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Role of All of the PRRSV Glycoproteins in Protective Immune Response

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RESEARCH REPORT
SWINE HEALTH

Title: Role of All of the PRRSV Glycoproteins in Protective Immune Response - **NPB # 08-253**

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Industry Summary:

Porcine reproductive and respiratory syndrome (PRRS) caused by PRRS virus (PRRSV) is of major economic significance to swine industry in the U.S.A. and worldwide. Currently, there is no effective vaccine available to combat PRRS. In previous studies, we have demonstrated that virus-neutralizing antibodies are important for protective immunity against PRRSV. These neutralizing antibodies constitute a significant correlate for evaluating the efficacy of a vaccine. Although four viral glycoproteins are present in PRRSV, their role in assembly of infectious virions, interactions with the cellular receptor, virus entry and uncoating, and immunological response *in vivo* remain poorly understood. Development of safe and efficacious vaccines to combat PRRSV infections requires a basic understanding of the role of these glycoproteins in virus biology. One of the objectives of the proposal is to investigate how the four glycoproteins interact with each other to form multiprotein complex and which of these proteins interact with the cellular receptor, CD163. Knowledge of these interactions will be important in developing strategies to inhibit the process of binding of the virus to the cells, so that virus infections could be blocked. Studies conducted by us and others have unambiguously

demonstrated that the glycoprotein GP5 is a major inducer of protective neutralizing antibodies. By genetic manipulation of PRRSV genome, we had previously demonstrated that elimination (through a process called "hypoglycosylation") of selected sugar moieties present on the surface of GP5 dramatically enhances the ability of a PRRSV strain to invoke a more robust response composed by PRRSV-neutralizing antibodies. As evidence indicates that other glycoproteins that make up the PRRSV may also be involved in the PRRSV-neutralizing response, another objective of the proposal is to generate PRRSVs containing hypoglycosylated forms of all the remaining glycoproteins (GP2, GP3, and GP4) and assess the effects of these changes on the ensuing PRRSV-neutralizing antibody response.

To carry out the studies in the proposed objectives, we examined interactions of PRRSV glycoproteins amongst each other and as well as with the receptor CD163. Our results show that strong interaction exists between GP4 and GP5 proteins, although weak interactions among the other minor envelope glycoproteins and GP5 have been detected. Both GP2a and GP4 proteins were found to interact with all the other GPs resulting in the formation of multiprotein complex. Our results further show that GP2a and GP4 proteins also specifically interact with the CD163 molecule. The carboxy-terminal 223 residues of CD163 molecule are not required for interactions with either the GP2a or the GP4 protein, although these residues are required for conferring susceptibility to PRRSV infection in BHK-21 cells. Overall, we conclude that the GP4 protein is critical for mediating interglycoprotein interactions and along with GP2a, serves as the viral attachment protein that is responsible for mediating interactions with CD163 for virus entry into susceptible host cell. Furthermore, mutations in the glycosylation sites of GP2, GP3, and GP4 proteins were introduced individually and mutant PRRSVs were generated from infectious clones containing these mutant glycoproteins. When inoculated into pigs and antibody response in the infected pigs were analyzed, we observed that there was a general down-regulation of neutralizing antibody response in pigs infected with the glycosylation mutant viruses. These results are contrary to our expectation of obtaining higher levels of neutralizing antibody response in these infected pigs. Overall, our results suggest that hypoglycosylation of the minor glycoproteins of PRRSV does not enhance neutralizing antibody response in pigs.

Since we have demonstrated interactions of GP2a and GP4 with the PRRSV receptor CD163, we are currently examining which regions of these glycoproteins interact with CD163. This will be important in obtaining specific antibodies or peptide mimetics against these regions of GP2a and GP4 that could potentially block PRRSV infections. Our studies supported by the NPB grant (#08-253) have been recently published in Journal of Virology (a copy of the paper is forwarded to B. L. Everitt).

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Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) contains the major glycoprotein, GP5, as well as three other minor glycoproteins, namely, GP2a, GP3, and GP4, on the virion envelope, all of which are required for generation of infectious virions. To study their interactions amongst each other and with the cellular receptor for PRRSV, we have cloned each of the viral glycoproteins and CD163 receptor in expression vectors and examined their expression and interaction with each other in transfected cells by co-immunoprecipitation (co-IP) assay using monospecific antibodies. Our results show that strong interaction exists between GP4 and GP5 proteins, although weak interactions among the other minor envelope glycoproteins and GP5 have been detected. Both GP2a and GP4 proteins were found to interact with all the other GPs resulting in the formation of multiprotein complex. Our results further show that GP2a and GP4 proteins also specifically interact with the CD163 molecule. The carboxy-terminal 223 residues of CD163 molecule are not required for interactions with either the GP2a or the GP4 protein, although these residues are required for conferring susceptibility to PRRSV infection in BHK-21 cells. Overall, we conclude that the GP4 protein is critical for mediating interglycoprotein interactions and along with GP2a, serves as the viral attachment protein that is responsible for mediating interactions with CD163 for virus entry into susceptible host cell. Additionally, using a series of glycosylation-site mutants of GPs, we have examined the ability of the hypoglycosylated forms of the protein to generate infectious PRRSV. Our results show that mutations at certain sites in various GPs are critical for production of infectious virus. Using several mutant PRRSVs with

hypoglycosylated minor GPs on the envelope, we have found that these viruses do not induce higher titers of neutralizing antibody response, contrary to our previous observations with the major glycoprotein, GP5.

Introduction:

The need for a new generation of PRRSV differential vaccine is well exemplified by the importance given by the pork industry to this topic. Since the launching of the PRRSV initiative in 2003, development of an efficacious and differential vaccine has been high on the list of research priorities for NPB research grant program. In addition, a joint review of PRRSV Initiative components (NPB and USDA-CAP programs) held in 2006 suggested that development of a new generation of PRRSV with higher safely and efficacy is very important. The research proposed in this project explores a way to significantly enhance the immunogenic efficacy of a potentially new generation of vaccines. The main finding that originates this proposal is our recently provided evidence that the poor, meager and sluggish neutralizing immune response invoked by PRRSV in vivo is, in a great part, due to the phenomenon of "glycan shielding" caused by the sugars that surround the antigenic sites of the surface glycoprotein GP5 of PRRSV. This shielding by the sugar moieties would preclude the host's antibodies to reach and neutralize the inmunogenic epitopes, which like the epitope B described on the GP5 of PRRSV, interact with the viral receptor on the host cell. The phenomenon of glycan shielding has been well described for HIV and SIV, and is possible that it occurs with other viruses such as influenza, hepatitis B virus and the arterivirus LDV. In the particular case of PRRSV, we verified that mutants of wt PRRSV that have been deprived of one or two sugar moieties on GP5 exhibited dramatically enhanced antigenicity, expressed as a sensibly enhanced capacity of being neutralized by regular sera from wt PRRSV-convalescent pigs. This enhancement of "neutralizability" is evidenced by the significantly increased end-point titer of these sera against the mutants, as compared with the regular end-point that is reached by the sera against wt PRRSV. This observation clearly suggests that the removal of one, and particularly two, of the sugar residues on GP5 increases the accessibility of the neutralizing epitope to specific antibodies. Most importantly, these results appear to indicate the presence of a significant amount of PRRSV-neutralizing antibodies in the wt PRRSV-infected convalescent sera that would otherwise be undetectable because of the typical use of wt

PRRSV containing fully glycosylated ("shielded") GP5 in SN assays. It is conceivable that these highly abundant, yet unnoticed antibodies in infected animals do not really contribute to protection because the wt PRRSV is fully glycosylated (thus shielded from neutralization). Certainly our most significant piece of new information for the future of PRRSV vaccinology and for this proposal, centers on the fact that the sugar-deprived mutants of PRRSV exhibited enhanced immunogenicity upon inoculation of pigs, to the point that the mutants can outperform the wt PRRSV in their ability to mount a sizable wt PRRSV-neutralizing response at 48 days PI. The mutants also developed an early and more robust homologous neutralizing antibody response than that induced by wt PRRSV, to the point where, in the case of the mutants, the characteristically sluggish and meager nature of PRRSV-neutralizing antibody response appears to have been corrected.

In this proposal, we continued these studies by assessing whether this effect of enhanced neutralizing antibody response against wt PRRSV can be further enhanced if deglycosylation is additionally conducted on the other glycoproteins of PRRSV. While it is known that GP5 plays a prominent role in neutralizing antibody induction, it has been suggested that other PRRSV glycoproteins, such as GP4 (which is known to be the target of a PRRV-neutralizing monoclonal antibody) may also have a role. On the other hand, nothing is known about the possible role of PRRSV GP2 and GP3 in neutralization of PRRSV. One of the objectives of the proposal is to examine if neutralizing antibody response can be enhances by hypoglycosylation of the minor glycoproteins (GP2a, GP3, and GP4) of PRRSV. In addition, another objective of the proposal is to examine interactions of various glycoproteins of PRRSV among themselves and also with the receptor, CD163. Knowledge about these interactions will aid in the design of principles to block and/or prevent PRRSV infections.

Stated Objectives from Original Proposal:

No.	Objective
1.	Delineate glycoprotein (GP)-GP interactions and identify which GPs interact with the PRRSV receptor, CD163
2.	Recover and characterize PRRSVs encoding hypoglycosylated forms of the glycoproteins and examine their ability to induce neutralizing antibody response to PRRSV in infected pigs.

Materials and Methods:

(i) Generation of mutant glycoproteins and examination of their expression in vitro

An intermediate pBR322-based plasmid containing a DNA fragment of ~4.9 kbp encompassing ORF2-7, and the entire 3'UTR of PRRSV from the PRRSV infectious cDNA clone (FL12) (EcoRV to BstZ17I fragment) was generated. Individual GP2, GP3, and GP4 coding sequences were also cloned in pGEM vector by PCR amplification of the corresponding sequences with gene-specific primers under the control of T7 RNA polymerase promoter so that expression of these proteins in transfected cells can be studied. The intermediate plasmid served as the template for mutagenesis to introduce mutations at the glycosylation sites in the glycoproteins. The following mutants were generated by PCR mutagenesis: GP2 (single site mutants: N178A, N184A, and double-site mutant: N178/184A, GP3 (single site mutants: N29A, N42A, N50A, N131A, N152A, N160A, and N195A and several double site mutants: N29-152A, N29-160A, and one triple site mutant N29-152-160A), and GP4 (single site mutants: N37A, N84A, N120A, and N130A and all possible double site mutants). In each of these mutants, the asparagine residue (N) of the glycosylation site at a particular position (identified by a number) in the protein backbone was altered to alanine (A). All mutant clones were sequenced to confirm the presence of the desired mutation and to make sure that unwanted changes were not introduced into the coding regions during mutagenesis. The mutant coding sequences were further subcloned into pGEM vector for protein expression studies. For protein expression studies, BHK-21 cells infected with vTF7-3 (which expresses T7 RNA polymerase in infected cells) were transfected with plasmids encoding the wild-type or mutant proteins under the control of T7 RNA polymerase promoter in pGEM vector. Transfected cells were radiolabeled with ^{35}S-amino acids and the viral proteins were immunoprecipitated from cell lysates with specific antibodies to GP2, GP3, and GP4. These monospecific rabbit antibodies were generated using peptides specific to these glycoproteins and they specifically immunoprecipitate the corresponding proteins from the transfected cells. The expressed proteins were detected by SDS-polyacrylamide gel electrophoresis and fluorography.

(ii) Construction of full-length cDNA clones encoding the mutant glycoproteins

The mutant coding sequences from the intermediate plasmid vector were then moved to the full-length infectious clone FL12 using the restriction enzyme sites EcoRV and BstZ17I. Again, the identity of the mutants

was confirmed by sequencing of the clones. Full-length cDNA clones of all of the single- and double-site glycosylation mutants of GP2a, GP3, and GP4 have been generated and used for recovery of infectious PRRSV.

(iii) Rescue of mutant viruses and examination of their in vitro growth properties

The full-length plasmids encoding various glycoprotein mutants were digested with AclI and the linearized DNAs were used as the template to generate capped RNA transcripts using the mMESSAGE mMACHINE Ultra T7 kit as per manufacturer's (Ambion, Austin, TX) recommendations and as described previously from our laboratory. The integrity of the in vitro transcripts was examined by glyoxal agarose gel electrophoresis followed by ethidium bromide staining.

Subsequently, MARC-145 cells were electroporated with approximately 5.0 μg of in vitro transcripts along with 5.0 μg of total RNA isolated from MARC-145 cells as described previously. About $2x10^6$ cells in 400 μl of DMEM containing 1.25% DMSO were pulsed once using Bio-Rad Gene Pulser Xcell at 250V, 950μF in a 4.0 mm cuvette. The cells, diluted in normal growth media, were plated in a 60-mm cell culture plate. A small portion of the electroporated cells were plated in a 24-well plate to examine expression of N protein at 48 hrs post-electroporation, which would indicate genome replication and transcription. Once expression of N protein was confirmed using indirect immunofluorescence assay (IFA), the supernatant from bulk of the electroporated cells in 60-mm plates were collected at 48 hrs post-electroporation, clarified and passed onto naïve MARC-145 cells. The infected cells were observed for cytopathic effect (CPE) along with the expression of N protein using IFA. The supernatants from infected cells showing both CPE and positive fluorescence were considered to contain infectious virus. After confirmation, high titer virus stocks were prepared in MARC-145 cells, titrated, and frozen at -80°C in small aliquots for further studies. In all the experiments, FL12 containing wt PRRSV genome and FL12pol$^-$ containing polymerase-defective PRRSV genome were used as controls.

Wt and mutant virus growth kinetics in MARC-145 cells were determined as described previously from our laboratory by plaque assay.

(iv) Animal experiments

Twenty-one-day old, recently weaned pigs were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. Four pigs per group were infected with either FL12 wt PRRSV or mutant viruses. In all cases, the inoculum consisted of 10^5 $TCID_{50}$ diluted in 2 ml and were administered intramuscularly in the neck. The rectal temperatures of the inoculated animals were monitored for 15 days post-inoculation (PI). Viremia was measured by regular isolation on MARC-145 cells at days 4, 7, and 14 post-inoculation. Serum samples were drawn weekly for a total period of 46 days post-inoculation. The serum samples were used to detect homologous neutralization titers for each of the mutants and wt PRRSV.

The titer of PRRSV-neutralizing antibodies in a serum sample was determined using the fluorescence focus neutralization assay. Serial dilutions of test sera were incubated for 60 min at 37ºC in the presence of 200 $TCID_{50}$ of the challenge virus, which consisted of either FL12 (wt PRRSV) or any of the mutant glycoprotein-containing viruses in Dulbecco's modified Eagle's medium containing 5% FBS. The mixtures were added to 96-well microtitration plates containing confluent MARC-145 cells which had been seeded 48 hrs earlier. After incubation for 24 hrs at 37ºC in a humidified atmosphere containing 5% CO_2, the cells were fixed for 10 min with a solution of 50% methanol and 50% acetone. After extensive washing with PBS, the expression of N protein of PRRSV was detected with monoclonal antibody SDOW17 using a 1:500 dilution, followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma) at a 1:100 dilution. Neutralization titers were expressed as the reciprocal of the highest dilution that inhibited 90% of the foci present in the control wells.

Results

Objective 1:

(i) GP5 protein interacts strongly with GP4 protein of PRRSV.

To examine if the envelope glycoproteins interact with each other, we co-transfected BHK-21 cells with two plasmids at a time encoding GP2a-Fl, GP3, GP4, or GP5. Approximately 16 hours after transfection, the cells were radiolabeled and the cell extracts were examined for protein interactions by co-IP assays using monospecific antibody against one of the proteins. Results show that the GP2a polyclonal antibody was able to co-immunoprecipitate GP3 or GP4 protein when expressed together indicating that GP2a interacts with both of

these proteins. No detectable level of interaction was observed between GP2a and GP5 under similar conditions in repeat experiments. Similarly, by the use of anti-GP3 polyclonal antibody, GP2a and GP4 proteins could be co-immunoprecipitated with GP3 in co-transfected cells, suggesting that GP3 interacts with GP2a and GP4. Interaction between GP3 and GP5 was not observed under these conditions. By the use of monospecific anti-GP5 antibody, GP4 protein could be efficiently immunoprecipitated from cells co-transfected with plasmids encoding the GP4 and GP5 proteins, indicating strong interaction between these two proteins. GP2a protein could be consistently detected at low levels when co-expressed with GP5 and immunoprecipitated with anti-GP5 antibody. However, undetectable to very low levels of GP3 protein could be seen in some but not all experiments when GP3 is co-expressed with GP5 and immunoprecipitated with anti-GP5 antibody. Similar results were also obtained using the anti-GP4 polyclonal antibody. Overall, the results from these co-IP studies suggest that GP2a protein interacts with GP3, GP4 and GP5 proteins, GP3 interacts with GP2a and GP4 but not with GP5, GP4 also interacts with all the three glycoproteins, whereas GP5 interacts with GP4 and GP2a proteins. Although the extent of interaction between the GPs is difficult to estimate from the co-IP studies, it is clear that the interaction of GP4 with GP5 appears to be much stronger than the interactions between the other GPs.

(ii) GP4 mediates interactions resulting in detection of multiprotein complex

To further examine if multiprotein glycoprotein complexes can be detected by co-IP assay, we transiently expressed multiple glycoproteins of PRRSV in BHK-21 cells and attempted to pull down all the interacting glycoproteins using one monospecific antibody by co-IP. We chose to use GP3 antibody in this study, as the use of this antibody in BHK-21 cell extracts resulted in less background bands. We observed that the GP3 antibody was not able to pull down GP5 protein when both of these proteins were co-expressed, confirming the results obtained previously. Furthermore, this antibody was able to pull down small amounts of GP2a protein, but not GP5 protein when these three proteins were co-expressed, indicating that GP3 interaction with GP2a is not sufficient to pull down GP5 protein in co-IP assay. However, when GP3, GP4, and GP5 proteins were co-expressed, all the three proteins could be specifically immunoprecipitated with anti-GP3 antibody, indicating that GP4 protein was important for generation of this tripartite glycoprotein complex.

Additionally, when all the four glycoproteins were co-expressed and immunoprecipitation was carried out with GP3 antibody, all the four proteins could be detected. The results suggest that the GP4 most likely mediates the interactions among PRRSV glycoproteins to generate the multiprotein glycoprotein complex.

(iii) Cloning, expression, and functionality of porcine CD163, the cellular receptor for PRRSV

To determine which of the PRRSV envelope glycoproteins interact with CD163, we first cloned in pcDNA3.1(+) vector, the full-length cDNA of CD163 from porcine alveolar macrophage (PAM) cells by RT-PCR amplification of total RNA from the cells using CD163 specific primers. Four clones were used to examine the expression of the encoded protein by transfection, radiolabeling, and immunoprecipitation with porcine anti-CD163 antibody. We observed that three of the clones expressed proteins that could be specifically immunoprecipitated with anti-CD163 antibody and possessed electrophoretic mobility (~130 kDa) corresponding to the full-length CD163. A smaller protein product of ~100 kDa encoded in another the clone was also detected by the CD163 antibody. Sequence analysis of the clones showed that CD163 in the clone that produced the smaller protein contained a premature termination codon at aa position 893, resulting in a truncated protein of 892 aa corresponding to the observed molecular mass. This truncated protein lacks the carboxy-terminal cytoplasmic domain, the transmembrane (TM) domain as well as the ninth repeat unit of the scavenger receptor cysteine-rich (SRCR) protein domain. We have termed this truncated protein as CD163ΔTM. The CD163 cDNAs in the other clones contained full-length sequences of 1115 amino acids with greater than 99% sequence identity with the reported sequence of the porcine CD163.

To determine if the cloned CD163 confers susceptibility to PRRSV infection, we transfected the plasmids into BHK-21 cells that are non-permissive to PRRSV infection. Subsequently, the cells at about 48 hours post-transfection were infected with PRRSV. Synthesis of N protein, indicative of PRRSV entry, transcription, and replication was examined in these cells by immunofluorescent staining of the cells at 48 hours post-infection with anti-N monoclonal antibody, SDOW1. Synthesis of the N protein was readily detected in the cells transfected with clones encoding the full-length CD163 (Fig. 2B), indicating that the CD163 encoded in these clones conferred susceptibility to PRRSV infection. The N protein was not detected in cells transfected

with the clone encoding CD163ΔTM, suggesting that this truncated protein is non-functional in conferring PRRSV susceptibility to the cells. The inability of CD163ΔTM to confer PRRSV susceptibility to the cells is not due to low levels of expression of the protein since under similar transfection conditions, CD163ΔTM is detected at least at levels similar to or greater than that of the full-length protein. The results indicate that the carboxy-terminal 223 amino acids of CD163 are required for the function of the protein in conferring PRRSV susceptibility. It is possible that the CD163ΔTM may have defective cellular localization or improper folding leading to loss of its function.

Immunofluorescent microscopic examination of cells transfected with one of the full-length CD163 clone showed that CD163 protein was seen localized on the plasma membrane as well as in the cytoplasm. Cells transfected with the clone encoding CD163ΔTM showed no surface expression of the protein, although the protein was expressed in the cytoplasm, indicating that CD163ΔTM is defective in plasma membrane localization. Empty vector transfected cells did not exhibit any immunofluorescent staining on the plasma membrane or in the cytoplasm.

(iv) GP2a and GP4 proteins of PRRSV interact with porcine CD163 receptor

We next wanted to examine which of the PRRSV envelope glycoprotein(s) interact with the receptor CD163. To perform the studies, the CD163 along with each of the four envelope glycoproteins were co-expressed in transfected cells and interactions were examined by co-IP with porcine CD163 monoclonal antibody. This antibody did not immunoprecipitate the individual glycoproteins when these glycoproteins were expressed alone, demonstrating that the antibody does not immunoprecipitate the proteins non-specifically. However, when the individual glycoproteins were co-expressed with CD163, GP2a and GP4 proteins could be specifically immunoprecipitated with anti-CD163 antibody in multiple repeat experiments. Interestingly, both the mature GP2a and partially glycosylated GP2a proteins could be immunoprecipitated with the CD163 antibody, indicating that both forms of GP2a interact with CD163. The use of GP-specific antibodies also showed that CD163 protein could be immunoprecipitated with anti-GP2a or anti-GP4 antibodies. The GP3 and GP5 proteins could not be detected by immunoprecipitation with anti-CD163 antibody from co-transfected cells.

These results suggest the GP3 and GP5 proteins do not interact with the CD163 or that their interactions can not be detected under conditions in which interactions with GP2a and GP4 could be readily detected. ***The results obtained from this objective have been published in J. Virology (the complete citation is given at the end of this report).***

Objective 2:

(v) Glycan addition at several sites in GPs is required for infectious PRRSV production.

Using in vitro transcripts from full-length cDNA clones encoding various glycosylation-site mutants of the viral glycoproteins, we have found that N184 of GP2a is critical for recovery of infectious PRRSV. Likewise, glycan addition at N42, N50, and N131 of GP3 are important for PRRSV recovery. Infectious PRRSV could be recovered from all single site glycosylation mutant clones, although clones containing double glycosylation site mutations did not support virus rescue. These results suggest that glycan addition at certain sites in the minor glycoproteins of PRRSV is important for recovery of infectious virus.

(vi) Mutation of N-glycosylation sites of minor envelope glycoproteins has no effect on neutralizing antibody production in piglets

Twenty one days old weaned piglets were infected with FL12 as well as mutant viruses generated for three different envelope glycoproteins. For GP3 sites, we had generated seven mutant viruses out of which three have mutation in single amino acid position (FL-GP3-N29A; FL-GP3-N152A; and FL-GP3-N160A) where as other three have mutation in two amino acid positions in different combinations (FL-GP3-N29,152A; FL-GP3-N29,160A; and FL-GP3-N152,160A). We also generated one virus harboring mutation in three amino acid positions of GP3 protein (FL-GP3-N29,152,160A). Four 21 days old piglets were grouped and injected with specific viruses. All the different groups of piglets were housed separately. Sham infected piglets were included as negative control. The neutralizing antibody titer was measured and shown in Table 1. The FL12 infected piglet sera was tested against FL12 infected cells (homologous titer) and compared with sera collected from mutant virus infected piglets. For GP2a and GP4, recovered single mutant viruses were used for the experiment. Based on the previous report on cumulative

TABLE 1. Serum neutralization titer of animal experiment

Groups	Days post infection	
	7 days	46 days
FL12	1	32
GP2a FL-N178A	1	9.5
GP3 FL-N29,152A	1	13.5
GP3 FL-N29,152,160A	1	16
GP4 FL-N37A	1	13.5
GP4 FL-N84A	1	22.6
GP4 FL-N120A	1	26.9
GP4 FL-N130A	1	38.1

The serum neutralization value of both wild type and mutant viruses are made 1 for 7^{th} day for comparison. The results are shown as of average of four animals per each group.

effect of mutations of N-glycosylation sites of a protein on glycan shielding, we used one of the double deglycosylated mutant virus (FL-GP3-N29,152A) and triple deglycosylated mutant virus (FL-GP3-29,152,160A) for inoculation in piglets. We did not observe any significant difference between neutralizing antibody response produced by the mutant viruses. This result shows that unlike GP5, N-glycosylation of GP2a, GP3, and GP4 is not required for conferring protection against host neutralizing antibody response in virus infected animals.

Discussion:

The data presented here support the contention that the multimeric complexes of the minor glycoproteins with or without the M and GP5 proteins are involved in direct interaction with the cell surface receptor. Since GP5 and M proteins are present in abundant amounts on the virion envelope, these two proteins were considered to play a major role in receptor interaction. However, this contention was challenged by the results from the studies using chimeric viruses, which suggested that the GP5 and M proteins do not play a role in receptor interaction. Our studies show that only the GP2a and GP4 proteins interact with CD163. It appears that the GP4 is a critical viral envelope protein that not only mediates interactions with other GPs on the virion envelope, but also along with GP2a, mediates interactions with the CD163 for virus entry. Since the minor envelope proteins are not required for particle formation and there are only few large multimeric glycoprotein complexes present on the virion envelope, we suggest that the major function of the minor glycoprotein complex on the virion envelope is to interact specifically with the cell surface receptor for virus entry. On the

other hand, the GP5 and M heterodimeric complexes, which are present in large amount and are uniformly distributed throughout the virion envelope, play major roles in virion assembly. The GP5-M complexes on the virion envelope may also play additional roles in nonspecific interactions with heparin-like receptors on PAM cells since M protein has been shown to bind to such molecules. The nonspecific interaction would allow initial virus binding to the cell surface followed by specific interaction with the receptor through GP2a and GP4 proteins for receptor-mediated entry of the virion. It is possible then that GP2a and GP4, by containing viral receptor-interacting domains, would potentially be involved in the establishment of protective immunity against PRRSV infection. Viral receptor-interacting proteins and domains are known to induce highly neutralizing antibodies and contribute important targets for vaccine and therapeutic development. While a single report so far suggests that GP4 contains at least one neutralizing epitope, the full potential of GP4 and GP2a to induce PRRSV-neutralizing antibodies and T-cell immunity should be further investigated.

Based on the results, we have proposed a tentative model in our recent publication for the multiprotein complex on PRRSV envelope and its interaction with the cell surface receptor CD163. Identification of regions of GP2a and GP4 that interact with CD163 would be of primary interest as this would allow development of principles to block PRRSV infections.

Publications:

Das, P. B., Dinh, P. X., Ansari, I. H., de Lima, M., Osorio, F. A., and **Pattnaik, A. K.** (2009). The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor, CD163. ***J. Virology***, 84: 1731-1740 (E-Publication ahead of Print, November 25, 2009).