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Subcellular localization and topology of porcine reproductive and respiratory syndrome virus E protein

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) open reading frame (ORF) 2a contains a small internal ORF (2b) capable of encoding a protein of 70 or 73 amino acids (aa), termed E protein. The function and biochemical information of the E protein are currently not clear. In the present investigation, it was shown that the E protein was mainly located in the endoplasmic reticulum (ER) and Golgi complex in MARC-145 cells. Deletion studies identified the N-terminal 15 residues as an ER localization sequence of the E protein, besides two other localization sequences within positions 23–50 and 50–73, and the N-myristoylation site significantly affected the subcellular localization of the N-terminal 15 residues. The membrane association assay demonstrated that the E protein was an integral membrane protein embedded in the phospholipid bilayer. However, neither the N-myristoylation site nor the hydrophilic C-terminal domain was essential to the membrane association of the E protein. The topology analysis revealed that this protein had N-terminus oriented toward the cytoplasm and C-terminus toward the ER lumen. Finally, immunofluorescence assay indicated that the E protein colocalized with GP2, GP3, GP4 and M protein in cotransfected cells, but not N protein.

1. Introduction

PRRSV belongs to the genus *Arterivirus* in the family *Arteriviridae* (Cavanaugh, 1997) and causes significant economic losses in the swine industry worldwide (Halbur et al., 1995). The viral genome consists of a positive single-stranded polyadenylated RNA molecule of approximately 15 kb in length and is composed of the 5′ untranslated region (UTR), nine ORFs (ORF1a, ORF1b, ORF2a, ORF2b, and ORF3-7) and the 3′ UTR (Meulenberg et al., 1995). Those ORFs encode 13 nonstructural proteins (ORF1a and ORF1b) and 7 structural proteins including GP2, E, GP3, GP4, GP5, M and N, respectively (Snijder and Meulenberg, 1998). Despite possessing similar virion morphology and genome organization, PRRSV is divided into two genotypes, the European genotype and the North American genotype, based on their antigenic and genetic characteristics (Meng et al., 1995; Nelsen et al., 1999).

The small envelope (E) protein is a hydrophobic protein, which is encoded by the ORF 2b starting from the +6 nucleotide position in mRNA2. The E protein consists of 73 and 70 amino acids for the North American and European type of PRRSV respectively and has an apparent molecular mass of 10 kDa (Snijder et al., 1999; Wu et al., 2001). The specific antibody against the E protein has been detected in PRRSV-infected pigs (Wu et al., 2001). It has been proposed that the E protein is essential to the virus infection, since deletion of the E protein would affect the incorporation of GP2, GP3 and GP4 into virions, possibly through forming multi-meric complex with other proteins to assemble into virions (Wissink et al., 2005). The E protein is also an ion-channel-like protein and maybe facilitates uncoating of virions (Lee and Yoo, 2006). However, although the E protein has been found exhibiting predominately perinuclear distribution when fused with enhanced green fluorescent protein (EGFP) (Wu et al., 2001), the exact subcellular localization of the PRRSV E protein in infected cells is not clear. In addition, mutation of the N-myristoylation site in the E protein could significantly reduce the growth of the virus (Du et al., 2010), but the mechanism is not known.

In the present study, the specific antibody against E protein peptide was developed to localize the E protein in the infected cells. The localization sequences of the E protein were identified by generating a series of truncated E proteins fused with EGFP in MARC-145 cells. Subsequently, ultra-centrifugation experiment with different

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extraction buffers was used to identify whether the E protein was an integral membrane protein embedded in the phospholipid bilayer. Then roles of the N-myristoylation site and the C-terminal domain in the subcellular localization and membrane association of the E protein were defined. Additionally, fluorescence protease protection (FPP) assay provided a useful means to establish a topology model of the E protein. Finally, colocalization between the E protein and the other viral structure proteins of the PRRSV was investigated by immunofluorescence assay.

2. Materials and methods

2.1. Cells and viruses

MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Inc., Gaithersburg, MD, USA) supplemented with 10% FBS (PAA, laboratories, Linz, Austria) at 37 °C with 5% CO_2. PRRSV VR-2332 strain was provided by Prof. Jiyong Zhou (Zhejiang University, Hangzhou, China) and propagated in MARC-145 cells.

2.2. Antibodies

The mouse anti-myc-tag (9E10) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse anti-FLAG (M2) antibody from Sigma–Aldrich (St. Louis, MO, USA), rabbit anti-GFP antibody from Proteintech Group Inc. (Chicago, IL, USA), monoclonal anti-Golgi 58K protein/Formimino-transferase Cyclodeaminase (FTCD) clone 58K-9 from Sigma–Aldrich (St. Louis, MO, USA) and anti-E polyclonal rabbit serum (raised against a PRRSV-specific peptide consisting of amino acids 58–71 of the E protein) was prepared with designed peptide. Antigenic determinant analysis was carried out using the Jamenson–Wolf algorithm in the protean program (DNA-STAR Inc., Madison, WI, USA). The secondary antibodies goat anti-rabbit IgG and anti-mouse IgG both conjugated to TRITC were from Zhongshan Golden Bridge Biotechnology (Beijing, China). The anti-rabbit IgG coupled HRP was from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Plasmid constructs.

The polymerase chain reaction (PCR) was used to amplify target genes from cDNA of the PRRSV VR-2332 strain. PCR amplifications were carried out using the TaKaRa Ex Taq system (Takara Bio Inc., Japan) with corresponding primers (Table 1). Oligonucleotides were designed to include codons to initiate and stop translation or not and to create restriction sites. PCR conditions were as follows: an initial denaturation of 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were purified, analyzed by agarose gel electrophoresis and subsequently inserted into corresponding vectors. The recombinant DNAs were used to transform DH5α-competent *E. coli* cells. Transformed cells were grown overnight at 37°C in Luria–Bertani medium in the presence of 100 μg/ml ampicillin or kanamycin. The resulting colonies were screened by digestion with appropriate restriction enzymes. The target genes in the corresponding plasmids were sequenced by the dideoxy method prior to transfection.

Plasmids E/pEGFP-N1 and E/pCDNA-flag contained the E gene in fusion with EGFP- and flag- tag respectively at the C terminus. Plasmids E/pCMV-myc contained the E gene in fusion with myc-tag at the N terminus. Plasmids GP2/pCMV-myc, GP3/pCMV-myc, GP4/pCMV-myc, GP5/pCMV-myc, M/pCMV-myc and N/pCMV-myc contained GP2, GP3, GP4, GP5, M and N genes in fusion with myc-tag at the N terminus. Plasmids E/pEYFP-C1 and E/pECFP-N1 contained the E gene with EYFP-tag at the N-terminus and ECFP-tag at the C-terminus separately. EG2A/pEGFP-N1 expressed the E protein with a mutation at position 2 from Gly to Ala. E15G2A/pEGFP-N1 expressed the first 15 residues having mutation at position 2 from Gly to Ala. pEGFP-N1, pCMV-myc, pECFP-N1, pEYFP-C1, pDsRed2-ER, pEYFP-MEM, pDsRed2-Mito and the Golgi marker pEYFP-Golgi were all from Clontech Laboratories (Mountain View, CA, USA). pCDNA3 was purchased from Invitrogen Corporation (Carlsbad, USA).

2.4. Sequence analysis and transmembrane domain prediction

The E sequence of the PRRSV VR-2332 strain cDNA (accession number EF442771) was used to retrieve all reported isolates by using the Blast program at NCBI. A final set of 235 sequences were downloaded and analyzed to construct Fig. 1. Multiple sequence alignment and consensus sequence determination were carried out with the Muscle program (multiple sequence comparison by log-expectation). Visualization of sequence alignment and plotting of the most frequently represented amino acid residues at each position were done using the Weblogo program.

Various methods were combined to predict the transmembrane sequence: TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), ConPred II (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), and HMMTOP (http://www.enzim.hu/hmmtop/). Signal peptide scanning was performed using the PSORT II program (http://psort.ims.u-tokyo.ac.jp/form2.html) (Nakai and Horton, 1999).

2.5. Transfection and fluorescence microscopy

MARC-145 cells were grown on coverslips in 24-well plates followed by transfection or infection. The plasmids were transfected into MARC-145 cells using Lipofectamine TM 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations. At given time points cells were rinsed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. For detection of the E protein and other viral proteins cells were incubated for 2 h with mouse anti-myc-tag antibody, anti-FLAG (M2) antibody or rabbit anti-E polyclonal antibody. After washed with PBS cells were incubated for 1 h with the secondary antibodies (TRITC-conjugated goat-anti-mouse polyclonal antibody or TRITC-conjugated goat-anti-rabbit polyclonal antibody). Fluorescence images were taken with a Leica confocal laser scanning microscope. For the ER colocalization study, cells were rinsed with HBSS (Gibco Europe Ltd., Paisley, UK) and stained with the prewarmed ER fluorescence marker ER-TRACKER BLUE-WHITE DPX (Invitrogen Molecular Probes, Carlsbad, USA) at a concentration of 500 nM in HBSS for 30 min at 37 °C/5%CO_2. Then the staining solution was replaced with fresh probe-free HBSS. Finally, cells were fixed with 4% paraformaldehyde for 20 min at 37 °C, washed twice with HBSS (5 min per wash) and viewed.

2.6. Membrane association assay

MARC-145 cells were transfected with pEGFP-N1 or plasmids containing the E protein mutants in fusion with EGFP. At 48 h post-transfection cells were washed with PBS and scraped in 250 μl of ice-cold 1:10 TES (20 mM Tris, pH 7.4; 100 mM NaCl; 1 mM EDTA) supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Samples were kept on ice throughout the duration of the procedure. Cells were incubated on ice for 30 min, lysed by 30 strokes in a Dounce homogenizer and then centrifuged