Veterinary Microbiology 164 (2013) 212–221

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Porcine epidemic diarrhea virus N protein prolongs S-phase cell cycle, induces endoplasmic reticulum stress, and up-regulates interleukin-8 expression

Xingang Xu [a,1], Honglei Zhang [a,1], Qi Zhang [a], Yong Huang [a], Jie Dong [a], Yabing Liang [a], Hung-Jen Liu [b,c,**], Dewen Tong [a,*]

[a] College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan
[c] Agricultural Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan

ARTICLE INFO

Article history:
Received 10 October 2012
Received in revised form 14 January 2013
Accepted 21 January 2013

Keywords:
PEDV
N protein
S-phase
ER stress
IL-8
NF-κB

ABSTRACT

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease of swine caused by porcine epidemic diarrhea virus (PEDV). The porcine intestinal epithelial cell is the PEDV target cell. In this study, we established a porcine intestinal epithelial cell (IEC) line which can stably express PEDV N protein. We also investigate the subcellular localization and function of PEDV N protein by examining its effects on cell growth, cycle progression, interleukin-8 (IL-8) expression, and survival. The results show that the PEDV N protein localizes in the endoplasmic reticulum (ER), inhibits the IEC growth and prolongs S-phase cell cycle. The S-phase is prolonged which is associated with a decrease of cyclin A transcription level and an increase of cyclin A degradation. The IEC expressing PEDV N protein can express higher levels of IL-8 than control cells. Further studies show that PEDV N protein induces ER stress and activates NF-κB, which is responsible for the up-regulation of IL-8 and Bcl-2 expression. This is the first report to demonstrate that PEDV N protein can induce cell cycle prolongation at the S-phase, ER stress and up-regulation interleukin-8 expression. These findings provide novel information on the function of the PEDV N protein and are likely to be very useful in understanding the molecular mechanisms responsible for PEDV pathogenesis.

1. Introduction

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease in swine characterized by severe enteritis, vomiting, and watery diarrhea and has high mortality in piglets (Ducatelle et al., 1981). PED is one of the most important causes of economic loss in many swine-raising countries. This is mainly due to its high prevalence compared to the rare incidence of transmissible gastroenteritis (TGE) and the asymptomatic characteristics of the rotavirus (RV) infections (Carvajal et al., 1995). The causative agent of PED is the porcine epidemic diarrhea virus (PEDV), which belongs to the family *Coronaviridae* and was first reported in 1978 (Pensaert and de Bouck, 1978). PEDV is an enveloped virus possessing a single-stranded positive-sense RNA genome approximately 28 kb in size with a 5′ cap and a 3′ polyadenylated tail. The genome comprises a 5′ untranslated region (UTR), a 3′ UTR, and at least seven open reading frames (ORFs) that encode 4 structural proteins

* Corresponding author at: College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China. Tel.: +86 29 8709 1622; fax: +86 29 8709 1032.
** Corresponding author at: Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan.
Tel: +886 4 22840485x243.
E-mail addresses: hjliu5257@nchu.edu.tw (H.-J. Liu), dwtong@nwsuaf.edu.cn (D.W. Tong).
[1] Both the two authors have contributed equally to this work.

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.01.034

[spike (S, 150–220 kDa), envelope (E, 7 kDa), membrane (M, 20–30 kDa), and nucleocapsid (N, 58 kDa)] and three non-structural proteins (replicases 1a, 1b, and ORF3); these are arranged on the genome in the order 5′-replicase (1a/1b)-S-ORF3-E-M-N-3′ (Song and Park, 2012; Kocherhans et al., 2001; Yeo et al., 2003).

PEDV N protein binds to virion RNA and provides a structural basis for the helical nucleocapsid. Also, it can be used as the target for the accurate and early diagnosis of PEDV infection (Song and Park, 2012). It has been suggested that N protein epitopes may be important for induction of cell-mediated immunity (Curtis et al., 2002; Saif, 1993). To date, no data exists on the subcellular localization of PEDV N proteins and its effects on cell growth and cell cycle progression. Porcine intestinal epithelial cells (IECs) are the cells targeted by PEDV and the epithelial cells in the gut serve as a physical barrier which restricts the movement of components and passage of potentially harmful microorganisms between the lumen and underlying mucosa (Schierack et al., 2005).

In the present study, we demonstrate for the first time that PEDV N protein induces endoplasmic reticulum stress and up-regulates NF-κB, Bcl-2, and interleukin 8. In addition to above findings, we also uncovered that PEDV N protein prolongs the S phase of stage cell cycle. The results have potentially important implications for understanding the molecular mechanisms of pathogenesis for this economically important porcine disease.

2. Materials and methods

2.1. Vectors, plasmids and cells

The pEGFP-N1 eukaryotic expression vector was purchased from Clontech (USA) and *Escherichia coli* DH5α used for cloning were purchased from Tiangen Biotech (China). In this study, the PEDV Shaanxi strain was isolated from intestinal tract contents of PEDV infected piglets in Shaanxi Province of China and N gene of PEDV was amplified as described previously (Honglei et al., 2012). The established swine intestinal epithelial cell lines (IEC), which were kindly provided by Prof. Yan-Ming Zhang, College of Veterinary Medicine, Northwest A&F University, were cultured as described previously (Jing et al., 2010). Briefly, IEC cells were grown in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated new born calf serum (Gibco BRL), 100 IU of penicillin and 100 μg of streptomycin per mL, at 37 °C in a 5% CO_2 atmosphere incubator.

2.2. Antibodies and reagents

Mouse monoclonal antibodies against cyclin A, GRP78, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, Inc., CA, USA). Mouse anti-GFP monoclonal antibody was purchased from Millipore (Millipore, Temecula, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Pierce (Pierce, Rockford, IL, USA). The MG132 proteasome inhibitor was purchased from Calbiochem (Calbiochem, San Diego, CA, USA) and the nuclear staining dye Hoechst33342 and ER-Tracker™ Red probe were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA).

2.3. Construction of recombinant plasmid and establishment of the stable cells expressing GFP-N and GFP protein

The primers used to amplify N gene of PEDV were as follows: forward primer (PEDV-XhoI), 5′-CCGCTCGAGATGGCTTCTGTCAGCTTTCA-3′ (26374–26393 of CV777 strain) and reverse primer (PEDV-Hind III), 5′-CCCAAGCTTATTTCCTGTATCGAAGAT-3′ (27679–27696 of CV777 strain). The restriction sites are underlined. The primers were designed according to the archived PEDV CV777 strain nucleotide sequence (GenBank: AF353511.1). The amplified product was cloned into the corresponding sites in the pEGFP-N1 expression vector. The recombinant plasmid was identified by enzyme digestion and DNA sequencing. The recombinant plasmid was named as pEGFP-N.

IEC cells were seeded into 6-well dishes 24 h before being transfected (up to 70–80% confluence). Cells were transfected with pEGFP-N and pEGFP-N1 control vector using Lipofectamine 2000 (Invitrogen) and maintained (up to 80–90% confluence) in selection media containing 1200 μg/mL G418 for two weeks. When all control cells had evidence of death in the presence of the selection agents, cultures transfected with pEGFP-N and pEGFP-N1 were propagated for two further weeks in medium containing 600 μg/mL G418. The resulting stably transfected cell lines expressing either GFP or GFP-N fusion proteins were used for subsequent analysis.

2.4. The observation of protein degradation characteristics

The stable cell lines expressing GFP-N protein and GFP were seeded respectively into 6-well dishes at a suitable concentration of cells each well. After incubation at 37 °C with 5% CO_2 for 24 h, the culture medium was replaced with fresh medium containing 20 μM MG132, and then incubated in a CO_2 incubator at 37 °C for 24 h. After 24 h incubation, the cells were washed with phosphate-buffered saline (PBS) for twice and incubated with Hoechst33342 at 37 °C for 15 min. Images were viewed after cells washed with PBS for twice by Fluorescence microscope (Model TE2000, Nikon, Japan).

2.5. Confocal microscopy

To examine the expression and subcellular localization of PEDV N protein, the cells expressing GFP-N protein or control cells (the cells expressing GFP and untransfected IEC cells) were grown on glass bottom dishes (35 mm) and washed with Hank's balanced salt solution (HBSS) and incubated with Hoechst33342 at 37 °C for 10 min, and then washed twice with HBSS. Cells were then incubated with ER-Tracker Red probe (Invitrogen) at 37 °C for 25 min and washed with HBSS for twice. Images were viewed by laser confocal scanning microscopy (Model LSM510 META, Zeiss, Germany).