table 1). The 285-bp fragment of the NS6 gene was amplified by RT-PCR and then cloned into a pET28a (+) expression vector. The successful construction of the recombinant plasmid, named pET28a-NS6, was verified using enzyme analysis and DNA sequencing.

4.4. Preparation of the NS6 protein

The recombinant plasmid pET28a-NS6 and empty vector pET28a (+) were separately transformed into Rosetta (DE3) cells and induced by 0.8 mM IPTG for 3–6 h. The bacteria were then collected and washed with phosphate-buffered saline (PBS), followed by cell lysis via the supersonic schizontysis. SDS-PAGE was performed to analyze the NS6 protein expression. The proteins were purified by the supersonic schizontysis method as described previously (Liu et al., 2009), and the concentration of purified proteins was subsequently determined by using a Trace ultraviolet spectrophotometer.

4.5. Immunization of mice

Three 6-week-old female BALB/c mice were inoculated via subcutaneous injection with purified NS6 protein (120 μg/mouse) mixed with the same amount of Freund’s complete adjuvant. Two weeks later, these mice were given a second immunization with the NS6 protein (100 μg/mouse) emulsified in an equal amount of Freund’s incomplete adjuvant. Two weeks after the second inoculation, mouse antisera were collected, and the titers of antibody in the sera were assessed by performing indirect ELISA. Subsequently, mice with higher antibody titers were given a booster immunization of NS6 protein (200 μg) by intraperitoneal injection 3–5 days prior to their use in hybridoma production.

4.6. Production of anti-NS6 protein MAb

Splenocytes from the mouse with the highest antibody titer following immunization with the NS6 protein were harvested and fused with SP2/0 myeloma cells under the condition of 50% polyethylene glycol (PEG4000) as a fusion agent. The resulting hybridoma cells were then cultured in 96-well plates at 37 °C in a humidified 5% CO₂ incubator in HAT screening culture medium with fetal bovine serum. One week later, positive hybridomas with coverage of one third to one half of the bottom of 96-well plates were filtered by indirect ELISA. Subsequently, the positive hybridoma cells were subcloned three times by the limiting dilution method. Finally, the stable hybridoma cells were passaged, and the resulting cell supernatants were collected for use in later experiments. Meanwhile, the stable hybridoma cells were stored in liquid nitrogen.

4.7. Indirect immunofluorescence assays (IFAs) and confocal microscopy

LLC-PK cells in 24-well plates with 80% confluence were transfected with the plasmid encoding the putative NS6 gene or an empty vector for 28 h. Separately, similar monolayers of LLC-PK cells in 24-well plates were mock-infected or infected with PDCoV and then cultured in the DMEM containing trypsin (10 μg/mL) at 37 °C. Both sets of cells were then fixed, followed by use in indirect IFAs with the following primary antibodies: NS6-specific MAb (4B9) and rabbit anti-HSP90β1 (GRP94, ER marker; Abclonal) polyclonal antibody, rabbit anti-GOSR1 (GS28, Golgi marker; Abclonal) polyclonal antibody, or rabbit anti-SEC31A (SEC31, ER-Golgi intermediate compartment marker; Abclonal) polyclonal antibody (1:100 dilution in PBS). Alexa Fluor 594-conjugated donkey anti-mouse (rabbit) IgG or Alexa Fluor 488-conjugated donkey anti-mouse (rabbit) IgG (1:500 dilution in PBST) was then used as secondary antibodies. Cells were treated with 4,6-diamidino-2-phenylindole (DAPI) to indicate the locations of the cell nuclei. Fluorescent images were obtained with a confocal laser scanning microscope (Fluoview ver. 3.1; Olympus, Japan).

4.8. Western blotting analysis

For the western blotting analysis, cells that had been transfected or infected as described above were lysed, collected, and separated by SDS-PAGE with 15% polyacrylamide gels. Subsequently, the separated protein was transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBST with 0.1% polysorbate-20. NS6-specific MAb were then incubated with the nitrocellulose membrane for 3 h at room temperature. After washing three times with PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution, in PBST) for 45 min at room temperature. After washing three times, the membrane was visualized by enhanced chemiluminescence reagents (ECL; BIO-RAD).

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