**An SH3 Binding Motif within the Nucleocapsid Protein of Porcine
Reproductive and Respiratory Syndrome Virus Interacts with the Host
Cellular Signaling Proteins STAM1, TXK, Fyn, Hck, and Cortactin**

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Running title: Interaction of PRRSV N PxxP motif with host proteins

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Words in Abstract: 250

Word Count: 4291

Figures: 4;

Supplementary figures: 1

Tables: 1

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) causes an economically-important global swine disease, and has a complicated virus-host immunomodulation that often leads to a weak Th2 immune response and viral persistence. In this study, we identified a Src homology 3 (SH3) binding motif, PxxPxxP, that is conserved within the N protein of PRRSV strains. Subsequently, we identified five host cellular proteins [signal transducing adaptor molecule (STAM)I, TXK tyrosine kinase (TXK), protein tyrosine kinase fyn (Fyn), hematopoietic cell kinase (Hck), and cortactin] that interact with this SH3 motif. We demonstrated that binding of SH3 proteins with PRRSV N protein depends on at least one intact PxxP motif as disruption of P53 within the motif significantly reduced interaction of each of the 5 proteins. The first PxxP motif appears to be more important for STAMI-N protein interactions whereas the second PxxP motif was more important for Hck interaction. Both STAMI and Hck interactions with PRRSV N protein required an unhindered C-terminal domain as the interaction was only observed with STAMI and Hck proteins with N-terminal but not C-terminal fluorescent tags. We showed that the P56 residue within the SH3 motif is critical for virus lifecycle as mutation resulted in a loss of virus infectivity, however the P50 and P53 mutations did not abolish virus infectivity suggesting that these highly conserved proline residues within the SH3 motif may provide a selective growth advantage through interactions with the host rather than a vital functional element. These results have important implications in understanding PRRSV-host interactions.

Keywords: Porcine reproductive and respiratory syndrome virus (PRRSV); N protein; Hck; Fyn; TXK; STAMI

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of porcine reproductive and respiratory syndrome (PRRS), is a major economic concern within the swine industry worldwide. PRRS increases the cost of swine production by \$114.71 per sow annually, or \$664 million per year in the United States alone (Derald Holtkamp, 2011). The current commercial vaccines for PRRSV are not very effective against heterologous strains (Han et al., 2014; Kimman et al., 2009; Meng, 2000).

PRRSV is a member of the *Arteriviridae* family in the order of *Nidovirales*. The virus consists of a single-stranded, positive-sense, RNA genome of ~15 kb. The genome encodes ten open reading frames (Conzelmann et al., 1993; Lee and Yoo, 2005; Meulenberg et al., 1993; Thiel et al., 1993). ORF1a and ORF1b comprise 75% of the genome and code for 14 different non-structural proteins including proteases, polymerase, and helicase (Fang and Snijder, 2010). The structural proteins (ORFs 2-7) are encoded by six nested subgenomic mRNAs (Lunney et al., 2010; Meng et al., 1996). The major structural proteins consist of a 25 kDa envelope glycoprotein (GP5), an 18-19kDa unglycosylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6, and 7, respectively. The N protein, a small 123 amino acid basic phosphoprotein found within the nucleus, nucleoli, and cytoplasm of infected cells (Rowland et al., 1999), is the most abundant viral protein within infected cells and comprises 20-40% of the virion (Snijder and Meulenberg, 1998).

Like other members of the *Arteriviridae* family, PRRSV has a narrow cell tropism preferring to infect cells of the monocyte/macrophage lineage such as alveolar macrophages (Van Breedam et al., 2010). The initial host defenses against PRRSV infection are mounted via innate immune responses in the lung particularly alveolar macrophage responses (Murtaugh et al.,

2002; Xiao et al., 2010) followed by T-helper 1 (Th1) and Th2 responses within the respiratory tracts of PRRSV-infected pigs (Thanawongnuwech et al., 2003). Activation of several cytokines including interferon- γ , which has PRRSV-inhibitory activity *in vitro* (Huang et al., 2014; Rowland et al., 2001; Wang and Zhang, 2014), has been reported during infection (Aasted et al., 2002; Johnson et al., 2002). The current thought is that the interferon (IFN)- γ response to PRRSV is inhibited or delayed by unknown factors leading to a poorly-neutralizing Th2-dependent response in many pigs thus facilitating viral persistence (Meier et al., 2003; Meier et al., 2004). Additionally, PRRSV N protein has been shown to have a direct immunomodulatory role as transient expression of N protein alone within pulmonary alveolar macrophages has been shown to up-regulate IL-10 production. This N protein-associated IL-10 production was also shown to increase the number of PRRSV-specific regulatory T cells potentially altering how the immune response to PRRSV infection progresses (Wongyanin et al., 2012).

SH3 domains are conserved amino acid sequences that interact with proline-rich peptides forming protein complexes. SH3 domains are involved in cell-to-cell communications and signal transduction pathways (Schlessinger, 1994). SH3 binding motifs are often distinguished via a conserved amino acid sequence consisting of X-P-p-X-P where “X” is an aliphatic amino acid, “P” is always a proline, and “p” is sometimes a proline although specificity of the interactions have been shown to involve amino acid variation within and surrounding the conserved PxxP motif (Mayer and Eck, 1995). We found that the N protein of PRRSV contains an amino acid motif of proline, glutamic acid, lysine, proline, histidine, phenylalanine, and proline (PEKPHFP) (**Fig. 1A and 1B**) within N protein amino acid residues 50 to 56 resembling the PxxPxxP motif found within SH3 domain-containing proteins.

One mechanism that PRRSV could be utilizing to alter the host immune response is to modify protein-protein interactions through disruption of Src homology binding domains changing cellular signaling. There are many instances of other viruses altering specific cellular signaling pathways to modify how the host immune function responds. The human immunodeficiency virus type 1 (HIV-1) Nef protein has been shown to bind to SH3 domains of the proto-oncogene tyrosine-protein kinase Src (Src) family kinases Hck, tyrosine protein kinase Lyn (Lyn), Fyn, and Src altering T-cell signaling (Collette et al., 1996; Lee et al., 1995; Saksela et al., 1995). The Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway via a SH3 binding motif preventing virus-induced apoptosis via caspase-9 (Shin et al., 2007). The hepatitis C virus NS5a protein interacts with several SH3 domain-containing proteins including Grb2, amphiphysin II, PI3K and members of the Src family including Fyn, Hck, Lyn, and lymphocyte specific kinase (Lck) to either modulate immune function or aid in viral replication (Macdonald et al., 2004; Macdonald et al., 2005; Street et al., 2004; Tan et al., 1999; Zech et al., 2003). Therefore, understanding how this conserved SH3 binding motif within the N protein of PRRSV is interacting with the host during PRRSV infection is important in delineating the mechanism of PRRSV pathogenesis in the future.

2. Materials and Methods

2.1 Expression vectors, plasmids, and cells.

Fluorescent vectors used for PRRSV N protein expression in this study were enhanced cyan fluorescent protein (eCFP-N1), enhanced green fluorescent protein (eGFP-N1), and enhanced yellow fluorescent protein (eYFP-N1) vectors (Clontech, Mountain View, CA). For

bacterial expression, the PRRSV *ORF7* gene was cloned into the pET24a+ bacterial expression vector (Novagen). The cell lines MARC145 and BHK-21 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and antibiotics.

2.2 Construction of recombinant vectors expressing the His-tagged and CFP, GFP, YFP, fluorescent-tagged PRRSV N protein and SH3 host proteins.

ORF7, encoding the N protein, was PCR-amplified from the PRRSV VR2385 strain using the pIR-VR2385-CA infectious cDNA clone as the template (Ni et al., 2011) with the forward primer spk85 and the reverse primer spk86 (**Table 1**). The PCR fragments were inserted into the pET24a+ vector using *HindIII* and *XhoI* sites (**Fig. 1E**). For insertion into the fluorescent vectors, the PRRSV *ORF7* gene was amplified from PRRSV infectious cDNA clone pIR-VR2385-CA using forward primer spk102 and reverse primer spk103 (**Table 1**). The PCR fragments were inserted into the N1 vectors using *HindIII* and *XhoI* restriction sites (**Fig. 1E**).

Primate *STAM1* was amplified from total RNA extracted from the MARC145 cell line using an RNeasy mini kit (Qiagen) via RT-PCR with forward primer spk365 and reverse primer spk366 (**Table 1**). The PCR fragments were inserted into the N1 fluorescent vectors using *XhoI* and *HindIII* restriction sites. The Hck, Fyn, TXK, and cortactin genes were generated as synthetic gene products (Biomatik Corp) using the respective *sus scrofa* gene sequences available in the GenBank database (TXK; XM_003360910.3, Fyn; NM_001080206.2, cortactin; XM_005654635.1, and Hck; XM_003134391). These synthetic genes were then cloned in frame with either GFP, YFP, or CFP, respectively (**Fig. 1E**).

2.3 Expression and purification of recombinant proteins from E. coli.

The pET24a+ plasmid encoding the PRRSV N gene, and cellular genes STAMI, Hck, Fyn, or TXK was each transformed into the BL-21 (DE3) strain of *E.coli* (Lucigen). Single bacterial colonies were used to inoculate 5 ml starter cultures, which were scaled up to 150 ml cultures 5 hours post-inoculation. Protein expression was achieved via autoinduction (Studier, 2005). Approximately 20 hours post-inoculation of the 150 ml culture or once bacterial growth reached the lag phase, cultures were aliquotted into 50 ml conical tubes and spun at 3,400 RPM for 20 minutes. Pellets were frozen at -80°C until processed. Bacteria were lysed using Bug Buster reagent (EMD) containing HALT protease inhibitors (Thermo). Insoluble fractions containing the protein of interest were solubilized using a protein refolding kit (EMD) and subsequently affinity-purified using nickel columns.

2.4 SH3 binding domain array.

Purified recombinant PRRSV N protein was incubated with the Panomics SH3 domain array 1 (Affymetrix, CA) according to manufacturer's instructions. The arrays contained 38 SH3 binding domain peptides spotted on a membrane in duplicate. A positive control class Ib SH3 ligand was run in parallel to validate that the assay was functional and specific. Arrays were imaged using a Chemidoc Imaging System (Biorad). Spot intensities were quantified using ImageJ software and plotted/analyzed using Graphpad Prism 5 software.

2.5 Construction of recombinant viral vectors.

Mutation of each proline residue within the PxxPxxP SH3 binding motif of the N protein in PRRSV pIR-VR2385-CA infectious cDNA clone was done via overlap extension PCR. For the N protein proline residue 50 to alanine mutation (P50A), initial PCR was performed using primers spk93 with spk232 and spk231 with

spk94 followed by a second round of PCR using the external primers spk231 and spk232. For the proline residue 53 to alanine mutation (P53A), primers spk95 with spk232 and primer spk231 with spk96 were used for initial PCR followed by a second round with spk231 and spk232, and for the proline residue 56 to alanine mutation (P56A), primer spk132 was used with spk232 and spk231 with spk133 followed by a second round of PCR using the external primers spk231 and spk232 (**Table 1**). The second round PCR products and the PRRSV pIR-VR2385-CA infectious cDNA clone plasmid were then digested using unique *HpaI* and *AclI* restriction sites and the PCR fragments were ligated into the pIR-VR2385-CA infectious clone plasmid. Mutations were confirmed via DNA sequencing.

2.6 Expression and infectivity of PRRSV cDNA clone pIR-VR2385-CA containing altered N protein with the proline to alanine mutations (P50A, P53A, and P56A).

BHK-21 cells were seeded in parallel on 6 well tissue culture dishes. Cells were transfected with 8 µg plasmid DNA per well of each of the P50A, P53A, and P56A PRRSV infectious clones using lipofectamine LTX according to the manufacturer's instructions. At 48 hours post-transfection, 1 plate of cells was fixed in cold 80% acetone for 30 minutes. Fixed cells were rinsed in phosphate buffered saline (PBS), incubated with mouse anti-PRRSV N protein (SDOW17, RTI, LLC), followed by FITC-labeled goat anti-mouse secondary antibody (KPL). The nuclei were stained with Hoechst nucleic acid stain (Sigma). The second plate of the transfected BHK-21 cells was freeze-thawed three times at -80°C at 72 hours post-transfection. The cellular debris was removed by centrifugation at 10,000 x g for 10 minutes. Equivalent volumes of cleared culture supernatant was then used to infect MARC145 cells on a 6-well dish for 2 hours at 37°C. Fresh primary growth media was added and cells were incubated in a 37°C,

5% CO₂ incubator for 48 hours. Infected cells were fixed and probed with anti-PRRSV N protein as described above. The stained cells were imaged using a Nikon-TE2000 inverted microscope equipped with a photometrics HQ-2 camera, using FITC and DAPI filter cubes.

2.7 Confocal microscopy and Forster resonance energy transfer (FRET).

MARC145 or BHK-21 cells were grown on No. 1.5 coverslips in 6 well dishes. Cells were transfected using lipofectamine LTX according to manufacturer's instructions. At 18-26 hours post-transfection, the cells were fixed in 4% paraformaldehyde and mounted in aquapolymount (Polysciences Inc). Cells were imaged using a Zeiss LSM510 confocal microscope using a plan-apo 63X objective oil immersion lens. YFP fluorophores were excited using the 514 nm channel of the krypton argon laser and CFP fluorophores were excited 458 nm using the same laser.

For Förster resonance energy transfer, cells were imaged, bleached for 11-16 iterations of 100% 514 nm laser intensity with imaging at each frame. Cells were bleached to 30% or less of initial YFP intensity. Post-acquisition fluorescence intensity was determined using Zen software (Carl Zeiss Microscopy). FRET intensity was calculated as (Donor Post corrected for non-specific bleaching-Donor Pre)/Donor Pre *100. A minimum of 16 total cells were analyzed for each construct from at least two separate transfections.

3. Results

3.1 PRRSV N proteins contain a conserved SH3 binding motif.

Alignment of the N proteins from different genotypes of PRRSV revealed that there exists a conserved PxxPxxP amino acid motif found in all known genotypes of PRRSV despite

significant sequence divergence among strains (Gao et al., 2004; Nelsen et al., 1999). Isolates of representative type 2 PRRSV strains VR2385 and MN184, representative type 1 strains Lena and Lelystad, and the Chinese pig high fever disease PRRSV isolate JXA1 all contain the conserved PxxPxxP motif (**Fig. 1B**). Of the 940 N protein sequences available in the GenBank database, all have at least one intact PxxP motif and the P53 residue is completely conserved. Of the 940 sequences studied, 23 virus strains had a mutation within the P50 location with leucine being the most common substitution (11 strains), followed by serine (9 strains), then arginine, glutamine, and phenylalanine each having a single strain containing that substitution (Fig. 1C). Only two of 940 strains analyzed have an amino acid other than proline at position 56, and the MN184B (Genbank: AAC23804) and a British genotype 1 strain (Genbank AFH96137) contained a leucine and a lysine in place of proline at position 56, respectively (Fig 1C). When aligning the PxxPxxP motif region with N proteins from other members within the *Arteriviridae* family, we found that proline 50 is conserved in lactate dehydrogenase-elevating virus (LDHV) and equine arteritis virus (EAV) but not in simian hemorrhagic fever virus (SHFV). Proline 53 is not found in any of the other species and P56 is only found in LDHV, suggesting that the PxxPxxP motif may be a specific adaptation to the porcine host or host cell type not encountered by the other species.

3.2 Bacterially-expressed recombinant N protein of PRRSV interacts with SH3 domains bound to nitrocellulose.

E. coli-derived, purified recombinant N protein was capable of interacting with five of the 38 tested SH3 domains using the Panomics SH3 domain array 1. Positive results were seen as noticeably darker spots on the array due to increased amounts of the recombinant His-tagged

PRRSV N protein binding to SH3 domain-containing proteins on the array subsequently binding more anti-His HRP-conjugated antibody (**Fig. 2A**). We quantified the binding intensities using densitometric analysis of each spot from two separate arrays, four total spots (**Fig. 2A**). Cellular genes TXK (785.5, $P = 0.0088$), STAM (818.0, $P = 0.0101$), SLK (Fyn) (783.0, $P = 0.0260$), Hck (789.0, $P = 0.0170$), and Cortactin (800.5, $P = 0.0070$) all produced interaction spots with significantly greater intensities than that of the N protein incubated with the negative control glutathione S transferase (GST) (758.5) (**Fig. 2A**).

3.3 PRRSV N protein and SH3 domain-containing proteins can be expressed as fluorescently coupled proteins in eukaryotic cells.

STAM1, Hck, Fyn, TXK, and Cortactin genes were synthesized based on the gene sequences available in GenBank and each cloned into expression constructs linking the full-length SH3 domain-containing proteins carboxyl- or amino-terminally to GFP, CFP or YFP (**Fig. 1E**). Expression of fluorescently tagged Fyn and TXK in MARC145 or BHK-21 cells resulted in fluorescence that was both diffusely cytoplasmic and nuclear. STAM1, Hck, and cortactin were predominantly diffuse within the cytoplasm while excluding the nucleus (**Fig. S1A**). STAM1 and TXK occasionally produced tiny punctate spots within the cytoplasm and cortactin occasionally produced one large perinuclear spot in some subsets of cells (**Fig. S1A**). Fluorescently tagged N protein produced several phenotypes including cytoplasmic and nuclear diffuse, nucleolar, and perinuclear punctate localization (**Fig. S1A**). The sizes of the expressed proteins in Western blotting from cellular lysates with anti-GFP antibody were 27.9kDa (YFP), 43.0kDa (NYFP), 63.9kDa (TXKYFP), 88kDa (FynYFP), 88.3kDa (STAMIYFP), 112.7kDa (cortactinYFP), and

97.8kDa (YFP^{Hck}). These molecular weights matched what was predicted for each protein attached to the YFP protein (**Fig. S1B**).

3.4 In situ confirmation and specificity of SH3 binding motif interaction using fluorescence resonance energy transfer (FRET) show that P50, P53, and P56 residues are involved to varying degrees with SH3 protein interactions.

FRET is a biophysical non-radioactive transfer of energy between fluorescent molecules with overlapping excitation and emission spectra that are brought within a distance of 1-10 nm of each other consistent with a biologically relevant physical interaction (Jares-Erijman and Jovin, 2006; Kenney et al., 2008; Sekar and Periasamy, 2003; Siegel et al., 2000). In general, FRET is superior to immunoprecipitation in that there is no possibility of non-specific bead or antibody binding and the interaction signals can be visualized within the cell itself. Free CFP and free YFP expressed within the same cell yielded a background FRET efficiency of 3.95% (**Fig. 3A**). The TXKYFP and FynYFP expressed with NCFP yielded FRET efficiencies of 7.24% and 9.78%, respectively, both significantly higher than CFP/YFP alone ($P = 0.003$ and 0.034 respectively). The STAM1YFP and cortactinYFP expressed with NCFP produced a FRET efficiency higher than CFP/YFP alone (5.09% and 7.55%), however it was not significantly different ($P = 0.414$ and $P = 0.08$) than CFP/YFP (3.95%). The HckYFP with PRRSVNCFP yielded a FRET efficiency of 2.81%, which was below the CFP/YFP background level of 3.95% (**Fig 3A**). Altering the terminus in which YFP was attached to the STAM1 or Hck proteins (C-terminus to N-terminus) resulted in a FRET efficiency of 9.78% and 13.50%, significantly higher than CFP/YFP ($P = .011$ and $P = 0.0019$) (**Fig. 3B**). We were unable to tag cortactin with YFP at its N-terminus and maintain observable protein expression (**Data not shown**).

Co-expressing YFPSTAMI with PRRSV N protein containing either P50A and P53A attached to CFP lead to a reduction of their FRET efficiencies to 4.15% and 5.13%, which is significantly reduced from YFPSTAMI expressed with wild-type PRRSV N protein CFP (9.93%, $P = 0.023$ and 0.048). Expressing YFPSTAMI with PRRSV N P56ACFP led to a reduction in FRET efficiency to 6.72% but was not significantly reduced from YFPSTAMI expressed with wild-type N protein CFP (9.93%, $P = 0.244$). Expressing TXKYFP with PRRSV N protein containing either P50A, P53A, or P56A attached to CFP reduced the observed FRET efficiency to 3.96%, 1.20%, and 1.51%, each no longer significantly higher than CFP/YFP FRET efficiencies (**Fig. 3D**). Expressing FynYFP with PRRSV N protein containing either P50A, P53A, or P56A attached to CFP reduced the observed FRET efficiency to 2.94%, 4.16%, and 1.03%, respectively, each not significantly higher than the FRET efficiency for CFP/YFP alone (3.95%) (**Fig. 3E**). Finally, expressing YFPHck with PRRSV N protein containing either P50A, P53A, or P56A attached to CFP reduced the observed FRET efficiency to 8.59%, 6.67%, and 5.21%. Each interaction was higher than CFP/YFP alone, however P53A and P56A yielded significantly less FRET than YFPHck with PRRSVNCFP ($P = 0.0242$ and 0.0052). YFPHck expressed with PRRSV N P50A CFP had a reduced FRET efficiency (8.59%) compared to YFPHck expressed with wild-type PRRSV N protein (13.5%) but this reduction was not statistically significant ($P = 0.089$) suggesting that there could be some residual binding of Hck with the PRRSV N protein containing a P50A mutation.

3.5 Viruses containing proline to alanine mutations at PRRSV N protein amino acids 50 and 53 but not 56 are infectious.

The wild-type PRRSV VR2385 infectious clone was used to assess whether mutant viruses containing proline to alanine mutations within the N protein at amino acids 50, 53, and 56 would retain the ability to infect cells. DNA-launched infectious clone plasmids containing each virus including wild-type VR2385, VR2385 N P50A, VR2385 N P53A, and VR2385 N P56A were each transfected into BHK21 cells. Supernatant from freeze-thawed cell lysates were then transferred to MARC145 cells overlaid with agarose (plaque assay). The VR2385 N P50A and VR2385 N P53A each produced plaques that were similar in number and size to the VR2385 wild-type virus, although the VR2385 N P56A failed to produce plaques (**Data not shown**). Wild-type virus produced titers of 6.6×10^5 tissue culture infectious doses 50 (TCID₅₀) per ml, P50A produced 4.91×10^5 TCID₅₀/ml, and P53A produced 3.16×10^5 TCID₅₀/ml at 48 hours post-transfection of a T25 flask, which was not significantly different from one another suggesting no major replication defects between these strains in cell culture whereas no detectable titers were obtained for P56A (**Data not shown**).

To further confirm whether VR2385 N P56A was indeed not infectious or simply failed to produce cytopathic effect, we again transfected BHK-21 cells with the DNA-launched infectious clones containing wild-type, P50A, P53A, or P56A N protein mutations. We performed an IFA on those transfected cells to show viral protein production (**Fig. 4, left panels**) and subsequently transferred the supernatant onto MARC145 cells to assess infectivity via IFA 48 hours post-infection. The wild-type VR2385, VR2385 N P50A, VR2385 N P53A, and VR2385 N P56A all produced an equivalent positive signal within transfected BHK-21 cells. Within infected MARC145 cells, the wild-type VR2385, VR2385 N P50A, and VR2385 N P53A produced cells positive for N protein, whereas VR2385 N P56A failed to produce a positive signal in the MARC145 cells confirming this virus is not infectious (**Fig. 4, P56A right panels**).

4. Discussion

Host immune modulation during the course of PRRSV infection is a dynamic process requiring virus and host protein interactions to create a favorable host environment in which the immune response is prevented from clearing the virus. The N protein of PRRSV, being the most abundant protein synthesized during the course of PRRSV infection, likely plays important roles in modifying the host cellular environment. In this study, we have identified a conserved SH3 binding motif within the N protein of PRRSV and also identified five cellular proteins that interact with this SH3 binding motif.

We have demonstrated that the PRRSV N protein interacts *ex vivo* with the host cellular proteins STAMI, TXK, Fyn, Hck, and cortactin. We further confirmed the interactions of N protein with STAMI, TXK, Fyn, and Hck *in situ*. The results showed that, in the case of STAMI and Hck interactions with the PRRSV N protein, binding appears to require an unhindered C-terminus as FRET efficiencies above background were not observed when YFP was attached to the C-terminus of these proteins but was significantly increased when the fluorescent tag was relocated to the N-terminus. Reductions in binding of the SH3 proteins with the PRRSV N protein were also dependent on at least one intact PxxP motif as disruption of any of the prolines within the motif significantly reduced interaction with Fyn and TXK. The STAMI appeared to be more tolerant of the P56A mutation suggesting that the first PxxP motif may be more important for STAMI N protein interactions. The Hck was more tolerant of the P50A mutation suggesting that the second PxxP motif may be more important for this interaction.

Amino acid residue P56 of the PRRSV N protein appears to be critical for the virus lifecycle potentially serving a conformational role within the N protein as mutation of this

proline residue to alanine results in no detectable infectious particles. It would be interesting to explore whether strains AAC23804 and AFH96137 (Fig. 1C) have some types of compensatory mutation or were simply amplified as a dead-end quasispecies as they lack the proline at position 56. The conservation of residues P50 and P53 do not appear to coincide directly with viral infectivity as mutations to alanine still result in infectious virus and there are at least 23 examples of naturally-occurring PRRSV strains altering P50 but none altering P53 or losing both PxxP motifs suggesting that these highly conserved proline residues within the sequence may be involved in virus-host interaction geared to a selective growth advantage rather than a vital functional element within the virus. There are a number of documented functions that each interacting SH3 protein plays in the host which could be altered during the course of PRRSV infection.

Each of the proteins that we have shown to interact with the PRRSV N protein play multiple roles in host cellular signaling and immunomodulation. Fyn and Hck are within the Src family of tyrosine protein kinases. Fyn is a tyrosine-specific phospho-transferase which phosphorylates proteins involved in a myriad of signaling pathways including: T and B cell receptor signaling (Brdicka et al., 2000; Manie et al., 1997; Palacios and Weiss, 2004; Qian et al., 1997; Zamoyska et al., 2003), differentiation of natural killer cells, oligodendrocytes (Klein et al., 2002) and keratinocytes, integrin-mediated signaling, growth factor and cytokine receptor signaling, platelet activation, cell adhesion, ion channel function (Hisatsune et al., 2004; Hou et al., 2002), among others. Hck is primarily found in cells of hemopoietic origin particularly of myeloid or B-lymphoid lineage. Hck is thought to help couple the Fc receptor to the activation of the respiratory burst along with roles in neutrophil migration and degranulation. Signal transducing adaptor molecule (STAM) interacts with Jak2 and Jak3 kinases which phosphorylate

STAM upon cytokine stimulation, suggesting that STAM is an adaptor involved in the downstream signaling of cytokine receptors (Asao et al., 1997; Takeshita et al., 1996; Takeshita et al., 1997; Tanaka et al., 1999). Cortactin recruits Arp2/3 complexes to F-actin acting as a nucleation site for more F-actin to be added (Daly, 2004). Cortactin is important in lamellipodia formation such as when macrophages are chemotaxically migrating towards a target (Weed and Parsons, 2001). Additionally cortactin has been connected to the formation of clathrin-mediated (Samaj et al., 2004) and clathrin-independent endocytosis (Sauvonnet et al., 2005). Tyrosine-protein kinase (TXK) plays a redundant role with IL2-inducible T-cell kinase (ITK), modulating the adaptive immune response (Haire et al., 1994; Readinger et al., 2009). TXK affects development, differentiation, and function of conventional T cells and nonconventional NKT cells. TXK acts as a Th1 cell-specific transcription factor, regulating the transcription of interferon gamma (Kashiwakura et al., 1999; Maruyama et al., 2007; Takeba et al., 2002). Therefore, whether PRRSV N protein interaction with Fyn, Hck, STAM, cortactin, or TXK is functioning through one or more of these known pathways to modulate PRRSV infection or through another pathway yet to be discovered remains to be seen.

The highly conserved nature of the PxxPxxP SH3 binding motif within the N protein of PRRSV and its interaction with the host cellular factors STAM, Hck, TXK, and Fyn warrant further study, beyond the scope of this present study, into the downstream effects these signaling pathways are playing in PRRSV infection. The results from this study lay the foundation for the PRRSV scientific community to further investigate the role of virus-host interactions in PRRSV pathogenesis.

Acknowledgements

389 This work was supported by a grant from the U.S. Department of Agriculture National Institute
390 of Food and Agriculture (USDA NIFA-2011-67012-30719). We thank Barbara Dryman, Lynn
391 Heffron, and Pablo Piñeyro for their technical assistance.

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Figure Legends

Figure 1. Alignment of PxxP motif in representative PRRSV strains and schematic of

expression constructs used in this study. (A). Representation of the full-length genome of

PRRSV with each protein coding region denoted. The focus of this study, the N protein, is

expanded. The amino acid residues 48-59 of the N protein is highlighted to show the PxxPxxP

motif. The mutations that were introduced are denoted. (B). Protein alignment of different

representative strains of PRRSV shows that the PxxPxxP motif is highly conserved in PRRSV N

protein. (C). Twelve partial N protein sequence alignments from the 23 out of 940 sequences

available in GenBank database that were lacking one of the prolines within the PxxPxxP motif

aligned to the PRRSV VR2385 strain. Conserved prolines are in bold and changes within the

conserved proline are underlined. (D). Sequence alignment of N proteins from selected members

within the *Arteriviridae* family including PRRSV VR2385, lactate dehydrogenase-elevating

virus (LDHV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV).

Genbank accession numbers: JX044140.1, NC_001639.1, AY349167.1, and NC_003092.2,

respectively. Prolines within the conserved PxxPxxP motif are in bold, absolutely conserved

residues are boxed, and a putative PxxP motif within EAV is underlined. (E). Recombinant

proteins are depicted as either N- or C- terminal fusion to fluorescent proteins or His and FLAG

epitopes depending on the experiment in which they were used.

Figure 2. Detection of protein-protein interactions utilizing Panomics SH3 domain array.

Densitometry quantification of spot intensity from two independent domain arrays incubated

with recombinant PRRSV N protein.

Figure 3. Detection of protein-protein interactions *in situ* using acceptor photobleaching Förster resonance energy transfer (FRET). (A). FRET efficiency for PRRSV N protein tagged with CFP transiently expressed in the same cell as SH3-containing proteins tagged with YFP at their C-terminus. (B). Comparison of N- versus C-terminally YFP tagged STAMI and Hck proteins and their interaction with PRRSV N protein CFP. (C) Comparison of PRRSV N protein PxxPxxP mutations on the FRET efficiencies of YFPSTAMI protein. (D). Comparison of PRRSV N protein PxxPxxP mutations on the FRET efficiencies of TXK YFP protein. (E). Comparison of PRRSV N protein PxxPxxP mutations on the FRET efficiencies of Fyn YFP protein. (F). Comparison of PRRSV N protein PxxPxxP mutations on the FRET efficiencies of YFP Hck protein. Asterisks (*) indicate values are significantly higher ($P < 0.05$) than CFP/YFP FRET values. Octothorpes (#) indicate FRET values are significantly reduced ($P < 0.05$) from YFP STAMI + wild-type PRRSV N CFP Fret values. Alpha symbols (α) indicate FRET values are significantly reduced ($P < 0.05$) from TXK YFP + wild-type PRRSV N CFP FRET values. Beta symbols (β) indicate FRET values are significantly reduced ($P < 0.05$) from Fyn YFP + wild-type PRRSV N CFP Fret values. Gamma Symbols (γ) indicate FRET values are significantly reduced ($P < 0.05$) from YFP Hck + wild-type PRRSV N CFP Fret values.

Figure 4. Infectivity of PRRSV VR2385 mutant viruses containing PxxPxxP mutations in the N protein *in vitro*. Left two panels are immunostaining of BHK-21 cells transiently transfected with a plasmid containing the DNA-launched PRRSV VR 2385 infectious clone (wild-type or indicated proline to alanine N protein mutation) with antibody against the PRRSV N protein. Right two panels depict MARC145 cells that were infected with the cell lysates from BHK-21 cells transiently transfected with respective constructs. Cells were fixed 3 days post-

440 infection and immunostained for PRRSV N protein using anti-PRRSV N SDOW17 monoclonal
441 antibody followed by anti-mouse FITC and stained with Hoescht nucleic acid stain to confirm
442 the presence of cells in fields with no FITC staining.

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Table 1		
Oligonucleotides used in this study.		
Name	Sequence (5'—3')	Function

Spk85	CATCAT <u>AAGCTT</u> ATATGCCAAATAACAACGGCAAG	ORF7 into pET24a+
Spk86	ATGATG <u>CTCGAGG</u> CATGCTGAGGGTGATGC	ORF7 into pET24a+
Spk102	CATCAT <u>CTCGAG</u> ATGCCAAATAACAACGGCAAG	ORF7 into GFP/CFP/YFP
Spk103	ATGATGA <u>AAGCTT</u> GCATGCTGAGGGTGATGC	ORF7 into GFP/CFP/YFP
Spk365	CATCAT <u>CTCGAG</u> ATGCCTCTTTTGGCCACC	STAMI into GFP/CFP/YFP
Spk366	CATCAT <u>AAGCTT</u> CTTTAGCAGAGCCTTCTGAGAATATG	STAMI into GFP/CFP/YFP
Spk231	GGAATTTACCC <u>AACGTT</u> CCTTGG	OE PCR External
Spk232	GCAATAAACAA <u>AGTTAA</u> CAACAACAATTGC	OE PCR External
Spk93	GAAGAAAAAC g CGGAGAAGCCC	P50A Mutagenic
Spk94	GGGCTTCTCC Gc GTTTTCTTC	P50A Mutagenic
Spk95	CGGAGAAG g CCCATTTTCCTC	P53A Mutagenic
Spk96	GAGGAAAATGGG c CTTCTCCG	P53A Mutagenic
Spk132	CCCATTTT g CTCTAGCGACTG	P56A Mutagenic
Spk133	CAGTCGCTAGAG c AAAATGGGG	P56A Mutagenic
Restriction sites for insertion into vectors are underlined. Mutagenic primer sequences that do not match the wild type sequence are lowercase and bold.		

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B

VR2385	44	KNKKKNFENHFFELATEDDVRHHFTP	60
Mn184	44	KSNKKSFENHFFELATEDDVRHHFTP	60
JXA1	44	KNKKKNFENHFFELATEDDVRHHFTP	60
Iena	48	QAKKKKFENHFFELAAEDDVRHHLTG	60
Lehystad	45	QAKKKKFENHFFELAAEDDIRHHLTQ	70
Consensus		--KKK--FENHFFELA--EDD--RHH--T--	
SH3 Binding		KKKKKKKKKKKKKKKKKKKKKKKKKKKK	

C

VR2385	KNKKKNPEKPHFPLATEDDDVRHHFTP
ABU43167	KSETKSLEKPHFPLATEDDDVRHHFTP
ABL98021	KNKKKNQEKPHFPLATEDDDVRHHFTP
ACU68888	KNKKKNSEKPHFPLATEDDDVRHHFTP
ACZ72695	KFNNNLEKPHFPLATEDDDVRHHFTP
ABR23628	KNKKKSLEKPHFPLATEDDDVRHHFTP
ADG80171	KNRKNLEKPHFPLATEDDDVRHHFTP
ACZ28848	A-KKKBEKPHFPLAAEDDIRHHLTQ
ADP37108	T-KRKRFEKPHFPLAAEDDIRHHLTQ
ADM34166	TKKKKS-EKPHFPLAAEDDIRHHTQT
AGI16116	AKKKRS-EKPHFPLAAEDDIRHHLTQA
AAC23804	KNKKKNPEKPHFPLATEDDDVRHHFTP
AFH98137	AKKKK-PEKPHKLAAEDDIRHHLTQ

D

VR2385	KNKKKNPEKPHFLATEDNRHHFTP
LDHV	QKKKKQF-KLHFFMAGPSDLRVMTF
EAV	--PRAQF--TQATIREPGDLREHLNQ
SHFV	--VKKPLNETHYVIREPGDLRVVLPG

Figure(s)

Figure(s)

Figure(s)

Transfected BHK-21

Infected Marc-145