RESEARCH REPORT
SWINE HEALTH

Title: Defining a Novel Structural Component of Porcine Reproductive and Respiratory Syndrome Virus and its Role in Disease Pathogenesis; - **NPB #13-196**

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Industry Summary:

The objective of this project was to characterize the makeup of nsp2, shown to be a new structural component of the PRRSV virion. Radiolabelled nsp2 protein, in the absence or presence of synthetic enclosed membranes (microsomes), was produced to understand if nsp2 could be part of the virion membrane. After synthesis, the membrane fraction was isolated by centrifugation. Nsp2 was found to be produced as several different sized products, just as was found in PRRSV infected cells. Some nsp2 products were found in tight association with the membrane fraction, but no shift in protein size was observed, suggesting no modification by membranes, such as sugar residue addition. The nsp2 protein orientation with respect to the artificial membranes, referred to as topology, was determined. Surprisingly, a small portion of the C-terminal of the nsp2 protein, when expressed in the in vitro system used, was protected. The unexpected result indicated that this domain of the protein would be oriented towards the outer part of the virion. Additional studies must be completed using the same nsp2 proteins expressed in cell culture to confirm this result.

The novel finding that nsp2 was a structural protein (part of the infecting virus) led us to further examine the nsp2 biochemical structure, post-synthesis additions such as sugar modification, as well as to determine the origin and potential cleavage of the different sized nsp2 products. Our unexpected results have led us to postulate that additional proteins, viral or cellular, may be needed in order to achieve correct membrane orientation. Thus, the next study will involve expression of the same constructs in cultured cells.

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Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped virus with eight recognized structural proteins encoded in the 3' end of the virus genome. Recently, nonstructural protein 2 (nsp2), part of the replicase polyprotein, was also shown to be a structural protein. Nsp2 has an extended hypervariable N-terminal domain with a recognized papain-like protease region, 4-5 predicted membrane spanning regions followed by a relatively conserved carboxyl (C)-terminus. In order to biochemically characterize the nsp2 protein independently of the virus and host cell, in vitro rabbit reticulocyte translation of nsp2 in the presence or absence of canine pancreatic microsomal membranes was used. The membranes core glycosylate proteins if a signal sequence is present, perform other post-translational modifications such as cleave signal sequences and phosphorylate residues, and protect portions of the protein that have traversed the membrane from degradation by proteases. In our experiments, we found no change in molecular weight with membranes present that would signify signal protein cleavage and core glycosylation, suggesting nsp2 is not post-translationally modified using the cell-free system. However, isoforms of nsp2 were readily seen within 30 minutes of in vitro synthesis. The insertion and topology of nsp2 was also assessed. Full-length nsp2 was found to strongly associate with membranes and, surprisingly, two additional large nsp2 isoforms of approximately 117 and 106 kDa were enriched within the membrane fraction. Membrane integration was further defined for full-length nsp2 through high-speed density fractionation, protection from protease digestion, and immunoprecipitation. The results demonstrated that nsp2 integrated into the membranes with an unexpected topology, where the N-terminal domain was located on the exterior of the membranes, corresponding to the interior of virions, and a C-terminal 15 kDa domain was located in the microsome lumen, corresponding to the extravirion space. Additional studies must be completed using the same nsp2 proteins expressed in cell culture to further probe this unexpected result.

Introduction:

The challenges of producing a broadly effective vaccine for porcine reproductive and respiratory syndrome virus include determining the exact composition and arrangement of the structural proteins on the virion. The very large nonstructural protein 2 (nsp2), 30% of the initial replicase polyprotein, was recently shown to be a structural component for both Type 1 and Type 2 strains of PRRSV (Kappes et al., J Virol. 87:13456-65 2013). Nsp2 codes for a papain-like protease (PLP2) near its N-terminus, a long hypervariable region showing insertions and deletions between strains, several predicted transmembrane-spanning regions and a somewhat conserved C-terminal end (Han et al, Virus Res. 122:175-82. 2006). Because there are predicted transmembrane-spanning domains, we have hypothesized that nsp2 is embedded in the viral membrane, yet we have not yet determined if this is the case and, if so, the portions of nsp2 that are on the surface of the virion (extravirion) and thus may interact with host cells and/or other viral glycoproteins. Therefore, the objective of this proposal was to examine the biochemical nature of nsp2. This core knowledge will be used to locate common epitopes between strains that are located on the extravirion, and design future vaccines to target nsp2.

Objectives:

Objective 1: The specific topological architecture and post-translational modifications of nsp2 in the membrane will be determined through the use of synthesized VR-2332 nsp2 protein and a well-defined nsp2 protein deletion constructswith and without membranes, followed by treatment with specific proteases. Post-translational processing will also be investigated using highly purified virions of PRRSV.

Objective 2. We will further characterize nsp2 cleavage isoforms by in vitro transcription/translation experiments using pNsp2 and full-length replicase polyprotein, including mutation of nsp2 OTU-specific cleavage sites, to delineate which bands are the products of recently identified frameshifting (Fang et al., 2012) and which may be due to the action of cell-specific proteases, viral proteases, previously uncharacterized OTU-specific cleavage at GG dipeptides dependent on three dimensional protein structure, or alternative post-translational processing. Mapping the cleavage isomers from infected cells and virions will be attempted.

Materials & Methods:

A. Diagram of ORF1a showing that there are four or five predicted transmembrane regions in nsp2, depending on the computer algorithm utilized (Figure 1A). Depending on the number of transmembrane regions, the possible topologies (protein structure in the membrane) are shown (Figure 1B).

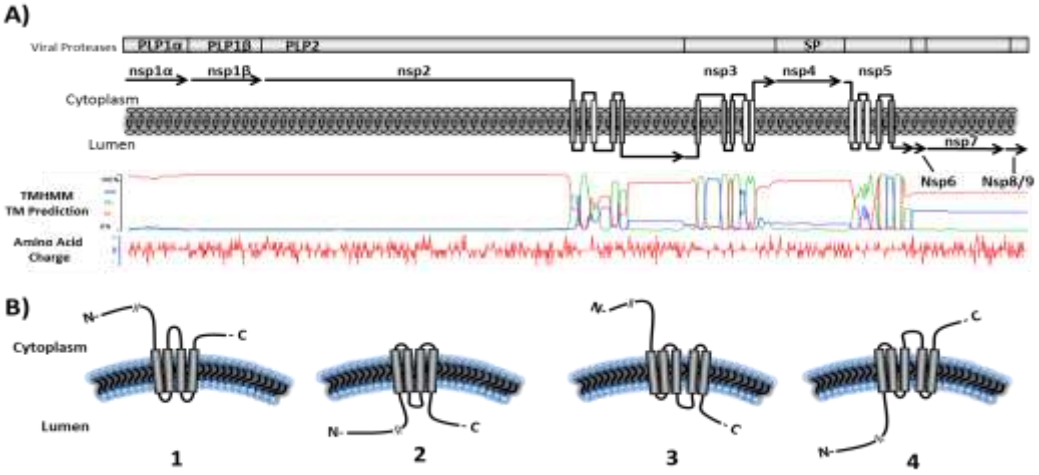

Figure 1. PRRSV ORF1a encodes putative multi-spanning transmembrane domains. Predicted hydrophobicity of the entire ORF1a coding region is depicted using the TMHMM prediction algorithm within the Geneious software platform. A) Top: graphical representation of pp1a noting the location of viral protease domains papain-like protease 1α (PLP1α), PLP1β, PLP2, serine protease (SP). Middle: model of membrane integration sites and topologies of pp1a based upon predicted transmembrane spanning helices. Regions where the putative transmembrane spanning domains are unclear are denoted in white. Bottom: plot of hydrophobicity strength scores (TOPCONS) and amino acid charge by position. B) Possible topologies of nsp2 dependent on even (1 and 2) or odd (3 and 4) number of transmembrane spanning elements and cytoplasmic versus luminal orientation.

B. **Nsp2 Protein Synthesis.** New constructs were designed and implemented to complete Objective 1 (see midterm report). Successfully developed pertinent to this report was VR-2332nsp2FL (full-length; pNsp2-FL) using the pcDNA3.0 cloning vector. In addition, N- and C-terminally FLAG tagged constructs of VR-2332nsp2FL were produced (pNsp2-N-FLAG, pNsp2-C-FLAG). All new clones clones of VR-2332nsp2 translated well in cell-free lysates radiolabeled with 35S-cysteine.

C. **Immunoprecipitation.** To facilitate immunoprecipitation assays, expression constructs of pNsp2-FL were engineered to encode an exogenous FLAG epitope (DYKDDDDK) either at the 5' termini (pNsp2-N-FLAG) or directly downstream of the nsp2 coding region (pNsp2-C-FLAG).

D. **Bioinformatic predictions.** pVR-2332 (DQ217415) nsp2 protein coding sequence was analyzed by the transmembrane prediction algorithms Phobius ['Normal prediction' method; (Kall et al., 2004)]; HMMTOP 2.0 (Tusnady and Simon, 2001); SCAMPI (Bernsel et al., 2008); TOPCONS (Bernsel et al., 2009); OCTOPUS (Viklund and Elofsson, 2008); PRO/PRODIV-TMHMM (Viklund and Elofsson, 2004); and MemBrain [TMH prediction; (Shen and Chou, 2008)] using default settings unless otherwise noted. Signal P 4.1 was used to determine if nsp2 had a detectable signal sequence at the N-terminus, indicating a standard method of membrane integration. No detectable signal sequence was found for eukaryotic proteins.

E. **Cell-free translation of nsp2.** Nsp2 expression constructs were translated using the TNT® T7 coupled transcription/translation reticulocyte lysate system (Promega). Reactions were assembled on ice using 1µg DNA template per 25µl translation reaction either with or without the addition of canine pancreatic microsomal membranes (microsomes; Promega). RNasin® Plus RNase inhibitor (Promega) was added to each reaction (1.6U/µl per rxn) to inhibit RNA degradation during the translation reaction. [35]-S cysteine (Cysteine L-[35S]; 10µCi/µl; PerkinElmer), thawed on ice, was added to the translation reaction after all other components (0.4mCi/ml final concentration). When used, microsomes were added to the translation reaction as a final step at a dilution of 1µl microsomes per 25µl translation reaction volume. The reaction was then mixed gently followed by incubation at 25°C for 1 hour. Lowering the incubation temperature to 25°C was found to aid expression of full-length nsp2 product. Translation products were quantified by measuring the amount of incorporated product (scintillation counts; Promega #TB126) prior to use in downstream assays.

F. **Polyacrylamide gel electrophoresis (PAGE) analysis.** Reduced and denatured samples were electrophoresed through NuPAGE® Novex® 4-12% Bis-Tris polyacrylamide gels (Life Technologies). PAGE separated [35]S-cysteine labeled products were imaged using BIOMAX MR High-Resolution Film (Carestream). Immunoprecipitation (IP) pull-down assays were completed with the use of the magnetic DYKDDDDK (FLAG) immunoprecipitation kit (ClonTech) per manufacturer's instructions.

G. **Isolation of the microsomal fraction.** Approximately 250,000 counts per translation reaction were diluted with chilled (4°C) PBS or tris-buffered sucrose (TBSS buffer; 25mM Tris-HCl pH7.5, 250mM sucrose). Reactions were diluted to a final volume of 10 ml and mixed thoroughly by gently pipetting. Diluted translation products were stabilized at 4°C for ≥1 hour, followed by pelleting at 150,000xg, 4°C, for 1 hour. Supernatants from high-

speed pelleting were discarded and the pellets were eluted in low volumes (15-25µl) of chilled PBS or TBSS buffer while keeping all components on ice.

H. **Protease protection assay.** Translated products were processed to isolate the microsomal fraction using TBSS buffer. Pelleted products were eluted in TBSS buffer and were stabilized at 4°C for ≥1 hour. Stabilized products (4°C) were then treated with proteinase K (Ambion) (0.6mg/ml) for 2 hours followed by proteinase K inactivation with fresh phenylmethylsulfonyl fluoride (PMSF) (10mM final concentration) and incubated at 4°C overnight. Protease protection assay (PPA) reactions were assessed by IP and PAGE analysis as described above.

Results:

Objective 1.

A. Transmembrane topology
   a. Translation of VR2332nsp2FL (pNsp2), pNp2-N-FLAG and pNp2-C-FLAG using cell-free translation in the absence (Figure 2; A and C) or presence of membranes (Figure 2; B and D). Isotopically labeled nsp2 translation products were shown to be predominantly full-length nsp2 of an approximate molecular mass of 130kDa (Fig. 2A), consistent with the expected product size of 129.4kDa. However, when overexposed, smaller protein products, presumably nsp2 isomers, were also made. Translation of nontemplate control (NTC) and vector control yielded no protein products, except in the presence of membranes (when overexposed) that have RNA and proteins associated with them.

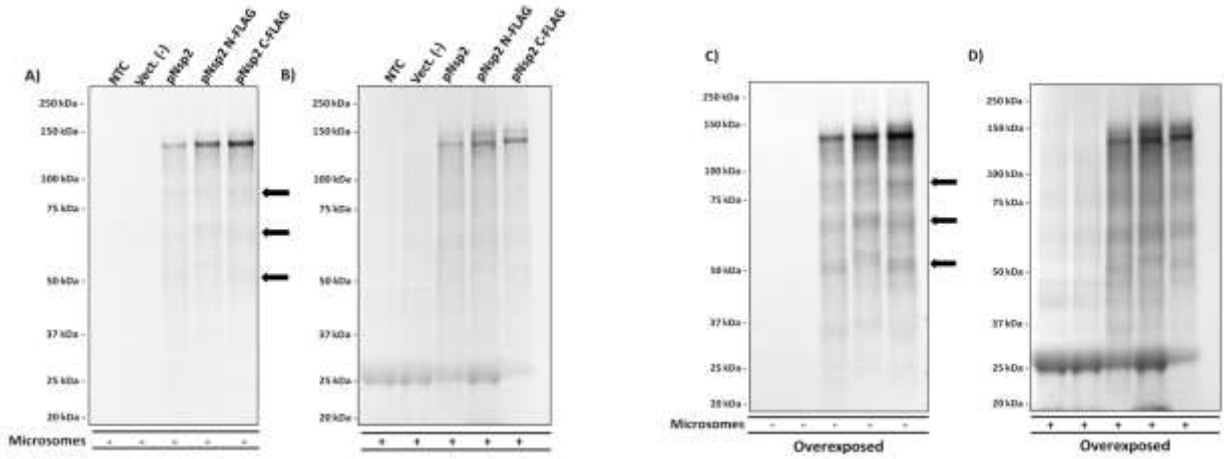

Figure 2. Cell-free translation of PRRSV nsp2. VR-2332 nsp2 coding region was isotopically labeled by transcription/translation coupled reaction in rabbit reticulocyte lysate with [35] S-cysteine either without (A and C) or with (B and D) microsomal membranes. [35]S-labeled translation products were loaded at 100,000 counts/ lane and PAGE separated under denaturing, reducing conditions in MES running buffer. C) Overexposed image of (A) to highlight the low molecular weight products; D) overexposed image of (B) to highlight microsomal cross-labeling and low molecular weight products. Lane 1 = Non-template control (NTC), Lane 2 = pcDNA vector control, Lane 3 = pVR-nsp2, Lane 4 = pVR-nsp2 N-FLAG, Lane 5 = pVR-nsp2 C-FLAG.

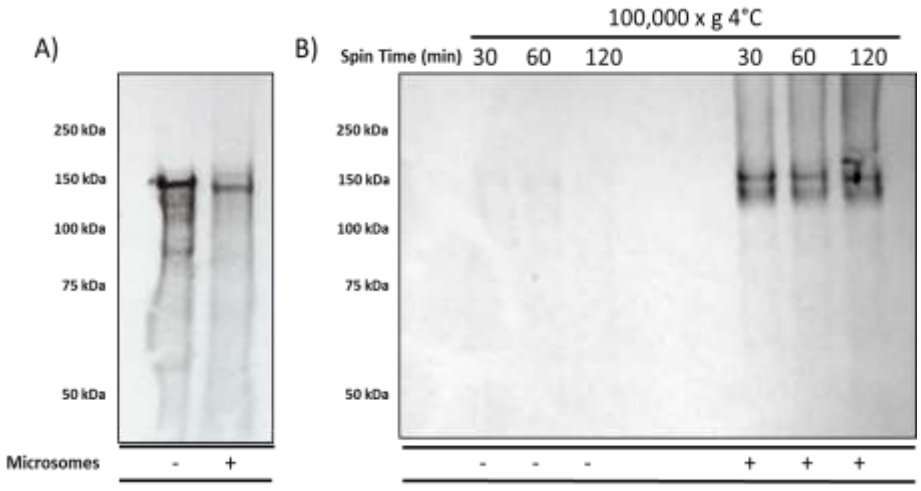

Figure 3. Nsp2 associates strongly with membranes. [35]S-labeled pNsp2 translation products were expressed either with (+) or without (-) the addition of microsomal membranes. A) Translation products at 100,000 counts per lane. B) Translation products equal to 100,000 counts/reaction were pelleted to enrich for the microsomal fraction. Eluted pellets were assessed by PAGE separation under reducing, denaturing conditions.

b. The membrane association of untagged nps2 (pNsp2) was assessed by isolating the membrane fraction of translation reactions by high-speed centrifugation (Figure 3). Figure 3A shows that predominantly full-length nsp2 was synthesized with and without the addition of microsomal membranes. When these preparations were centrifuged for 30, 60 or 90 minutes to pellet membranous fractions, no proteins were seen in the pellet when membranes were not included in the translation (Figure 3B, left panel). However, when membranes were present during protein synthesis, full-length nsp2 as well as an additional one or two slighty lower protein bands were seen (Figure 3B, right panel). Equivalent results were obtained using Nsp2-N-FLAG and Nsp2-C-FLAG (data not shown).

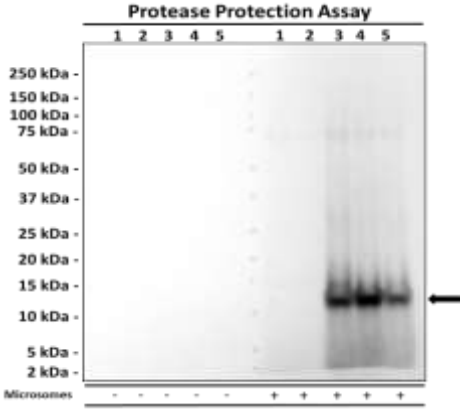

Figure 4. Nsp2 fragments is protected from protease degradation by membrane insertion. [35]S-labeled translation products were expressed either with or without canine microsomal membranes. Products were pelleted at 150,000 x g (4°C) 1hr. Membrane associated nsp2 was subjected to protease protection assay (proteinase K digestion) as outlined in the materials and methods and assessed by PAGE separation under reducing, denaturing conditions. Lane 1 = Non-template control (NTC), Lane 2 = pcDNA vector control, Lane 3 = pVR-nsp2, Lane 4 = pVR-nsp2-N-FLAG, Lane 5 = pVR-nsp2-C-FLAG.

c. Protein domains that have either embedded within the membrane or that have traversed the bilayer and are enclosed within the luminal surface of the microsomes are protected from protease digestion. To test whether nsp2 functions as an integral membrane protein, the isolated membrane fraction of each translation product was subjected to proteinase K digestion. The 35[S]-cysteine labeled products protected from proteinase K were assessed by PAGE (Figure 4). A single 13-15kDa 35[S]-protein product was observed to be protected from the digestion, and N-FLAG, C-FLAG, and untagged nsp2 membrane associated products were equally protected (Figure 4, lanes 3-5). These results demonstrate nsp2 integrates within microsomal membranes and that an approximately 15kDa product is protected from digestion either through integration within the membrane bilayer (transmembrane helices) or through orientation within the luminal surface of the microsomal compartment.

d. To determine which part of the protein may lie within the microsome and thus be protected from degradation by proteinase K, the protected products were further subjected to immunoprecipitation (IP) analysis (Figure 5A). IP analysis of protected fragments clearly demonstrated the 15kDa fragment originated from the C-terminal domain (Figure 5A). The protected fragment from untagged nsp2 construct was not immunoprecipated, demonstrating the IP reaction specifically targets FLAG-tagged products (Figure 5A; lane 3). While the N-FLAG tagged nsp2 PPA product failed to

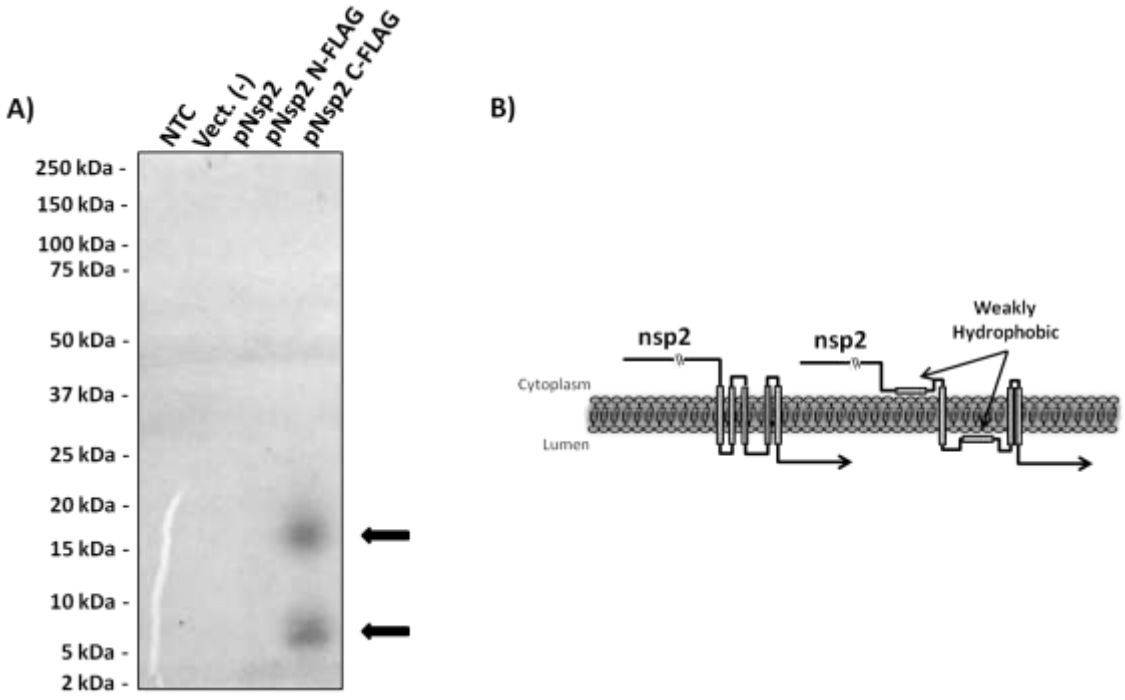

Figure 5. The C-terminus of nsp2 is protected from protease degradation by membranes. A) Translation products were expressed in the presence of microsomes. [35]S-labeled translation products were stabilized in TBSS and an enriched microsomal fraction was generated through pelleting at 150,000xg 1hr. The pelleted fraction was digested with proteinase K (protease protection assay) and further processed by immunoprecipitation, targeting the exogenous FLAG epitope (α-FLAG). Lane 1 = Non-template control (NTC), Lane 2 = pcDNA vector control, Lane 3 = pVR-nsp2, Lane 4 = pVR-nsp2 N-FLAG, Lane 5 = pVR-nsp2 C-FLAG. B) Graphical representation of the predicted membrane associated functions of the weakly hydrophobic putative transmembrane domains TM1 and TM3.

precipitate by α-FLAG (Figure 5, lane 4), two C-terminal FLAG tagged nsp2 products of approximately 15kDa and 5kDa were identified. It is unknown if the 5kDa protein was enriched within the IP reaction or if it is a partially digested product. Partially digested products could result from low amounts of incompletely inactivated proteinase K incubated for long time points (≥18hr) during the IP reaction. These results clearly demonstrate the C-terminal domain of nsp2 adopts a transmembrane or luminal topology. Further, the large hydrophilic N-terminal domain (PLP2 & HV; ~90kDa) was not observed to be protected from digestion, indicating a cytoplasmic orientation. These results define an unexpected topology of nsp2 where the large N-terminal domain is maintained on the cytoplasmic surface and the C-proximal hydrophobic region integrates within the membrane orientating the C-terminal domain within the luminal surface (Figure 5B).

B. Post-translational modifications. Translation of VR2332nsp2FL in the presence of microsomes revealed no discernable difference in protein product size, such as N-glycosylation. We conclude that post-translational modifications are not evident using the cell-free translation system. The post-translational modification analysis of purified virions was not completed, due to time and financial constraints.

Objective 2.

A. Nsp2 Autocleavage Assay. VR2332nsp2FL is translated in vitro by rabbit reticulocyte lysates in the absence of membranes into one major full-length product and several minor products at different times and temperatures. The faint products do not intensify compared to the full-length band over time. Therefore, autocleavage (by encoded papain-like protease 2; PLP2) of full-length nsp2 does not efficiently occur in cell-free translation experiments. In addition, a variety of cell lysates (uninfected, VR-2332-infected, HP-PRRSV-infected) generated under a range of conditions did not efficiently process nsp2 into the smaller isomers (data not shown). It was concluded that nsp2 does not appear to autoprocess itself using its protease (PLP2) domain without additional viral domains, and cell proteases also do not process nsp2.

B. As stated in our midterm report, the mutation of nsp2 OTU-specific cleavage sites was not completed, as the encoded PLP2 protease did not efficiently process full-length nsp2 into smaller products as was expected. Mapping the cleavage isomers from infected cells and virions was also not completed, again due to time and financial constraints brought on by additional cloning and optimization.

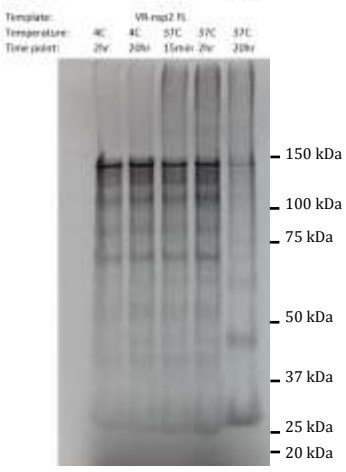

Figure 6. Autocleavage Assay. VR-nsp2-FL [35]S-labeled translation products were incubated at 4C for 2h and 20h, as well as at 37C for 15 min, 2h and 20h. Autocleavage of full-length nsp2 to smaller isoforms was not detected.

Discussion:

The nsp2 protein has been previously defined to be a critical component in PRRSV infection and the recent discovery of nsp2 packaging within the virion of PRRSV has broad implications to subsequent PRRSV immunology research and provides a unique target for novel vaccine design. However, basic nsp2 biochemical characterization had not been completed, needed for the intelligent design of future vaccination strategies. We undertook this characterization using a minimalist system, in vitro protein expression in the absence and presence of artificial membranes, so that the nsp2 protein would not be subject to other viral or host cell components. Under these conditions, nsp2 was not subject to further proteolytic processing by its self-encoded enzyme, and we could find no evidence of post-synthesis protein modifications. Surprisingly, we found that the nsp2 protein was oriented so that the C-terminal end was located on what would equate to the outside of the virion. This information will be used in the near future to design and complete similar experiments using cultured cells.

The research is relevant to the U.S. pork producer because a target for novel treatment strategies potentially was be localized. The research also identified conserved nsp2 features, and defined the underlying basis for nsp2 cellular and viral processing that support PRRSV infection or immune subversion. Future benefits, after additional studies are completed, may result in the development of a more broadly protective vaccine resulting in a reduction of morbidity and mortality ensuing from PRRSV infection.