Influence of N-linked glycosylation of minor proteins of porcine reproductive and respiratory syndrome virus on infectious virus recovery and receptor interaction

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**A B S T R A C T**

It has been proposed that the N-linked glycan of the minor proteins of porcine reproductive and respiratory syndrome virus (PRRSV) is important for the production of infectious virus. In this study, we showed that N-linked glycosylation of GP2 is not essential for virus viability and none of the individual glycosylation sites in the minor proteins is critical for the susceptibility of mutants to neutralizing antibody.

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**Introduction**

The porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory and syndrome in the swine population (Paton et al., 1991). The disease was first reported in Europe and in the USA in the early 1990s and it has since become a problem to the swine industry worldwide (Collins et al., 1992; Wensvoort et al., 1991). PRRSV belongs to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*. Other related viruses of the family *Arteriviridae* are equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992; Snijder and Meulenberg, 1998). PRRSV is divided into two genotypes, the European, or type I, virus and the American, or type II, virus. The two genotypes show approximately 60% genome sequence homology (Fang and Snijder, 2010; Forsberg, 2005).

PRRSV contains a positive-stranded RNA genome of approximately 15.4 kilobases and has nine open reading frames (ORFs). The ORF1a and ORF1b encode viral replicase polyproteins that are translated immediately upon viral entry, and processed proteolytically by virally encoded proteinases into 13–14 nonstructural proteins (NSPs) (Fang and Snijder, 2010). The NSPs are also responsible for replication of genomic RNA, transcription of subgenomic RNA, and some of them could be antagonism of IFN and TNF-α (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Subramaniam et al., 2010). The ORFs 2–5 encode the glycosylated membrane proteins GP2–GP5, ORF6 encodes a non-glycosylated membrane protein (M), and ORF7 encodes the nucleocapsid (N) protein. The ORF2b is fully embedded in ORF2 and encodes the small, non-glycosylated E protein. The recently discovered ORF5a protein may be a novel small structural protein, of which the coding gene overlaps partially the 5' end of ORF5 (Firth et al., 2011; Johnson et al., 2011). All of these structural proteins except the ORF5a protein are required for the generation of infectious virions (Firth et al., 2011; Molenkamp et al., 2000; Wissink et al., 2005), which indicates that they play critical roles in virion assembly and/or interactions with the cell surface receptors to allow virus entry. The major components of the PRRSV envelope are GP5 and M, which are known to form disulfide-linked heterodimers and that formation of such heterodimers is required for viral assembly and infectivity, and also for binding to the cellular receptor pig sialoadhesin (pSNA) (Mardassi et al., 1996; Van Breemd et al., 2010). The interaction between the M/GP5 glycoprotein complex and pSNA is critically dependent on the sialic acid-binding capacity of pSNA, as well as on the presence of sialic acids on GP5 (Van Breemd et al., 2010). The minor envelope glycoproteins GP2a, GP3, GP4 and the unglycosylated 2b interact with each other to form a heterotetrameric complex in infected cells and that formation of this complex is required for the generation of infectious virions (Das et al., 2010; Mardassi et al., 1996; Wissink et al., 2005). The GP4 and GP2a proteins also may serve as...
viral attachment proteins that interact specifically with the CD163 molecule to allow virus entry into susceptible host cells (Das et al., 2010).

N-linked glycosylation of minor proteins of PRRSV plays diverse functions in virus infectivity, receptor binding and stimulation of the immune response. The GP2 protein of North American type II PRRSV contains two predicted N-glycosylation sites, at positions N178 and N184. It has been shown that both glycosylation sites are used for the addition of glycan (Das et al., 2011).

Previous studies based on the type II FL12 virus have shown that glycan addition at position N184 is required for production of infectious virus, and that glycosylation at position N178 has no effect on growth of the mutant virus in MARC-145 cells. The study also showed that glycosylation of at least one site in the GP2 protein is necessary for efficient interaction with the receptor CD163 (Das et al., 2011). In contrast, loss of the N173- or 179-linked glycan, corresponding to the N178 and N184 site in type II PRRSV, in a mutant of type I Lelystad virus does not prevent the virus from displaying efficient growth in MARC-145 cells, and mutations at these two glycosylation sites does not affect virus recovery (Wissink et al., 2004). The GP3 protein has seven potential N-linked glycosylation sites, at positions N29, N42, N50, N131, N152, N160, and N195. It has been shown that all the glycosylation sites except N195 are used for glycosylation, and that glycosylation at positions N42, N50, and N131 is critical for the production of infectious virus (Das et al., 2011). The virus named PRRSV-01 lacks two N-glycosylation sites naturally in its envelope glycoproteins, one in GP3 at position N131 and the other in GP5 at position N51, is sensitive to neutralizing antibody (Vu et al., 2011). It is suggested that PRRSV performs immune evasion through glycan shielding that involves both GP5 and GP3 (Ansari et al., 2006; Vu et al., 2011). The GP4 protein contains four predicted N-glycosylation sites, at positions 37, 84, 120, and 130. It has been shown that the four glycosylation sites are used for glycan addition, and that mutation of single glycosylation sites in GP4 does not affect infectious virus recovery but multiple mutations are lethal. The glycosylation of GP2 and GP4 is believed to be required for efficient interaction with CD163 (Das et al., 2011).

Previous studies on glycosylation ablation in the minor proteins of PRRSV did not address whether removal of the glycosylation per se or the associated structural changes in the proteins accounted for the observed deleterious effects on the replication of PRRSV. The latter scenario is supported by the existence of natural PRRSV isolates devoid of N-glycosylation in the minor proteins, which implies that the existence of some minor protein-associated glycans is not vital to the virus life cycle. In this study, we showed that the N-linked glycosylation of GP2 is not essential for virus viability. None of the individual glycosylation sites in GP3 has a vital effect on the recovery of infectious virus. Moreover, the mutations of single or double glycosylation sites in GP4 are not critically important for production of infectious virus but triple or quadruple mutations are lethal. The BIFC results showed that GP4 but might be not GP2 is involved in interactions with the cellular receptor CD163, for which glycosylation of GP4 might not play a vital role in interaction with CD163. The study further revealed that removal of glycosylation site at N131 in GP3 does not render the virus susceptible to convalescence serum.

Results

N-linked glycosylation of GP2 is not essential for virus viability

The GP2 protein of North American type II APRRSV strain contains two predicted N-glycosylation sites, at positions N178 and N184 (Fig. 1). It has been shown that both glycosylation sites are used for glycan addition, and that addition of the glycan at amino acid position N184 is critical for recovery of infectious virus (Das et al., 2011). However, some strains of PRRSV have no such specific N-linked glycosylation site at position N178 or N184 in GP2, including the strains EDRD-1 (GenBank accession No: AB288356) and PrimePac PRRS vaccine (GenBank accession: AF066384), in which no glycosylation site at positions 178 and 184, respectively, are found. This implies that the existence of GP2-associated glycan is not vital to the virus life cycle. Based on the full-length infectious cDNA clone pAPRRSasc (Tian et al., 2011), full-length cDNA clone mutants were generated in which codons for relevant Asn residues at positions 178 and 184 were replaced by codons for Try and Asp, which were present in the strains EDRD-1 and PrimePac PRRS vaccine, respectively. Three recombinant full-length cDNA clones were constructed and designated as pCGP2N178Y, pCGP2N184D, and pCGP2N178Y/184D. The recombinant full-length cDNA clones, as well as the parent plasmid pAPRRSasc, were transfected into BHK-21 cells. At 48 h post-transfection (h.p.t.), the supernatant of the transfected cells was harvested to infect fresh MARC-145 cells. The infected cells were analyzed by immunofluorescence assay (IFA) using antibody (D5-4) against the PRRSV N protein. All mutants expressed PRRSV N protein and spread into the neighboring cells at 48 h post-inoculation (h.p.i.), as shown in Fig. 2a. These results showed that infectious viruses were recovered readily from the cells transfected with full-length CMV-launched DNA clones containing mutations at N178, N184 or N178/184 in GP2. Recombinant viruses carrying single or double glycosylation sites mutation replicated and propagated well in MARC-145 cells, which suggests that N-linked glycosylation of GP2 is not essential for virus viability.

To determine the replication characteristics of wt and mutants carrying single or double glycosylation sites mutation in the GP2 protein, we analyzed first whether the mutant viral particles were less infectious. To this end, we infected MARC-145 cells with 0.01 MOI of wt (vAPRRSasc) and mutant viruses (vCGP2N178Y, vCGP2N184D, and vCGP2N178Y/184D). Multi-step growth kinetics were analyzed by titrating the supernatants of infected cells. The supernatants were harvested at the indicated time points (12, 24, 48, 72, 96, and 120 h) from MARC-145 cells infected with wt and mutants. The amount of infectious particles was determined by titrating the supernatants of infected cells. The results revealed that the growth kinetics of the recovered viruses carrying a single glycosylation site mutation was similar to that of the wt virus, and the overall yield of mutants did not show significant differences when compared with wt. However, the multiple-step growth curve of the mutant carrying mutations at two glycosylation sites had different growth behavior from the wt. As shown in Fig. 2b, the mutants reached their peak titer at 96 h.p.i., about 24 h delayed with respect to wt. The overall yield of mutants was nearly one log less than that of wt. The viral plaque assay was performed on MARC-145 cells to monitor the plaque phenotype of the mutant viruses. As shown in Fig. 2c, the majority of the plaques generated by wt and the variants containing a single glycosylation site mutation were clear and distinct. However, plaques produced by mutants carrying double glycosylation sites mutation were smaller than those produced by
These results indicate that single glycosylation site mutation in GP2 does not affect the recovery of infectious virus and removal of the two glycans from GP2 affect virus growth and production of infectious virus.

To confirm that mutations introduced into the viral RNA were maintained in the replicating viruses, the RNA of the serially passaged viruses (P3) was purified from viral particles and used in RT-PCR and nucleotide sequencing analysis of the ORF2 to 7. We found that all of the engineered mutations in GP2 were retained in the recombinant viruses, and no conspicuous mutations in the flanking regions were found (data not shown).

None of the individual glycosylation sites in GP3 is essential for virus viability.

The GP3 protein has seven potential N-linked glycosylation sites, at positions N29, N42, N50, N131, N152, N160, and N195. It has been shown that all the glycosylation sites except N195 are used for glycosylation, and glycosylation at positions N42, N50, and N131 was reported to be critical for infectious virus production (Das et al., 2011). However, previous investigation has not established whether the growth-defective phenotype is attributable to the loss of glycosylation per se, or to structural alteration to the GP3 protein that results from the amino acid substitution. In addition, some PRRSV field strains, such as the strain PRRSV-01 (Genbank accession: JF422072), bear Asn by Ser amino acid mutations at position N131, which suggests that the glycan at N131, and other glycosylation sites on GP3, may be not critically important for the virus life cycle.

In this study, the mutation Asn to Ser was selected in the seven potential glycosylation sites, because this substitution was likely to introduce few modifications of the protein structure. To this end, we generated recombinant clones with specific mutations in each of the potential glycosylation sites in GP3. These glycosylation sites were removed by changing the Asn to Ser in a cDNA clone of PRRSV. The wt and mutated clones carrying the N29S, N131S, N152S, N160S, and N195S substitutions (pCGP3N29S, pCGP3N131S, pCGP3N152S, pCGP3N160S, and pCGP3N195S) were used to transfect BHK-21 cells. At 48 h.p.t., the supernatant of the transfected cells was harvested to infect fresh MARC-145 cells. Viral replication in MARC-145 cells was analyzed by IFA using antibody against the PRRSV N protein (D5-4). As Fig. 3a shows, most of the monolayer cells were IFA positive at 48 h.p.i. This result indicated that none of the individual glycosylation sites in GP3 is essential for virus viability.

The results of multiple step growth curve analysis showed that the growth kinetics of the recovered viruses that carried a glycosylation site mutation at positions N29, N131, N152, N160, and N195 (vCGP3N29S, vCGP3N131S, vCGP3N152S, vCGP3N160S, and vCGP3N195S) were similar to that of the wt virus, and the overall yield of mutant viruses did not show significant differences from wt (Fig. 3b). However, the overall yield of mutants carrying a glycosylation site mutation at position N42 or N50 (vCGP3N42S, vCGP3N50S) was nearly one log less than that of wt. The plaque phenotype assay showed that the majority of the plaques produced by wt and the variants that contained a single glycosylation site mutation at positions N29, N131, N152, N160, or N195 were clear and distinct. However, the plaques produced by mutants carrying N42 or N50 mutation gave rise to smaller plaques than wt and the other variants (Fig. 3c). These results indicate that the loss of glycan at N42 or N50 somehow affects virus growth kinetics.
Mutation of single and double glycosylation sites in GP3 does not have a vital effect on the recovery of infectious virus

The GP4 protein contains four predicted N-glycosylation sites, at positions 37, 84, 120, and 130 (Fig. 1). It has been shown that the four glycosylation sites are used for glycan addition, and that mutation of single glycosylation sites in GP4 does not affect infectious virus recovery but multiple mutations are lethal (Das et al., 2011). In this study, to generate recombinant clones with specific mutations in each of the potential N-glycosylation sites in GP4, these glycosylation sites were destroyed by changing the Asn to Ser in a cDNA clone of PRRSV. The wt and mutants carrying the N37S, N84S, N120S, and N130S substitution (pCGP4N37S, pCGP4N84S, pCGP4N120S, and pCGP4N130S) were transfected into BHK-21 cells. At 48 h.p.t., the supernatant of the transfected cells was harvested to infect fresh MARC-145 cells. Viral replication in MARC-145 cells was analyzed by IFA using antibody (D5-4) against the PRRSV N protein to analyze the replication of the mutants. The result showed that all the double glycosylation site mutants expressed the PRRSV N protein and had spread into neighboring cells at 48 h.p.i., as shown in Fig. 4a. This suggests that double glycosylation sites mutation in GP4 does not have a vital effect on infectious virus particles. The result showed that all the double glycosylation site mutants, but not the triple and quadruple glycosylation site mutants, expressed the PRRSV N protein and had spread into neighboring cells at 48 h.p.i., as shown in Fig. 4a. This suggests that double glycosylation sites mutation in GP4 does not have a vital effect on infectious virus recovery, but triple and quadruple glycosylation sites mutation in GP4 are lethal. Subsequently, we analyzed the multiple-step growth curve and performed the viral plaque assay on the mutant viruses (vCGP4N37S/84S, vCGP4N37S/120S, and vCGP4N37S/130S) carrying a single glycosylation site mutation at N37, N84, N120, or N130 showed no apparent difference from those of the wt virus, which suggests that a single glycosylation site mutation at these positions in GP4 has no effect on growth of the mutant virus in MARC-145 cells. However, the overall yield of the viral particles produced by the variant (vCGP4N37S) with the glycosylation mutation at N37 was 5-fold less and the plaques were smaller than those of wt, which suggests that this mutation affects the production of infectious virus particles.

To generate mutations at two or more glycosylation sites in GP4, the single glycosylation site constructs were used as templates, and clones carrying multiple glycosylation site mutations were generated. The double (pCGP4N37/84S, pCGP4N37/120S, pCGP4N37/130S, and pCGP4N84/120S), triple (pCGP4N37/84/120S, pCGP4N37/84/130S, pCGP4N37/84/120/130S, and pCGP4N84/120/130S), and quadruple (pCGP4N37/84/120/130S) glycosylation site mutants were transfected into BHK-21 cells. At 48 h.p.t., the supernatant of the BHK-21 cells was collected to infect MARC-145 cells. The IFA was performed on the infected MSC-145 cells using an antibody against PRRSV N protein to analyze the replication of the mutants. The result showed that all the double glycosylation site mutants, but not the triple and quadruple glycosylation site mutants, expressed the PRRSV N protein and had spread into neighboring cells at 48 h.p.i., as shown in Fig. 4a. This suggests that double glycosylation sites mutation in GP4 does not have a vital effect on recovery of infectious virus, but triple and quadruple glycosylation sites mutation in GP4 are lethal. Subsequently, we analyzed the multiple-step growth curve and performed the viral plaque assay on the mutant viruses (vCGP4N37/84S, vCGP4N37/120S, vCGP4N37/130S, and vCGP4N84/120S). The multiple-step growth curve of the mutants carrying double glycosylation site mutations had similar growth behavior to that of wt. However, the overall yield of the mutants was nearly one log less than that of wt, as shown in Fig. 4c. Plaques produced by the mutants carrying double glycosylation site mutations were indistinct and smaller than those of wt, as shown in Fig. 4d. These results indicate that double glycosylation site mutations in GP4 are not critical for virus recovery but they affect the production of infectious virus. To assess their genetic stability, the ORF2-3'UTR regions of the mutant viruses were amplified by RT-PCR and
sequenced to confirm the presence of desired mutations and the absence of any other unplanned mutations (data not shown).

GP4 but not GP2 might be involved in interaction with cellular receptor CD163

The GP2 and GP4 proteins have been shown to interact specifically with the CD163 molecule, which can function as a receptor for PRRSV entry (Das et al., 2010). Furthermore, it has been demonstrated by the Co-immunoprecipitation (Co-IP) assay that the glycosylation of GP2 and GP4 is important for efficient interaction with CD163 (Das et al., 2011). However, in this study, we showed that the glycosylation sites in GP2 are not critically important for virus infectivity, which suggests that the glycans of GP2 may not play a critical role in the interaction with CD163. To confirm the result of the Co-IP performed previously by Das et al. (2011), we carried out BiFC (bimolecular fluorescence complementation) analysis in BHK-21 cells. To obtain the BiFC construct, the venus protein was spliced between amino acid residues 173 and 174, which resulted in the fragments VN (N-terminal 173 residues) and VC (C-terminal 66 residues). The “Target” protein was fused to the N terminus of VN or VC with a linker sequence to generate the BiFC pair, as shown in Fig. 5a. To assess the specificity of the BiFC method, fragments VN and VC, or α-tubulin gene-fused VN and GP2, GP3, and GP4 fused with VC, were co-transfected into BHK-21 cells. As shown in Fig. 5b, no fluorescent signals were observed at 24 h.p.t., which suggest that BiFC can be used to study the interaction between CD163 and the minor proteins. Subsequently, BiFC was used to investigate the interaction between CD163 and the minor proteins. The minor proteins GP2, GP3, and GP4 were fused to the N-terminus of the VC fragment, respectively, and CD163 was fused to the N-terminus of the VN fragment. The BHK-21 cells were co-transfected with plasmids encoding the fusion proteins CD163-VC and GP2-VN, GP3-VN, or GP4-VN. The results showed that the specific BiFC signal was found in cells co-transfected with GP4-VN and CD163-VN, which suggests that GP4 co-localize with CD163. However, BHK-21 cells co-transfected with CD163-VN and GP2-VC or GP3-VC showed no BiFC signal under the same conditions, as shown in Fig. 5b. No significant BiFC fluorescence was detectable in cells transfected with CD163-VN, GP2-VC, GP3-VN, or GP4-VN alone (data not shown). The BiFC analysis thus suggests that GP4, but might be not GP2 or GP3, has a specific interaction with CD163 at the cytoplasm circumference in BHK-21 cells.

To determine whether glycosylation of GP4 is involved in the interaction with CD163, BiFC analysis was performed. Full-length ORF4 cDNA carrying the single, double, triple, or quadruple glycosylation site mutations was amplified by PCR and inserted into BiFC vectors as fusions with the VC fragments. BHK-21 cells were co-transfected with VC fused with GP4 carrying the

**Fig. 4.** Mutation of single and double glycosylation sites in GP4 does not have a vital effect on infectious virus recovery, but triple or more mutations are lethal. (a) IFA analysis of MARC-145 cells at 24 h.p.i. with wt and mutants bearing single or double glycosylation sites mutation in GP4. The IFA analysis was performed at 24 h.p.i. using a monoclonal antibody (D5-4) against PRRSV N protein. Images were taken at × 200 magnification. (b) Multi-step growth kinetics of wt and variants bearing the single mutation in the GP4 protein in MARC-145 cells. Cells in six-well plates were infected with PRRSV at an MOI of 0.01, culture supernatants were collected at the indicated time after infection, and virus titers were determined by TCID₅₀. Average titers with standard deviations (error bars) from three independent experiments are shown. (c) Multi-step growth kinetics of wt and variants bearing double glycosylation site mutations in the GP4 protein in MARC-145 cells. Average titers with standard deviations (error bars) from three independent experiments are shown. (d) Viral plaque morphology. Ten-fold serially diluted mutants with individual glycosylation site mutations in GP4 were inoculated into fresh MARC-145 cells in six-well plates, then cultured in EMEM containing 1% agarose overlay, fixed at 4 d.p.i. and stained with 1% crystal violet.
glycosylation mutations and CD163-VN. The result showed that significant BiFC fluorescence was detectable in cells co-transfected with CD163-VN and VC fused with GP4 carrying glycosylation mutations, which suggests that the glycosylation of GP4 may not play a vital role in the interaction with CD163.

**Influence of hypoglycosylation of minor proteins on PRRSV’s ability to be neutralized by convalescence serum**

It has been shown that PRRSV performs immune evasion through glycan shielding that involves both GP5 and GP3 (Ansari et al., 2006; Vu et al., 2011). N-glycan at position N131 in GP3 of a type II PRRSV isolate (PRRSV-01) is as important as N-glycans in GP5 regarding its role in protection of the virus from antibody neutralization. However, the engineered viruses carrying glycosylation mutations in the minor proteins are not sensitive to convalescent serum (Das et al., 2011). In this study, we obtained mutant viruses lacking one or two glycosylation sites in the minor proteins, which failed to be rescued in the previous study. The ability of these mutants to be neutralized by convalescent antiserum needed further investigation. To this end, convalescent antiserum collected from pigs infected with vARRSV or vAJXM, high titers of homologous (vAPRRS-specific serum) and heterologous neutralizing antibodies (vAJXM-specific serum) were detected in the sera collected at 49 d.p.i. However, the differences observed among the mean titers of serum from vARRSV- or vAJXM-infected pigs against the hypoglycosylated PRRSV mutants and wt were not statistically significant ($p < 0.5$), which suggests that the removal of these glycans from the minor proteins, including the N131-linked glycan from GP3, do not increase the accessibility of the neutralizing epitope to convalescent antisera.

**The removal of glycosylation site at N131 in GP3 does not render the virus susceptible to antibody neutralization**

It has been shown that the absence of N-glycosylation site at in the hypervariable region upstream of the neutralization epitope in the GP5 protein of engineered mutants and field isolate increases the susceptibility of the virus to antibody neutralization (Ansari et al., 2006; Faaberg et al., 2006). In our study, the vAPRRSASC which lacks the glycosylation site in the upstream of the N44 is also sensitive to the antibody neutralization. To rule out the possibility that the

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**Fig. 5.** Bimolecular fluorescence complementation (BiFC) analysis of minor proteins involved in the interaction with CD163. (a) Schematic diagram of BiFC constructs. Venus protein was spliced between amino acid residues 173 and 174, resulting in fragments VN (N-terminal 173 residues) and VC (C-terminal 66 residues). The “Target” protein was fused to the N-terminus of VN or VC with a linker sequence to generate the BiFC pair. (b) Fragments VN and VC or α-tubulin gene-fused VN and minor proteins fused with VC were co-expressed in BHK-21 cells, and the fluorescent signals were examined to assess the specificity of the BiFC method. Plasmids encoding the fusion proteins CD163-VC and GP2-VN, GP3-VN, GP4-VN, or GP4-VN carrying glycosylation site mutations were co-transfected into BHK-21 cells for 24 h. The BiFC fluorescence was imaged at 24 h.p.i.
Supernatants were collected at the indicated time points after infection, and the cells. Cells in six-well plates were infected with PRRSVs at an MOI of 0.01, culture kinetics of wt and mutants (vCGP5S34N and vCGP3N131SGP5S34N) were constructed and transfected into MARC-145 cells was analyzed by IFA. As Fig. 6 shows, mostly the wt was harvested to infect fresh MARC-145 cells. Viral replication in virus-A and virus-B (vCGP3N50S, vCGP3N37/84S, vCGP3N42S, vCGP3N42/120S, vCGP3N131SGP5S34N) were constructed and transfected into BHK-21 cells. At 48 h.p.i., the supernatant of the transfected cells were harvested to infect fresh MARC-145 cells. Viral replication in MARC-145 cells was analyzed by IFA. As Fig. 6a shows, most of the monolayer cells were IFA positive at 48 h.p.i. This result indicated that the introduction of a glycosylation site at N34 in GP5 does not affect infectious virus recovery. The multiple step growth curve analysis showed that the growth kinetics of the recovered viruses that carried a fully glycosylated GP5 (vCGP5S34N, vCGP3N131SGP5S34N) were similar to that of the wt virus, and the overall yield of mutant viruses did not show significant differences from wt (Fig. 6b). The ORF2-3'UTR regions of the mutant viruses were amplified by RT-PCR and sequenced to confirm the presence of desired mutations and the absence of any other unplanned mutations (data not shown).

To analyse whether the introduction of N-glycosylation site in GP5 affect the susceptibility of the viruses (vCGP3N50S, vCGP3N131SGP5S34N) to antibody neutralization, the FFN were performed. The results showed that the introduction of a glycan at position N34 in the GP5 deduce the two viruses sensitivity to the antibody neutralization. Low titers (~203) of homologous and heterologous neutralizing antibodies were detected in the sera (Table 1). The mutant vCGP3N131SGP5S34N with deletion of N-glycosylation site at N131 is not sensitive to antibody neutralization compared to mutant vCGP5S34N. This suggests that introduction of N34S into the GP5 makes the viruses less sensitive to neutralization by an antiserum and removal of glycosylation site at N131 in GP3 does not render the virus susceptible to antibody neutralization.

Discussion

Previous studies based on the type II FL12 virus have shown that the addition of glycan at position 184 in GP2 and glycosylation at positions N40, N52, and N131 in GP3 are required for the production of infectious virus. The mutation of individual glycosylation site in GP4 does not affect recovery of infectious virus, but multiple mutations are lethal. The glycosylation of the GP2 and GP4 proteins is required for efficient interaction with CD163 (Das et al., 2011). Our results showed that the N-linked glycosylation of GP2 is not essential for virus viability. Moreover, none of the single glycosylation sites on GP3 has vital effect on infectious virus recovery. The mutation of single and double glycosylation sites in GP4 is not critical for infectious virus production, but triple and more mutations are lethal. The BiFC results also showed that GP4, but might be not GP2, is involved in interaction with the cellular receptor CD163, and that glycosylation of GP4 might not play a vital role in interaction of the virus with CD163. The study further revealed that glycosylation of the minor proteins, including the N-linked glycosylation at position 131 in GP3, which has been shown previously to have an effect on the susceptibility of the virus to neutralization antibody, is not sensitive to convalescent serum. These results made significant contributions to better understand the virus replication and virus neutralizing mechanisms.

Previous studies based on the type I FL12 virus have shown that addition of glycan at position N184 is required for the production of infectious virus in MARC145 cells (Das et al., 2011). Our present study shows clearly that this is not the case. We found that loss-of-N178 or N184-linked glycan variants of PRRSV can display efficient growth in MARC-145 cells, and that mutations at two glycosylation sites do not have a vital effect on virus recovery in MARC-145 cells. The data we obtained are consistent with those of the type I Lelystad virus (Wissink et al., 2004). Therefore, these results indicate that the removal of the glycans in GP2, per se, is not critical for the recovery of infectious virus. We assume that the structural changes in the GP2 protein caused by the amino acid substitution introduced to abolish glycosylation accounted for the observed growth defective phenotypes. In our studies, variants with replacement of Asn184 by Asp, a smaller hydrophilic amino acid residue, retained efficient growth. We also observed that the growth kinetics and the plaque phenotype of the virus carrying the N184 glycosylation site

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</tr>
<tr>
<td>vCGP5S34N</td>
<td>32</td>
<td>25.3</td>
</tr>
<tr>
<td>vCGP3N131SGP5S34N</td>
<td>25.3</td>
<td>25.3</td>
</tr>
</tbody>
</table>

Anti-serum was obtained at 49 day post-infection from pigs that had been infected with vAPRRS or vAJXM. Neutralization titers were expressed as the reciprocal of the highest dilution that showed 90% or greater reduction in the number of fluorescent foci presenting in the control wells. The differences in the mean titers of serum from pigs infected with vAPRRS or vAJXM against the hypoglycosylated PRRSV mutants and wt (vAPRRS) were not statistically significant, respectively (p > 0.5).
mutation were similar to those of the wt. Similarly, replacement of N179 with Gln, a larger hydrophilic amino acid residue, did not affect the infectivity of the type I Lelystad virus significantly in PAM (Wissink et al., 2004). However, replacement of N184 with Ala, a small hydrophobic residue, was detrimental to infectious virus recovery in the FL-12 virus in MARC-145 cells (Das et al., 2011). In summary, it is suggested that the structural changes in the GP2 protein introduced by the amino acid substitution that destroys glycosylation, but not the glycan itself at position N184, may account for the observed growth-defective phenotypes. In contrast, the replacement of N178 with Ala in FL12 virus, or with Tyr, a hydrophilic aromatic amino acid, and replacement of N173 in type I Lelystad virus with Gln, a larger hydrophilic amino acid residue, did not affect the infectivity of the virus. This suggests that the glycosylation at position N178 and the nature of the amino acid residues (polar or nonpolar) introduced at this site are not critical to infectious virus production. The observation of the N-glycosylation of GP2 of type I and type II PRRSV is not critically important for virus infectivity in MARC-145 or PAM challenge the previous result that glycosylation of at least one site in GP2 protein is necessary for efficient interaction with the receptor CD163 (Das et al., 2011). Furthermore, our result showed that the GP2 might be not involved in interaction with CD163 by performing BIFC in present study. It is also contrast with the finding of previous study (Das et al., 2010). Nevertheless, it is clear that more biochemical information is required to clarify the exact role of the glycosylation of GP2 claimed to be involved in interaction with CD163.

We also found that the glycosylation sites at N40, N52, and N130 in GP3 are not critically important for recovery of infectious virus. This is in contrast with a previous study, which showed that the introduction of an Ala residue at positions N40, N52, and N130 in GP3 is lethal for infectious virus production (Das et al., 2011). The discrepancy in the effect of the N-glycosylation sites at N40, N52, and N130 on the growth of PRRSV may be due to differences in the way in which the N-glycosylation sites are abolished. In our study, the mutation of Asn to Ser, a hydrophilic residue that has a similar conformation to Asn, was selected in these potential glycosylation sites. This substitution is likely to introduce few modifications to the protein structure. In addition, some field strains of PRRSV, such as the strain PRRSV-01 (Genbank accession: JF422072), bear the Asn to Ser amino acid mutation at position N131, which suggests that this glycan is not critically important in the life cycle of the virus (Vu et al., 2011). This notion is also supported by the observation that the mutant bearing a glycosylation mutation at N131 did not show altered virus infectivity in our study. The growth kinetics and the plaque phenotype of the mutant carrying the N131 glycosylation site mutation were similar to those of the wt. We also observed that the glycosylation mutation at N40 or N52 was not critically important for recovery of infectious virus. However, the overall yield of the mutants was nearly one log lower, and the plaques were smaller than those of the wt. These results indicate that loss of glycan at residues 42 or 50 affect virus growth and infectious virus production.

We observed that a single glycosylation site mutation in GP4 is not critical for recovery of infectious particles. The growth kinetics and virus plaques of the recovered variants that carried single glycosylation mutations at N84, N120, and N130 showed no apparent difference from those of the wt virus. However, the glycosylation mutation at N37 affected virus growth. This observation on the growth characteristics of the variants is different from that of FL12 virus, in which single glycosylation mutation in GP4 resulted in variants with improved replication characteristics (Das et al., 2011). It is possible that the replacement of Asn with Ala in GP4 produces a better conformational structure for virus replication than replacement with Ser. The observation that mutation of double glycosylation sites in GP4 does not have a vital effect on the recovery of infectious PRRSV is intriguing. It has been shown previously that mutation of double glycosylation sites in FL12 virus prevented infectious virus recovery, despite the fact that single glycosylation site mutation in GP4 give rise to mutants with better growth characteristics (Das et al., 2011). Thus, it appears that the nature of the amino acid substitutions introduced to abolish multiple N-linked glycosylation sites may account for differences in the requirement for glycosylation of GP4 in the production of infectious virus. We also observed that the overall yield of mutants carrying two glycosylation site mutations in GP4 was nearly one log lower and the plaques were indistinct and smaller than those of wt. Most important of all, we also found that triple or quadruple glycosylation mutations in GP4 were lethal, which suggests that the presence of two glycosylation sites on GP4 is essential for virus viability. It is well known that N-linked glycosylation is important for the correct folding, targeting, and biological activity of proteins (Braakman and van Anken, 2000; Chackerian et al., 1997; Doms et al., 1993; Shi and Elliott, 2004; Wei et al., 2003). That improper folding of the mutant protein caused by the ablation of N-linked glycans affects virus infectivity has been reported for many enveloped viruses (Hanna et al., 2005; Kim et al., 2008; Lee et al., 2010). In this study, the deletion of three or more glycosylation sites in GP4 may cause improper folding of the mutant protein, which affects the recovery of infectious virus.

BIFC is a newly emerged technique that is used to study protein–protein interactions. It is a powerful tool to detect in vivo weak or otherwise transient protein–protein interactions with high levels of specificity and sensitivity (Hu and Kerppola, 2003; Kerppola, 2006; Shyu and Hu, 2008). In this study, BIFC was performed to increase understanding of the interaction of CD163 and the minor proteins of PRRSV. The observation that GP4 but not GP2 might have a specific interaction with CD163 in BHK-21 cells is surprising. It contrasts with the data obtained by Co-IP that GP2 is involved in an interaction with CD163 (Das et al., 2011). Our data also showed that the GP4 protein interacts with CD163. However, the addition of glycan to GP4 might not play a critical role in the efficient interaction with CD163. In BHK-21 cells co-transfected with CD-163-VC and wt, and with mutant GP4-VN, the interaction between CD163 and GP4 was found to be independent of the extent of glycosylation of GP4. A similar intensity of BIFC fluorescence was shown in the co-transfected BHK-21 cells, which suggests that the mutant that carries a glycosylation site mutation interacts with CD163 as successfully as the wt GP4. Like other assay systems, BIFC analysis has its limitations. The role of GP2 and GP4 and their glycosylation in the interaction with CD163 requires further confirmation by different methods.

Our study showed that the wt (vAPRRSasc) that lacks the N-glycosylation site in the hypervariable region upstream of the neutralization epitope in GP5 naturally was sensitive to convalescent antisera collected from pigs infected with vARRSV or vAJXM. This is consistent with previous data showing that the FL virus and natural isolate that carry a glycosylation site mutation at N34 in GP5 is sensitive to convalescent antisera (Ansari et al., 2006; Faaberg et al., 2006). Our study also showed that the variants with mutations at two glycosylation sites in GP2 or GP4, as well as other single glycosylation site mutations in the minor proteins, are not more sensitive to the convalescent serum than wt. It is suggested that the glycosylation of minor proteins does not have an influence on the response of viruses to antibody neutralization. This corroborates the previous finding that mutant PRRSVs bearing hypoglycosylated forms of minor proteins are not sensitive to convalescent serum (Das et al., 2011). We also concerned that the increase in susceptibility to neutralization by antibody conferred by deglycosylation of GP5 of vAPRRSasc is likely to interfere with any effects of deglycosylation of the minor proteins. To solve this issue, two mutants with fully glycosylated GP5 were constructed.
Mutant vCGP5S34N with fully glycosylated GP5 is less sensitive to neutralization by an antiserum that vAPRRSasc. It is consistent with previously studies showing that N-glycan addition at this site in GP5 help the virus escape neutralization by the host antibodies through “glycan shielding” (Ansari et al., 2006; Faaberg et al., 2006). We also observed that the mutant vCGP3N131SGP5S34N with fully glycosylated GP5 but carrying N-glycosylation site mutation at N131 in GP5 is not more sensitive to convalescent serum than vCGP5S34N. This suggests that the removal of glycosylation site at N131 in GP5 does not render the virus susceptible to antibody neutralization (Vu et al., 2011). The type II PRRSV isolate (PRRSV-01) plays an important role in protection contrasts with the previous finding that the N-glycan at N131 in GP3 of a mutant vCGP5S34N with fully glycosylated GP5 but carrying N-glycosylation site mutation at N131 in GP5 is less sensitive to antibody neutralization (Vu et al., 2011). The discrepancy between the two studies might be due to other amino acid substitutions in the envelope proteins of PRRSV-01, rather than the glycosylation site in GP3 at position N131, which affect the sensitivity of the mutant to antibody neutralization.

Materials and methods

Cells and antibodies

MARC-145 (ATCC, Manassas, VA) and baby hamster kidney cells (BHK-21; ATCC CCL10) were maintained at 37 °C in EMEM (Gibco) plus 10% FBS and DMEM (Gibco) supplemented with 10% FBS, respectively. Monoclonal antibody (DS-4) against the N protein of type I PRRSV was provided generously by Dr. Shaoying Chen (Fujian Academy of Agricultural Sciences, China).

Plasmids

The PRRSV infectious cDNA clone pAPRRSasc, which contains an Asc I restriction enzyme recognition site immediately upstream of the ORF2 start codon (Tian et al., 2011), is a derivative of the infectious clone pAPRRS (Yuan and Wei, 2008). The intermediate vector, named pCBC23456, which contains the sequences covering the ORF2–6 region was constructed from the infectious clone pAPRRSasc. Glycosylation site mutations were introduced at the glycosylation sites of the minor proteins in the intermediate vector pCBC23456 using standard site-directed PCR mutagenesis. The primers used in PCR mutagenesis are listed in Table 2. After restriction enzyme map identification and sequence analysis, the fragments that harbored the desired mutations were excised with Asc I and Xba I, and transferred subsequently into the full-length clone pAPRRSasc to generate mutant plasmids.

DNA transfection and recovery of mutant viruses

Mutant plasmids were prepared using a QIAprep Spin Mini-prep kit (Qiagen, Hilden, Germany), followed by identification by

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Primers used in this study.</th>
</tr>
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<tbody>
<tr>
<td>Name</td>
<td>Sequence (5′-3′)</td>
</tr>
<tr>
<td>GP2N178YP</td>
<td>GCCGATGACGGGGTCTATGTAACCTGATGATAT</td>
</tr>
<tr>
<td>GP2N178YR</td>
<td>ATATACTATGTTCACATATGACCCTCCTATGCCC</td>
</tr>
<tr>
<td>GP2N184DF</td>
<td>ATGTACATCATGATATGCTGGACTTTAAGTGACT</td>
</tr>
<tr>
<td>GP2N184DR</td>
<td>CTAATCCAAATTTGTTATCTACATATCATTCAGTAT</td>
</tr>
<tr>
<td>GP3N29SF</td>
<td>GTATGACCGACTGACCAGCAAAAG</td>
</tr>
<tr>
<td>GP3N31SF</td>
<td>CCGCGGAGACGGGTCACTGAAAAAC</td>
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<tr>
<td>GP3N31FS</td>
<td>CTGCAGCTTTACCTCCATCCGAA</td>
</tr>
<tr>
<td>GP3N152SF</td>
<td>CATAGTGGCCAGCAACACCAGCTTGC</td>
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<tr>
<td>GP3N152SR</td>
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<td>CCACCGACACTTAAAACCCCAACA</td>
</tr>
<tr>
<td>GP4N37SF</td>
<td>ACATCAAGACAGCAACACCCCGACG</td>
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<td>GP4N37SR</td>
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<tr>
<td>GP4N84SF</td>
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<td>GP4N84SR</td>
<td>CTCTGCTGCTCCGTGGTGAATG</td>
</tr>
<tr>
<td>GP4N120SF</td>
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<td>GP4N120SR</td>
<td>ATGGTACGACAGCACAAATAACAC</td>
</tr>
<tr>
<td>GP4N130SF</td>
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</tr>
<tr>
<td>GP4N130SR</td>
<td>AGCTCGATAAAAAAGACACCCCGACGC</td>
</tr>
<tr>
<td>CD163SRhol</td>
<td>AACTTCGATGCTTACCTCGAGCCTGTGCTTCCATTGAGGATT</td>
</tr>
<tr>
<td>CD163SFecoRl</td>
<td>TTACGTTGGTGAAGTCTGCTGCTG</td>
</tr>
<tr>
<td>SF12163</td>
<td>TTATAGCGGATTTAGGCGCGGCAATG</td>
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<tr>
<td>SR14780</td>
<td>GACTCAGAGGTGATGAACTCTCAACGTGTCTATG</td>
</tr>
<tr>
<td>GP2SFYHtI</td>
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<td>GP2SRhGtI</td>
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<td>GP3SFecoRl</td>
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<tr>
<td>SF12004</td>
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</tr>
<tr>
<td>QNT</td>
<td>GAGTTGACGAGGGAGCGGTACGGTTTAAAAATTTT</td>
</tr>
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</table>

Primer names are organized in groups. Prefixes: SF, forward PCR primer; SR, reverse PCR primer. The restriction endonuclease sites are indicated in italic letters.
electrophoresis, restriction enzyme map identification and quantification by spectrophotometry. BHK-21 cells were grown to 70% confluence on six-well plates and the DNAs were transfected with 3.75 μg of each plasmid, using 18.75 μl Lipofectamine™ LTX and 3.75 μl Plus Reagent (Invitrogen) according to the manufacturer's instructions. Rescued mutant viruses were harvested on BHK-21 cell monolayers which were remarked as primary passage (P0), and were used to infected MARC-145 cells for three passages (P1 to P3) by 10⁻² dilution at each passage.

Indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) was performed for the detection of protein expression in cells infected with the corresponding virus. Confluent monolayers of MARC-145 cells were infected with the mutant viruses. After 48 or 24 h post-inoculation, the infected cells were washed twice with phosphate-buffered saline (PBS), followed by fixation in cold methanol, and blocked in 1% BSA at room temperature. The cells were incubated with antibody against the PRRSV N protein at 37 °C for 2 h. After extensive washing with PBS, the cells were incubated with anti-mouse Alexa-568-labeled secondary antibody (Sigma) for 1 h. The cells were washed five times with PBS. Finally, the fluorescence was visualized under an Olympus inverted fluorescence microscope equipped with a camera.

Multi-step growth curves

Subconfluent MARC-145 cells in a six-well plate were infected with equal amounts of wt and the mutated viruses. A multiplicity of infection (MOI) of 0.01 was used in each case as indicated. After 1 h incubation at 37 °C, the cells were washed three times with PBS and incubated into 3 ml of EMEM containing 2% FBS and 3.75 μg of each plasmid, using 18.75 μl Lipofectamine™ LTX and 3.75 μl Plus Reagent (Invitrogen) according to the manufacturer's instructions. Rescued mutant viruses were harvested on BHK-21 cell monolayers which were remarked as primary passage (P0), and were used to infected MARC-145 cells for three passages (P1 to P3) by 10⁻² dilution at each passage.

Viral plaque assay

To examine the plaque morphology of the glycosylation mutant viruses, 10-fold serially diluted virus suspensions were incubated with MARC-145 cells in six-well plates. After adsorption for 1 h, the cell monolayers were washed off, and then overlaid with a mixture of EMEM medium containing 2% FBS and 1% low melting agarose (Cam-brex, Rockland, ME, USA). When the agarose overlay had solidified, the plate was inverted (bottom up) in a humidified CO₂ incubator at 37 °C for 4 days. The resulting plaques were stained with crystal violet (5% w/v⁻¹ in 20% ethanol).

RT-PCR and nucleotide sequence

Viral RNA was isolated from P1–P3 using a QiaAmp Viral RNA kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instruction. Aliquots of 1 μg of RNAs were used to synthesize first-strand cDNA using Reverse Transcriptase XL (AMV) (TakaRa Dalian, China) and the anti-sense primer QNT. The fragment containing the ORF2-ORF7 region was amplified using PfuUltra II Fusion HS DNA Polymerase (Stratagene) with the forward primer SF12004 and reverse primer QNT under the following conditions: denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 3 min, followed by a final extension at 72 °C for 10 min.

The PCR product was purified using a TIANgel Mini Purification Kit (TianGen) and sequencing.

Serum neutralization assays

The titer of PRRSV-neutralizing antibodies in each serum sample was determined as described previously (Ansari et al., 2006). Twofold dilutions of convalescent antisera, which were heat-inactivated for 30 min at 56 °C prior to the neutralization assay, were each mixed with an equal volume of culture medium containing 200 TCID₅₀ of test PRRSV mutants and wt. The mixture was incubated at 37 °C for 1 h and added to MARC-145 cells in 96-well tissue culture plates at a density of 1.2 × 10⁴ cells/well. After incubation for 36 h at 37 °C in a humidified atmosphere containing 5% CO₂, the cells were fixed in cold methanol, and blocked in 1% BSA at room temperature. The cells were incubated with antibody against the PRRSV N protein (D5-4) at 37 °C for 2 h. After extensive washing with PBS, the cells were incubated with anti-mouse Alexa-568-labeled secondary antibody (Sigma) for 1 h. Subsequently, the cells were washed five times with PBS. Finally, the fluorescence was visualized under an Olympus inverted fluorescence microscope equipped with a camera. Neutralization titers were expressed as the reciprocal of the highest dilution that inhibited 90% of the foci present in the control wells.

Bimolecular fluorescence complementation analysis

The BiFC plasmids were constructed as described previously (Nagai et al., 2002). Briefly, the sequences encoding fluorescent protein Venus residues 1–173 (VN) or residues 174 to 239 (VC) were connected by a C-terminus linker (GGGGS)₃. Porcine CD163 cDNA was amplified using PCR from the total RNA isolated from PAM cells using specific primers (Table 2) and fused to the N-terminus of VN via a (GGGGS)₃ linker. The coding regions of GP2, GP3 and GP4 carrying glycosylation site mutations were amplified and fused to the N-terminus of VC via a (GGGGS)₃ linker. The alpha-tubulin gene from the MARC-145 cell line was amplified and fused to the N-terminus of VN to produce the negative control. BHK-21 cells in six-well plates with about 70% confluence were co-transfected with 500 ng of each BiFC plasmid using the Lipofectamine™ LTX and Plus Reagent (Invitrogen). At 24 h.p.i., live cells were visualized using an Olympus inverted fluorescence microscope.

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References


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