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Glycosylation of minor envelope glycoproteins of porcine reproductive and respiratory syndrome virus in infectious virus recovery, receptor interaction, and immune response

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ABSTRACT

The role of *N*-glycosylation of the three minor envelope glycoproteins (GP2, GP3, and GP4) of porcine reproductive and respiratory syndrome virus (PRRSV) on infectious virus production, interactions with the receptor CD163, and neutralizing antibody production in infected pigs was examined. By mutation of the glycosylation sites in these proteins, the studies show that glycan addition at N184 of GP2, N42, N50 and N131 of GP3 is necessary for infectious virus production. Although single-site mutants of GP4 led to infectious virus production, mutation of any two sites in GP4 was lethal. Furthermore, the glycosylation of GP2 and GP4 was important for efficient interaction with CD163. Unlike PRRSVs encoding hypoglycosylated form of GP5 that induced significantly higher levels of neutralizing antibodies in infected piglets, PRRSVs encoding hypoglycosylated forms of GP2, GP3 or GP4 did not. These studies reveal the importance of glycosylation of these minor GPs in the biology of PRRSV.

Introduction

The porcine reproductive and respiratory syndrome virus (PRRSV) causes porcine reproductive and respiratory syndrome in swine population. The disease is characterized by respiratory distress in piglets and late term reproductive failure and associated complications in pregnant sows (Snijder and Spaan, 2007). This disease was first reported in the United States and later in the Netherlands and other parts of the world (Benfield et al., 1999; Paton et al., 1991). The PRRSV belongs to the order *Nidovirales*, family *Arteriviridae* and genus *Arterivirus*. Other related viruses of family *Arteriviridae* are equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDHV) and simian haemorrhagic fever virus (SHFV) (Snijder and Meulenberg, 1998). Based on their geographic location and genome sequences, PRRSVs are classified as European (type I) or North American (type II) genotypes. These two genotypes share approximately 60% genome similarity (Forsberg, 2005; Hanada et al., 2005).

PRRSV has a positive stranded RNA genome of approximately 15.4 kilobases and contains nine open reading frames (ORFs). ORF1a and ORF1b synthesize polyproteins which are processed to produce 13–14 non-structural proteins (NSPs) (Meulenberg, 2000; van Aken et al., 2006). The NSPs are involved in viral genome replication and polyprotein processing (Kroese et al., 2008; Meulenberg, 2000; Snijder and Meulenberg, 1998). Several of the NSPs, namely, the NSP 1α, NSP 1β, NSP 2, NSP 4, and NSP 11 are also involved in IFN and TNF-α antagonism (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Subramaniam et al., 2010). ORFs 2 to 7 encode seven structural proteins, four of which are glycoproteins [GP2 (previously called GP2a), GP3, GP4, and GP5] that are present on the virion envelope. The protein E (or 2b) and the membrane protein (M) are also present on the envelope, but are not glycosylated. The GP5 is present on the virion envelope in abundant amounts and therefore is called the major envelope glycoprotein whereas the GP2, GP3 and GP4 are called minor envelope glycoproteins as they are present in less abundant amounts. The GP2, GP3 and GP4 interact with each other and GP5 interacts with both GP4 and M protein (Das et al., 2010; Mardassi et al., 1996). These interactions are critical for formation of multiprotein complexes that are required for assembly of infectious PRRSVs (Wissink et al., 2005). Additionally, GP5 and M proteins interact to form heterodimers (Mardassi et al., 1996). GP2 and GP4 proteins have been shown to specifically interact with CD163 molecule (Das et al., 2010), a receptor for PRRSV entry (Calvert et al., 2007; Van Gorp et al., 2008).

The *N*-glycosylation of proteins of many different families of viruses have been shown to be important for tissue tropism, receptor

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interactions, viral entry, protein folding, targeting, secretion, assembly and egress, immune evasion, and pathogenesis (Beasley et al., 2005; Daniels et al., 2003; Fournillier et al., 2001; Hanna et al., 2005; Luftenegger et al., 2005; Mondotte et al., 2007; Shi et al., 2005; Shi and Elliott, 2004; Vigerust and Shepherd, 2007). The *N*-glycosylation of simian immunodeficiency virus, human immunodeficiency virus, Bunyamwera virus, hepatitis C virus, and Ebola virus not only promotes viral replication and infectivity, but also provides a glycan shield against host neutralizing antibodies and thereby facilitates virus spread and influence pathogenic outcome (Lin et al., 2003; Reitter et al., 1998; Scanlan et al., 2007; Shi et al., 2005; Vigerust and Shepherd, 2007; Wei et al., 2003). Previous studies have shown that glycan addition at certain sites in GP5 protein of PRRSV is required for infectious virus production (Ansari et al., 2006; Wissink et al., 2004); glycan addition at other sites in GP5 help the virus escape neutralization by the host antibodies through "glycan shielding" mechanism (Ansari et al., 2006). Additionally, hypoglycosylation of GP5 was also shown to induce significantly higher neutralizing antibody response in PRRSV-infected pigs (Ansari et al., 2006). The role of glycosylation of the minor envelope GPs in infectious PRRSV production and immune response has not been examined yet. Only one report using the Lelystad virus (type I PRRSV) showed that the two *N*-glycosylation sites of GP2 are dispensable for infectious virus production (Wissink et al., 2004).

In this communication, we have performed studies to examine the role of glycosylation of the minor envelope GPs of PRRSV, the GP2, GP3, and GP4 proteins in infectious virus generation, the effect of glycosylation on interaction with the receptor CD163 and if the host neutralizing antibody response can be modulated by hypoglycosylation of the GPs. Our results show that in contrast to the type I Lelystad virus, glycosylation at N184 of GP2 protein of the FL12 virus (a type II PRRSV) is required for infectious virus production. Additionally, glycosylation at residues N42, N50 and N131 of GP3 protein of PRRSV is shown to be critical for infectious virus production. While none of the single glycosylation site mutations in GP4 had any effect on infectious PRRSV recovery, introduction of two or more mutations was found to be lethal. The studies also show that glycosylation of GP2 and GP4 proteins is required for efficient interaction with CD163. The results further reveal that glycosylation of the three minor envelope glycoproteins does not appear to play any role in the neutralizing antibody response mounted by the infected animals.

Results

Expression of minor envelope glycoproteins in transiently transfected and virus-infected cells

PRRSV has four envelope glycoproteins, GP2, GP3, GP4, and GP5 encoded by the open reading frames (ORF) 2a, 3, 4, and 5, respectively (Fig 1A). Bioinformatic analyses using SignalP 3.0 and DAS TMpred programs suggested that each of these GPs has a cleavable signal sequence (SS) and a transmembrane (TM) domain, which are shown in Fig. 1B. The calculated molecular weights of the unglycosylated GP2, GP3, and GP4 following signal sequence cleavage are approximately 25 kDa, 26 kDa, and 17 kDa, respectively. The NetNGlyc 1.0 program also predicted several N-linked glycosylation sites in these proteins, which are schematically depicted in Fig. 1B. In the current study, we wanted to examine the role of glycosylation of the minor envelope glycoproteins in infectious progeny production. Examination of potential glycosylation sites in these proteins of the infectious clone derived FL12 virus suggested that GP2 has two potential glycan addition sites at positions 178 and 184; GP3 has seven such sites at positions 29, 42, 50, 131, 152, 160, and 195; and GP4 has four sites at positions 37, 84, 120, and 130 (Fig. 1B). These sites are somewhat conserved in strains of North American type II PRRSVs. We had

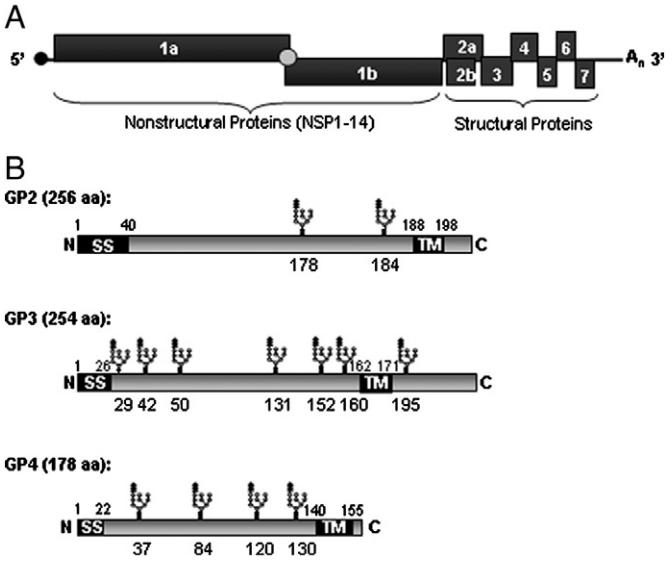

Fig. 1. PRRSV genome organization and the three minor envelope glycoproteins. (A) Schematic representation of PRRSV genome organization. The ORFs are shown as solid rectangles from 5' to 3' end with their names shown. The 5' cap is shown with a dark-filled circle; the ribosomal frame-shifting position between ORF1a and ORF 1b is shown with a gray-filled circle. (B) Linear structures of various minor envelope glycoproteins (GPs). The length of each of the proteins in amino acids and the potential *N*-glycosylation sites are shown. The length of the predicted signal sequences (SS) and transmembrane regions (TM) of the proteins are shown on top of the rectangles. N, amino-terminus; C, carboxy terminus.

previously shown that glycosylation of GP5 at amino acid position 44 is required for infectious virus production (Ansari et al., 2006). For the North American type II PRRSV and in particular, for the FL12 virus, it is not known which of the potential glycosylation sites are indeed used for glycan addition in these proteins.

Toward this goal, we first examined expression of the individual minor envelope GPs in cells transfected with plasmids encoding these proteins as well as in cells infected with PRRSV. In MARC-145 cells infected with infectious clone (FL12) derived virus or in baby hamster kidney-21 (BHK-21) cells transfected with GP2-encoding plasmid, the mature form of GP2 (identified by a white dot) was synthesized as an approximately 32 kDa protein (Fig. 2A). However, under both experimental conditions, the major species of the protein migrated with a molecular mass of approximately 29–30 kDa. This protein species is most likely the GP2 protein having *N*-glycan addition in only one of the two predicted sites. Thus, it appears that glycan addition at one site in GP2 is more efficient or that the addition of glycan at the second site is slow. Unlike GP2 protein, the major species of GP3 synthesized in virus-infected cells or in plasmid-transfected cells corresponded to a 42 kDa protein (Fig. 2B). However, significantly reduced amounts of a number of faster migrating protein species could also be detected. The identity of these proteins is unknown but could be non-specific cellular proteins immunoprecipitated by the GP3 antibody. The GP4 protein synthesized in PRRSV-infected cells was detected as a single major species of approximately 29 kDa but in addition to this protein, smaller proteins, possibly representing partially glycosylated forms of GP4 or some cellular proteins, were also detected in GP4 encoding plasmid-transfected cells (Fig. 2C).

The observation that single and multiple glycosylation mutant proteins co-migrated with the smaller protein species (see Figs. 3A and 5A) and the fact that endoglycosidase H digestion of the proteins resulted in detection of single protein species (see Das et al., 2010) suggest that the smaller species are partially glycosylated forms of the proteins and argue against the possibility that they are the degradation products of the full-length proteins.

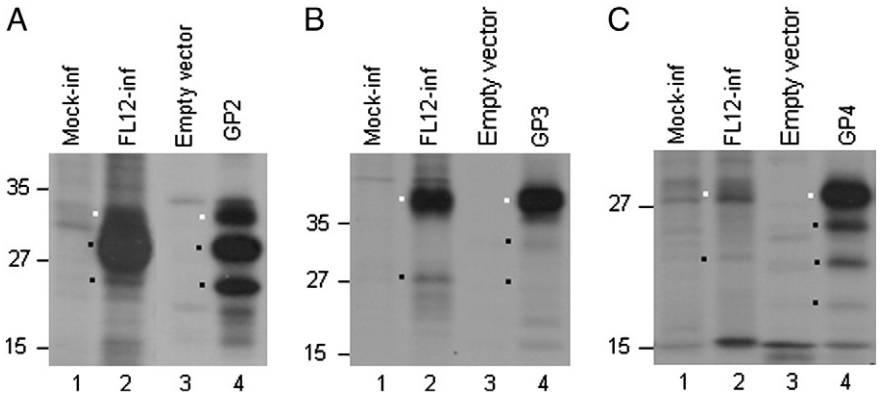

Fig. 2. Expression of minor envelope GPs in virus-infected and plasmid-transfected cells. MARC-145 cells were mock-infected or infected with wt FL12 virus (MOI of 1) or BHK-21 cells were infected with vTF7-3 virus and subsequently transfected with an empty vector or the vector encoding individual envelope GPs as described in materials and methods. The FL12 virus-infected MARC-145 cells were radiolabeled with ^{35}S after 48 hpi (lanes 1 and 2) whereas the transfected BHK-21 cells were radiolabeled with ^{35}S after 16 h post-transfection (lanes 3 and 4). The radiolabeled proteins from the cell extracts were immunoprecipitated with anti-GP2 (A), anti-GP3 (B) or anti-GP4 (C) antibody, resolved in SDS–10% PAGE and detected by fluorography. The fully glycosylated GPs are denoted by white dots whereas the partially glycosylated proteins are denoted by black dots. The relative mobilities of the molecular mass markers in kDa are shown on the left of each panel.

Glycan addition at amino acid position 184 of GP2 protein is important for infectious virus recovery

The GP2 protein of European type I PRRSV contains two *N*-glycosylation sites and it has been shown that glycan addition at these sites is dispensable for infectious virus production (Wissink et al., 2004). The GP2 of North American type II virus (FL12 virus) has two predicted glycosylation sites at positions 178 and 184. To examine if one or both of these sites are used for glycan addition and whether glycosylation of these sites are required for infectious virus

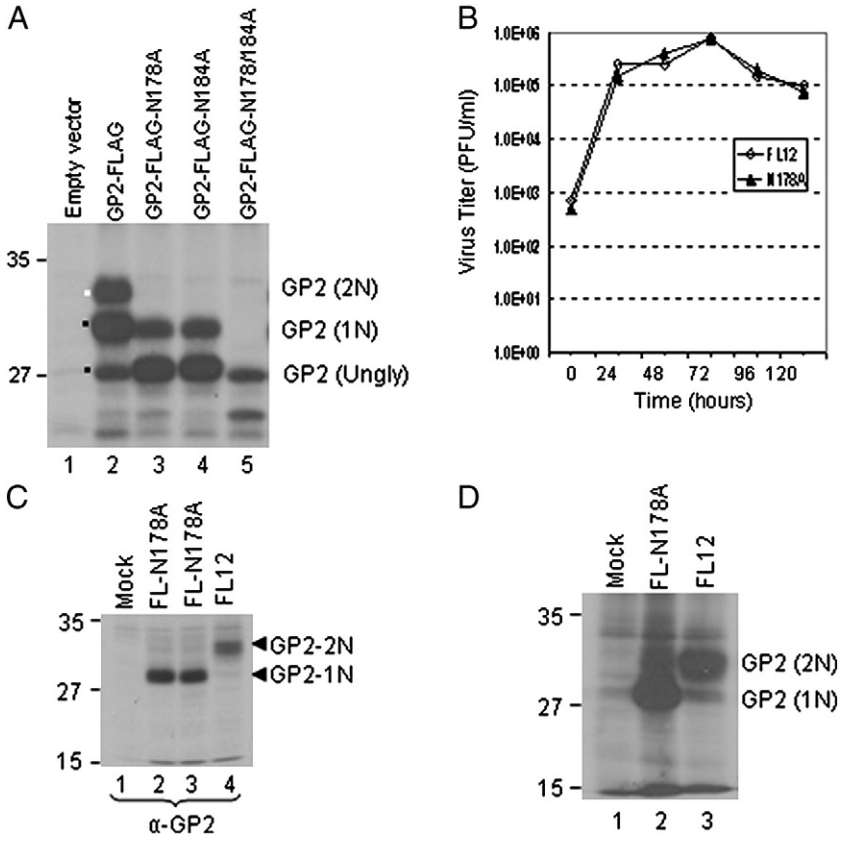

Fig. 3. *N*-glycosylation of residue N184 of GP2 is required for infectious virus production. (A) BHK-21 cells were infected with vTF7-3 virus and transfected with empty vector (lane 1), vector encoding GP2 (lane 2) or its *N*-glycosylation site mutants (lanes 3–5). Cells were radiolabeled for 4 h at 16 h post-transfection, and the proteins were analyzed by immunoprecipitation with anti-FLAG antibody, resolved in SDS-12%PAGE and detected by fluorography. The fully glycosylated wt GP2 is indicated by white dot whereas, the partially glycosylated or unglycosylated forms are indicated by black dots on the left of lane 2. Various glycosylated forms of GP2 are shown on the right side of the fluorogram. (B) Representative multi-step growth kinetics of wt FL12 and FL12-N178A mutant virus. (C) Detection of mutant GP2 protein in cells infected with 2nd (lane 2) or 10th (lane 3) passage FL-N178A virus or wt FL12 (lane 4) virus. MARC-145 cells were infected with the passaged virus at an MOI of 1, cells were radiolabeled as described in the materials and methods, the proteins were immunoprecipitated with anti-GP2 antibody, resolved by SDS–10% PAGE and detected by fluorography. (D) Detection of GP2 in cells infected with virus recovered from sera after 21 days post-inoculation of piglets with FL12-N178A or wt FL12 virus. The experiment was performed similarly as described for virus-infected cells in Fig. 2, except that the sera collected from animals that were sham-infected (lane 1), infected with FL12-N178A virus (lane 2), or FL12 virus (lane 3) were used for infection of naive MARC-145 cells. The relative mobilities of molecular mass markers in kDa are shown on the left side of each of the fluorograms.

production, the asparagine (N) residues at these positions were replaced with alanine (A) residues to generate two single (N178A and N184A) and one double (N178/184A) glycosylation mutants. These mutations were generated using a carboxy terminal FLAG-tagged GP2 construct. In cells transfected with plasmids encoding the mutants, synthesis of various mutant proteins could be readily detected (Fig. 3A). Although the FLAG-tagged wt GP2 migrated as a 33 kDa protein, the GP2-FLAG-N178A and GP2-FLAG-N184A proteins migrated with a molecular weight of around 31 kDa (Fig. 3A, lanes 3 and 4), the GP2-FLAG-N178/184A double glycosylation mutant protein migrated with a molecular mass of 28 kDa (Fig. 3A, lane 5), suggesting that both the glycosylation sites are used for glycan addition in GP2.

The mutant GP2 coding sequences (without FLAG-tag) were then inserted into full-length infectious clone FL12 (Truong et al., 2004) genome and virus rescue was performed by electroporation of in vitro transcribed RNA into MARC-145 cells. Four to five days after electroporation, cytopathic effect (CPE) was only observed in cells transfected with RNA from FL12-GP2-N178A construct but not in cells transfected with RNA from either FL12-GP2-N184A or FL12-GP2-N178/184A mutant constructs. Multiple attempts using at least two independent clones to recover virus from FL12-GP2-N184A or FL12-GP2-N178/184A mutant construct failed, indicating that glycan addition at position 184 in GP2 is critical for recovery of infectious virus. Following rescue of FL12-N178A mutant virus, examination of multi-step growth kinetics of the mutant virus showed no apparent difference from that of the wt virus (Fig. 3B), suggesting that glycosylation at position 178 has no effect on growth of the mutant virus in MARC-145 cells.

Since no differences were observed in growth kinetics of the mutant virus, we examined if the mutant virus still maintained the N178A amino acid substitution in the genome. The presence of correct mutation in FL-N178A recovered virus was confirmed by RT-PCR amplification and sequencing of the entire GP2 coding region. Furthermore, no other mutations were detected in GP2. In cells infected with the passage 2 and passage 10 mutant viruses, the GP2-N178A protein migrated with a mobility that correlated with loss of one glycosylation site (Fig. 3C). The GP2 proteins in these mutant virus-infected cells (lanes 2 and 3) clearly migrated faster than the GP2 protein synthesized in wt FL12 virus-infected cells (lane 4), demonstrating the stability of the mutant virus under in vitro passage conditions. Similarly, the cells infected with the virus present in sera collected on 21 days post-inoculation from FL12-N178A infected piglets also synthesized GP2-N178A protein which migrated with a mobility that correlated with loss of one glycan site (Fig. 3D, lane 2) as compared to GP2 synthesized in cells infected with the virus present in sera collected from piglets infected with wt FL12 virus (Fig. 3D, lane 3). These results suggest that the mutant FL12-N178A virus is stable under in vitro and in vivo growth conditions.

Glycosylation at several sites in GP3 is critical for infectious virus production

The GP3 protein contains seven potential N-linked glycosylation sites (Fig. 1B). To determine which of the sites are used for glycan addition, we mutated N to A at these sites. The wt or mutant GP3 sequences were cloned and expression of the proteins was examined in cells transfected with the plasmids encoding the proteins. While the wt GP3 migrated with a molecular weight of approximately 42 kDa (Fig. 4A, lane 2), the proteins expressed from the single glycosylation mutant clones (GP3-N29A, GP3-N42A, GP3-N50A, GP3-N131A, GP-3N152A, GP3-N160A) migrated with a molecular weight of approximately 39–40 kDa (lanes 3–8). On the other hand, one mutant construct GP3-N195A produced GP3 that co-migrated with wt GP3 (lane 9). These results indicate that six sites at positions N29, N42, N50, N113, N152, and N160 are used for glycan addition whereas N195 is not used for glycosylation.

We subsequently introduced the individual glycosylation site mutants of GP3 into the FL12 infectious clone to rescue infectious PRRSV. Upon transfection of MARC-145 cells with in vitro transcripts from each of the clones, CPE was observed in cells 5 days after electroporation of RNA from FL12-GP3-N29A, FL12-GP3-N152A and FL12-GP3-N160A, indicating successful recovery of these mutant viruses. However, following multiple repeat experiments using two independent clones, viruses encoding mutations at the positions N42, N50 or N131 could not be recovered, suggesting that glycan addition at these sites is essential for infectious particle formation. The recovered mutant viruses demonstrated similar multi-step growth kinetics as the wt PRRSV (Fig. 4B), although the FL12-GP3-N29A and FL12-GP3-N152A viruses grew reproducibly to similar or slightly higher titers than the wt FL12 whereas the titers of FL12-GP3-N160A virus were lower. All the three viruses were found to be stable for at least 10 passages in MARC-145 cells as they were found to retain the inserted mutation and synthesized GP3 in infected cells with a mobility that correlated with the loss of one glycosylation site (Fig. 4C, lanes 3–5). These results show that glycosylation at amino acid positions 42, 50 and 131 of GP3 is important for infectious virus production whereas glycan addition at residues 29, 152, and 160 is not required.

Since individual glycan addition at residues 29, 152, and 160 was found to be dispensable for infectious virus recovery, we next examined whether mutations at two or more of these sites would still lead to virus recovery. To address this, we simultaneously mutated these residues in various combinations to obtain GP3-N29/152A, GP3-N29/160A, GP3-N152/160A, and N29/152/160A mutant proteins. All the four mutant proteins were expressed in transfected BHK-21 cells at levels similar to the wt or single-site mutants and possessed electrophoretic mobility that correlated with the loss of two or three glycan moieties (Fig. 4D) Infectious viruses encoding each of the glycosylation mutant GP3 could be readily recovered from cells transfected with corresponding RNA. The recovered viruses were found to retain the incorporated mutation even after 10 passages in MARC-145 cell (data not shown). In multi-step growth kinetics studies, the triple mutant virus (FL12-GP3-N29/152/160A) grew slowly and to significantly reduced titers as compared to the double-mutant viruses (Fig. 4E), indicating that glycosylation of at least two of these sites is needed for optimal generation of infectious viruses. The triple mutant virus (FL12-GP3-N29/152/160A) growth could only be detected at 96 hpi, and the virus growth decayed significantly by 120 hpi. Also, in FL12-GP3-N29/152/160A virus-infected cells, the onset of CPE was significantly delayed. The GP3 proteins synthesized in these mutant virus-infected cells migrated with molecular sizes consistent with loss of two or three glycan moieties and also migrated faster than the GP3 protein synthesized in FL12 virus-infected cells (Fig. 4F). Overall, these results show that simultaneous loss of glycans at residues 29, 152 and 160 affected optimal virus growth and infectious virus production.

Mutation of individual glycosylation sites in GP4 does not affect infectious virus recovery but multiple mutations are lethal

GP4 protein has four predicted *N*-glycosylation sites at positions 37, 84, 120, and 130 (Fig. 1B). These sites were mutated individually to obtain the clones GP4-N37A, GP4-N84A, GP4-N120A, and GP4-N130A. In plasmid-transfected cells, the proteins encoded in these mutant clones migrated as approximately 26 kDa proteins, as compared to the wt GP4 that possessed an electrophoretic mobility of 29 kDa (Fig. 5A). This result indicated that all the four potential glycosylation sites in GP4 are indeed used for the modification. To determine if the glycan addition at all four sites is necessary for recovery of infectious PRRSV, we introduced the mutations in GP4 coding regions of infectious clone FL12 and found that PRRSVs encoding the single glycosylation site mutant GP4 could be readily recovered. However, insertion of mutations at

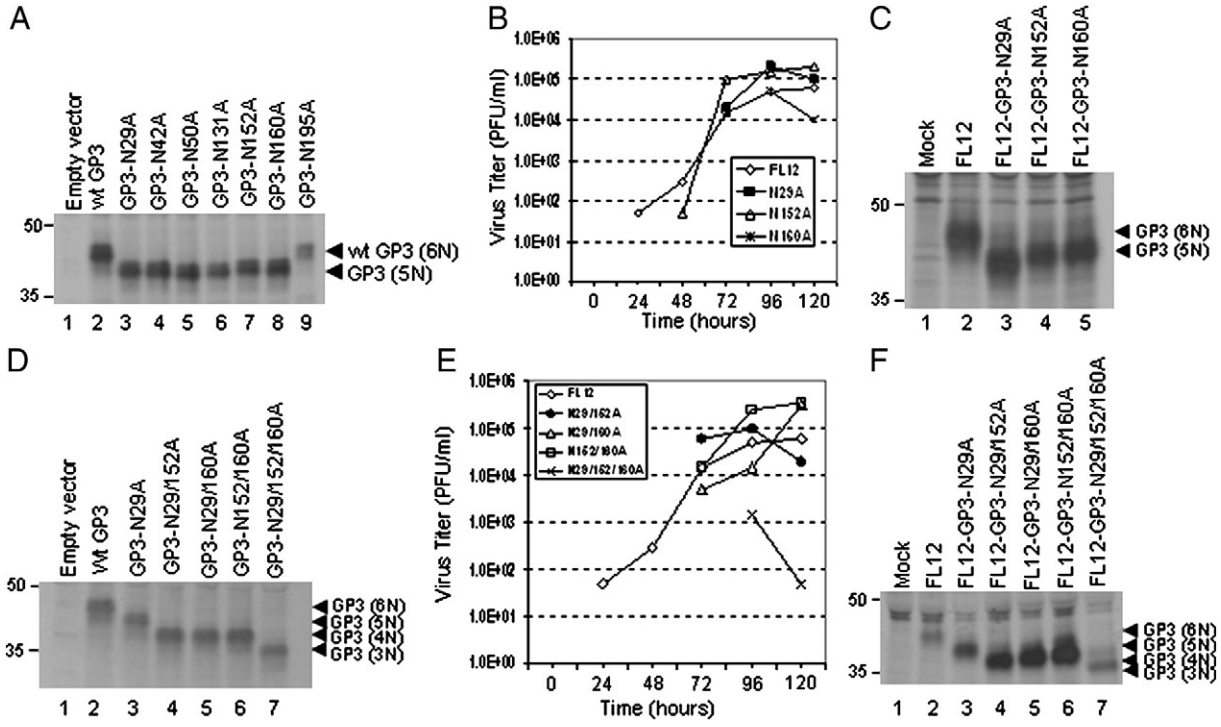

Fig. 4. Glycan addition at several sites in GP3 is required for infectious virus production. (A) BHK-21 cells were infected with vTF7-3 and transfected with pGEM3 (empty vector, lane 1), the plasmid encoding wt GP3 (lane 2) or its glycosylation site mutants (lanes 3–9). Cells were radiolabeled for 4 h at 16 h post-transfection, and the proteins were analyzed by immunoprecipitation with anti-GP3 antibody, resolved in SDS-12%PAGE and detected by fluorography. (B) Representative multi-step growth kinetics of viruses encoding GP3 single-site glycosylation mutants. (C) Synthesis of GP3 protein in cells infected with the wt FL12 or GP3 mutant viruses. The experiment was performed with passage 10 mutant viruses. (D) Expression of GP3 double and triple *N*-glycosylation mutants in BHK-21 cells. The experiment was performed as described in panel A and various glycosylated forms of the protein are identified on the right. (E) Representative multi-step growth kinetics of viruses encoding GP3 double and triple glycosylation mutants. (F) Expression of wt or mutant GP3 in cells infected with wt FL12 or mutant viruses encoding double or triple glycosylation mutant GP3 viruses. Tenth passage virus was used and the experiment was performed as described in panel C. The relative mobilities of molecular mass markers in kDa are shown on the left side of each of the fluorograms.

more than one site was found to be lethal for virus recovery. The multi-step growth kinetics analysis (Fig. 5B) as well as infectious virus yield assays indicated that all the mutant viruses had higher titers than the wt virus. Unlike the wt FL12 virus, which typically grows to its highest titer at 72 hpi, the GP4 single mutant viruses grew to their highest titers at 48 hpi. At this time point, the titers of FL12-GP4-N120A and FL12-GP4-N130A mutant viruses were almost 10- to 50-fold greater than that of wt FL12. The reason(s) for the better growth of these mutant viruses are not known at this time but could be related to more favorable interactions with the virus receptor in MARC-145 cells or more efficient formation of multi-glycoprotein complex, which is required for infectious virus production.

In the MARC-145 cells infected with the single glycosylation site mutant viruses, GP4 proteins migrated with a mobility that correlated with the loss of one glycosylation site (Fig. 5C, lanes 2–5). Furthermore, in MARC-145 cells infected with sera collected from pigs at 21 days post-inoculation, GP4 protein from the four GP4 single glycosylation mutants migrated with mobility faster than that of the wt protein (data not shown). Sequence analysis of the viral genomes isolated from the sera of infected pigs or from the culture supernatants of in vitro passaged virus showed that the introduced mutations were present and no other additional mutations were detected in the GP4 coding regions, indicating that the mutant viruses were stable under in vivo and in vitro passage conditions.

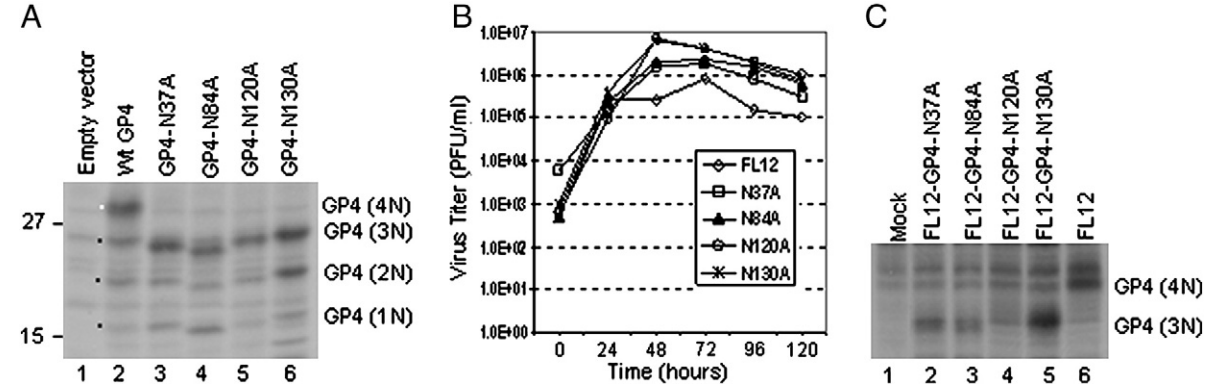

Fig. 5. GP4 proteins with mutation of single glycosylation sites do not affect infectious virus production. (A) Expression of GP4 single glycosylation mutants in transfected cells. The experiment was conducted as described in Fig. 4A. Proteins were detected using anti-GP4 antibody. The fully glycosylated GP4 proteins is identified by white dot, whereas the partially glycosylated forms of the proteins are identified by black dots, on the left side of lane 2. The relative mobilities of molecular mass markers in kDa are shown on the left side of the fluorogram. (B) Representative multi-step growth kinetics of GP4 single glycosylation mutant viruses. The experiment was performed as described in Fig. 3B. (C) Expression of GP4 proteins in cells infected with tenth passage GP4 glycosylation mutant viruses. The experiment was conducted as described in Fig. 4C using anti-GP4 antibody.

Glycosylation of GP2 and GP4 is required for efficient interaction with the receptor, CD163

We had previously shown that porcine CD163, a receptor for PRRSV, interacts specifically with GP2 and GP4 proteins of PRRSV (Das et al., 2010). Therefore, we wanted to examine if glycosylation of these two proteins plays any role in their interactions with the CD163 molecule by co-IP assays as described previously (Das et al., 2010). The receptor CD163 was co-expressed with the FLAG-tagged wt or glycosylation mutant GP2 proteins in plasmid-transfected cells. The interacting protein complexes were co-immunoprecipitated with anti-FLAG polyclonal antibody or anti-CD163 monoclonal antibody and resolved in SDS–10% PAGE gel. By using anti-FLAG antibody, CD163 could be readily detected by immunoprecipitation of cell lysates co-expressing CD163 and GP2-FLAG, GP2-FLAG-N178A or GP2-FLAG-N184A proteins (Fig. 6A, lanes 9–11). But, CD163 molecule could not be detected when it was co-expressed with completely unglycosylated form of GP2 (GP2-FLAG-N178/184A) protein (Fig. 6A, lane 12). When we carried out the converse experiment using anti-CD163 monoclonal antibody, the results showed that CD163 antibody could detect the wt and single glycosylation mutant GP2 proteins, but none to very low levels of completely unglycosylated GP2 was detected (data not shown). These results suggest that glycosylation of at least one site in GP2 protein is necessary for efficient interaction with the receptor CD163.

A similar study was performed to examine the role of *N*-glycosylation of GP4 protein in its interaction with CD163 (Fig. 6B). Since the single glycosylation mutant GP4 supported recovery of infectious PRRSVs, it was presumed that these mutants will interact with CD163. Therefore, in these studies, we used only one double mutant (GP4-N37/84A) and the quadruple mutant (GP4-N37/84/120/130A) that synthesizes the completely unglycosylated form of GP4 protein for their interactions with CD163. In cells co-expressing CD163 and the wt or the mutant GP4 proteins, interaction between CD163 and GP4 was found to be dependent on the extent of glycosylation of GP4. Unlike the wt GP4, which interacts well with CD163 (Das et al., 2010), the GP4 mutant with only two glycan moieties interacted less well with CD163 (Fig. 6B, lane 8). Further, the interaction of the unglycosylated GP4 with CD163 was very inefficient as only a very small amount of CD163 could be immunoprecipitated with GP4 antibody (Fig. 6B, lane 9). Similar results were obtained in multiple repeat experiments using these mutants as well as using the other double glycosylation mutant GP4 proteins, indicating that the extent of glycosylation of GP4 is critical for efficient interaction with CD163.

Mutant PRRSVs encoding hypoglycosylated forms of minor GPs do not induce enhanced neutralizing antibody response

We previously demonstrated that PRRSVs encoding hypoglycosylated forms of the major envelope glycoprotein GP5 induced significantly higher levels of neutralizing antibody response in infected piglets (Ansari et al., 2006). To determine if PRRSVs encoding hypoglycosylated forms of the minor envelope GPs can also induce higher neutralizing antibody response in infected animals, we used the wt FL12 or several mutant viruses encoding hypoglycosylated minor GPs. Groups of four 21-day-old piglets were injected with the wt or the mutant viruses as described previously (Ansari et al., 2006). Following infection, sera were collected from each animal at days 7 and 46 post-infection. Neutralizing antibody titers in sera collected at days 7 and 46 post-infection were measured using the fluorescent focus neutralization assay as described (Ansari et al., 2006; Wu et al., 2001). Results (not shown) revealed no significant differences in neutralizing antibody titers produced in wt or mutant virus-infected animals, indicating that hypoglycosylation of any of the minor envelope GPs do not induce higher neutralizing antibody response in infected animals.

Discussion

Viruses use host cell machinery for glycosylation of the viral envelope proteins (Vigerust and Shepherd, 2007). PRRSV has four envelope glycoproteins GP2, GP3, GP4, and GP5. Our previous study on GP5 protein had shown that glycan addition at residue N44 is required for infectious virus production whereas glycans at residues N34 and N51 suppress host neutralizing antibody response in infected piglets (Ansari et al., 2006). Those studies also revealed that PRRSVs encoding hypoglycosylated forms of GP5 are exquisitely sensitive to neutralization by antibodies and that the mutant viruses induce significantly higher amounts of neutralizing antibodies against homologous mutant viruses as well as against wt PRRSV (Ansari et al., 2006). The current study was undertaken to determine if glycosylation of the other envelope glycoproteins, namely, the GP2, GP3, and GP4 play any role in production of infectious virus, interactions with the cell surface receptor, CD163, and in neutralizing antibody response by the host. These studies led us to conclude that (i) glycan addition at N184 of GP2, N42, N50, and N131 of GP3 is required for infectious virus production, (ii) glycosylation of any three of the four sites in GP4 is necessary for recovery of infectious PRRSV, (iii) optimal interactions with CD163 is

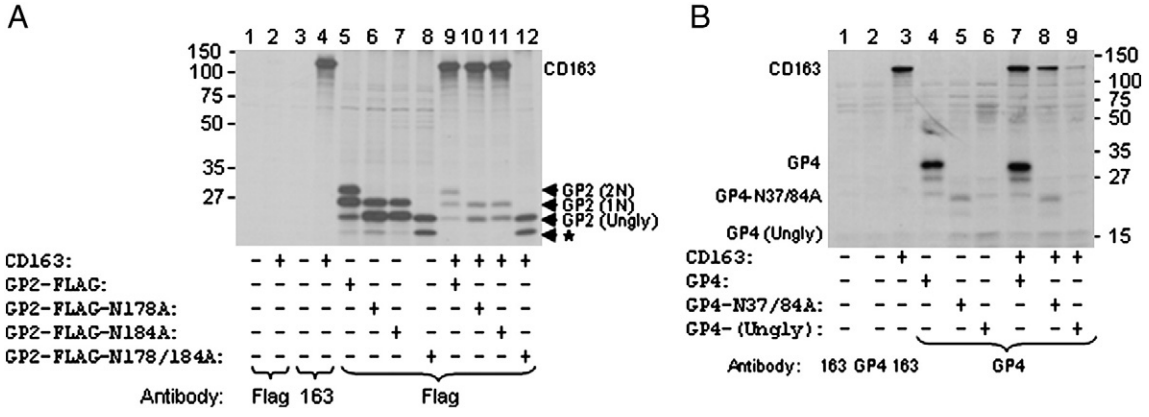

Fig. 6. Glycosylation of GP2 and GP4 proteins is required for efficient interaction with CD163. (A) Examination of CD163 interaction with wt or mutant GP2. BHK-21 cells were infected with vTF7-3 virus and transfected with empty vector (lanes 1 and 3) or the plasmid encoding CD163 and wt GP2-FLAG or glycosylation mutant GP2-FLAG proteins. − and + indicate without or with the plasmid shown on the left. The cells were radiolabeled and immunoprecipitated with specific antibodies as shown below the lanes. The proteins were resolved in SDS–10% PAGE and detected by fluorography. CD163 and different glycosylated forms of GP2 proteins are identified on the right. The band identified with an asterisk is possibly the signal sequence cleaved unglycosylated GP2. (B) Examination of CD163 interactions with wt or mutant GP4 proteins. The experiment was performed as described in panel A, proteins were immunoprecipitated with antibodies shown below the lanes. − and + indicate without or with the plasmid shown on the left. CD163 and various GP4 mutants are identified on the left. The relative mobilities of the molecular mass markers in kDa are shown on the right side of each panel.

dependent on glycosylation of GP2 and GP4 proteins, and (iv) *N*-glycosylation of minor envelope proteins do not play any measurable role in viral immune evasion against host neutralizing antibody response or enhance host neutralizing antibody response.

Examination of GP2 expression in virus-infected cells and in plasmid-transfected cells indicates that one of the two *N*-glycosylation sites is preferentially glycosylated. However, with the use of individual glycosylation site mutants, it was not possible to identify the site that is preferentially glycosylated as the amount of monoglycosylated GP2 was found to be similar in both of these mutants (Fig. 3A). It is plausible that glycosylation at one sequon in GP2 influences glycosylation at the second sequon, as has been observed for rabies virus G protein (Wojczyk et al., 2005). It is unknown how glycosylation at one sequon affects glycan addition at another sequon in the same protein. Studies addressing this question have so far yielded conflicting results. Glycan addition at one sequon in soluble rat CD4 did not influence glycan addition at the other sequon (Ashford et al., 1993); insertion or deletion of novel or existing sequons in the alpha subunit of human chorionic gonadotropin was shown to have no effect on glycan addition at other sequons in the protein (Furuhashi and Suganuma, 2003). In contrast, insertion of a novel sequon at two distinct but closely located positions in human tissue plasminogen activator had disparate effects on glycan addition at another site in the protein (Pfeiffer et al., 1994). Since it has been proposed that the glycosylation site must be at least 75 codons upstream of the termination site for efficient glycan addition (Ruiz-Canada et al., 2009), it is possible that one of the two sites (N178 or N184) in GP2 passes through the oligosaccharyl transferase active site on the ER membrane without being efficiently glycosylated. Further studies will be required to gain a better understanding of glycosylation of GP2 and how glycosylation at one site affects glycan addition at the other site.

Our study has revealed that out of the seven predicted potential glycosylation site in GP3, N195 is not glycosylated. The bioinformatics analysis shows that this residue has an intracytoplasmic orientation, perhaps making it unavailable for glycosylation by the host enzymes in the lumen of the ER. Studies with prototype foamy virus had revealed that the glycan addition site present on the signal peptide is not glycosylated (Luftenegger et al., 2005). The results obtained from our studies concur with the recent findings from the analysis of *N*-glycoproteome (Zielinska et al., 2010), which suggests that the sites that are glycosylated, always orient toward the lumen of the ER, Golgi, lysosome, or peroxisome but not toward the cytoplasmic face.

The observation that glycosylation at N184 of GP2 is required for infectious PRRSV production is intriguing. The FL12 virus used in our study is a type II PRRSV. A previous study with GP2 protein of Lelystad virus (type I PRRSV) showed that both the *N*-glycosylation sites in GP2 (N173 and N179) are dispensable for infectious virus production (Wissink et al., 2004). Therefore, these results indicate that there are differences in the requirement for glycosylation of GP2 for infectious virus production in these two genotypes of PRRSV. Differences in the requirement of glycosylation of GP5 proteins of these two types of viruses for infectious virus production have also been demonstrated previously (Ansari et al., 2006; Wissink et al., 2004). Thus, it appears that these two viruses may have evolved to have different requirements for glycosylation of their GPs for production of infectious viruses. Whether such differences exist for the other GPs and whether these differences contribute to their growth and pathogenic potential remains to be investigated.

Glycan addition at several sites (N42, N50, and N131) located at the amino-terminal half of GP3 is required for infectious virus production. Since glycosylation of residues located within the first 50 residues at the amino-terminus of a protein has been proposed to play critical roles in proper folding of the nascent protein through interactions with the ER chaperones (Helenius and Aebi, 2001), it is possible therefore, that without glycans at these positions, the GP3 adopts a protein conformation that is not favorable for infectious particle production. Alternatively, the mutant may exhibit altered interactions with the other viral GPs in a manner that affect multiprotein GP assembly required for infectious particles. Likewise, glycan addition at N184 of GP2 may be required for proper folding of the protein or its interactions with the other GPs for infectious virus production. Although individual mutations at any of the glycosylation sites in GP4 were found to be dispensable, double glycosylation mutations were lethal for infectious virus recovery. These results further concur with studies from other viruses that by mutating single or multiple glycosylation sites in the viral GPs, proper folding of the mutant protein is impaired in such a manner that affects infectious virus assembly. Defects in protein folding, intracellular trafficking, virion assembly, or egress have been observed for a number of enveloped viruses including influenza virus, Hantaan virus, Dengue virus and Japanese encephalitis virus when their envelope glycoproteins were mutated at the glycosylation sites (Kim et al., 2008; Mondotte et al., 2007). It will be interesting to determine how the partially glycosylated forms of the GPs affect interactions with other GPs of PRRSV. It is known that the PRRSV GPs, when expressed individually, are glycosylated with high-mannose sugars and are retained in the ER whereas in virus-infected cells, the GPs form heteromultimers and are further processed in the Golgi (Ansari et al., 2006; Wissink et al., 2004) for packaging in the virions. It is then possible that the mutant viruses may have altered heteromultimer formation and this may influence infectious progeny production.

The observation that single glycosylation site mutants of GP4 grew consistently to higher titers than the wt virus is reminiscent of the results with Sindbis virus (Knight et al., 2009), in which single glycosylation mutations in the E2 glycoprotein resulted in viruses with better replication characteristics. This was correlated with higher affinity of the mutant viruses for binding to glycosaminoglycan heparan sulfate for initial interaction with the host cell. Although the single glycosylation mutant GP4 proteins did not bind to the PRRSV receptor CD163 with increased affinity (data not shown), it is possible that these mutant proteins fold in a manner that is more favorable for infectious virus production as compared to the wt GP4. This could potentially be achieved through increased interactions with other viral GPs, incorporation into viral envelope, and/or interactions with other host cell molecules that mediate PRRSV entry and replication. Further studies will be needed to determine how the single glycosylation mutant GP4 proteins enhance virus infectivity or virus production.

Our results indicate that glycan addition at the two sites in GP2 may have different role for the functions of the protein. While glycosylation at N184 is required for infectious progeny production, glycan addition at N178 is not required. On the other hand, GP2 interaction with CD163 requires glycosylation at any one of these two sites. The glycan addition in GP4 may also have a similar role in its functions. Although infectious virus could not be recovered with GP4 containing mutations at any two of the four sites, one of the double mutants tested in our study interacted with CD163, albeit less efficiently.

Glycosylation of GP5 of PRRSV was previously reported to be required for conferring protection against host neutralizing antibody response (Ansari et al., 2006). The studies reported here reveal that there is no such effect of glycosylation of GP2, GP3, and GP4 proteins on protection against neutralizing antibody response and/or enhanced induction of neutralizing antibodies. It may be due to the fact that these three minor envelope glycoproteins are not abundantly expressed on the surface of the virion. Alternatively, potentially 'malleable glycan shield' as proposed for HIV-1 (Wei et al., 2003) could exist, in which case the glycan moieties on GP5 may block access of the neutralizing antibodies to the minor GPs even if they are unglycosylated. Glycan shield generally requires co-operative packing of entire envelope protein complex to prevent antibody access (Wei et al., 2003) due to steric hindrance, thus preventing binding of a neutralizing antibody to an epitope. Our results suggest that either these three GPs do not contain PRRSV neutralizing epitopes or that the glycan shielding mechanisms do not operate in these GPs. It should be noted, however, that the GP3 of North American type I strains seem to possess neutralizing epitopes (Jiang et al., 2007; Kim and Yoon, 2008; Yang et al., 2000). So the latter possibility appears to be more likely.

Materials and methods

Cell cultures and viruses

Growth and maintenance of BHK-21 and MARC-145 (a derivative of MA-104) cells have been described previously (Das et al., 2010). Recovery of infectious clone derived FL12 wt and mutant PRRSVs was described before (Truong et al., 2004). Preparation of stocks of recombinant vaccinia virus (vTF7-3) expressing the bacteriophage T7 RNA polymerase (Fuerst et al., 1986) was described before (Ansari et al., 2006).

Reagents and antibodies

The mMESSAGE mMACHINE ultra T7 kit for preparation of transcripts in vitro was purchased from Ambion Inc. (Austin, TX). The Pfu-turbo DNA polymerase enzyme was purchased from Stratagene (La Jolla, CA). The monoclonal antibody SDOW17 against the nucleocapsid (N) protein of type I PRRSV (Nelson et al., 1993) was purchased from National Veterinary Services Laboratory (Ames, Iowa). The monoclonal porcine CD163 antibody was purchased from AbD Serotec (Raleigh, NC). Anti- FLAG polyclonal antibody was purchased from Sigma-Aldrich. The anti-GP5 antibody was kindly provided by Dr. Carl Gagnon (University of Montreal, Canada). Polyclonal antibodies for GP2, GP3, and GP4 were reported before (Das et al., 2010; de Lima et al., 2009). Secondary antibody Alexa fluor-488 goat anti-mouse immunoglobulin G (IgG) antibody was purchased from Molecular Probes.

Plasmid construction

The intermediate vector carrying the sequences spanning the structural protein coding region and the 3'-UTR from the infectious clone pFL12 (Truong et al., 2004) has been described previously (Ansari et al., 2006; Das et al., 2010). This intermediate vector was used for subcloning of mutants of GP2, GP3, and GP4 into pFL12 infectious clone. Additionally, the coding regions of the individual GPs were amplified by polymerase chain reaction (PCR) and cloned in pGEM3 vector (Promega Biotech, Madison, WI) under the control of T7 RNA polymerase promoter. Glycosylation site mutants [changing asparagine (N) to alanine (A) residue] of various GPs were generated by the megaprimer-based PCR approach (Sarkar and Sommer, 1990) using the primers described in Table 1 and either the intermediate vector or the vector carrying the individual GPs. The coding regions of the GPs harboring the mutations were further subcloned into pFL12 using appropriate restriction enzymes as described previously (Ansari et al., 2006; Das et al., 2010). The carboxy terminal FLAG (DYKDDDDK)-tagged GP2 (GP2-FLAG) has been described before (Das et al., 2010). The GP2-FLAG containing N to A mutation was further amplified by using the primers GP2-NheI-For and GP2-FLAG-NheI-Rev (Table 1) and cloned in a modified pGEM3 vector which in addition to T7 RNA polymerase promoter also contains cytomegalovirus (CMV) promoter from pCDNA3.1(+) as described (Das et al., 2010). Mutations in GP3 and GP4 were introduced similarly using specific primer (Table 1) and the mutant coding sequences were transferred to the infectious clone FL12 through the intermediate vector. Constructs carrying multiple glycosylation site mutations were generated by fragment exchange using appropriate mutant constructs. The plasmid encoding the porcine CD163 has been described before (Das et al., 2010). Further details of the construction of various mutants can be obtained from the corresponding author.

Transfection, in vitro transcription, and electroporation

Transfection of plasmids containing genes under T7 RNA polymerase promoter was performed using Lipofectamine2000 as described before (Ansari et al., 2006; Das et al., 2010). The in vitro transcripts were generated using mMESSAGE mMACHINE ultra T7 kit as per the manufacturer's protocol. In vitro transcription reaction and electroporation of MARC-145 cells was performed as described before

Table 1
Primers used in this study.

Primer name	Primer nucleotide sequence
GP2-EcoRI-For	ATATATGAATTCGCCACCATGAAATGGGGTCCATGC
GP2-SphI-Rev	ATATATGCATGCTCACCGTGAGTTCGAAGG
GP2-NheI-For	ATATGCTAGCGCCCGCCACCATGAAATGGGGTCCATGC
GP2-FLAG-EcoRI-Rev	ATATGAATTCTCACTCGAGCTTGTCATCGTCGTCCTTGTAGTCCATCCGTGAGTTCGAAGGAAAAATTGC
GP2-N178A For	CATGACAGGGTCA**GCT**GTAACCATAG
GP2-N184A For	GTAACCATAGTGTAT**GCT**AGTACTTTG
GP2-N178/184A For	CATGACAGGGTCA**GCT**GTAACCATAGTGTAT**GCT**AGTACTTTGAATC
GP3-EcoRI-For	ATATAGAATTCGCCACCATGGCTAATAGCTGTGC
GP3-BamHI-Rev	TATATGGATCCCTATCGCCGCGCGGC
GP3-N29A-Rev	CAGTACGTAGT**AGC**GGAATCCGC
GP3-N42A-Rev	GAGTTCGAAGGAAAA**AGC**GCCCCTAAC
GP3-N50A-For	TCCTTCGAACTCACGGTG**GCT**TACACGGTG
GP3-N131A-For	GGGATAGGG**GCT**GTGAGTCAAG
GP3-N152A-For	CGACGGGGAG**GCC**GCCACCTTGCC
GP3-N160A-For	CGTCATGAC**GCT**ATTTCAGCCG
GP3-N195A-For	GTTGGTTTTA**GCT**GTTTCTTGG
GP4-EcoRI-For	ATATAGAATTCGCCACCATGGCTGCGCCCCTTC
GP4-BamHI-Rev	TATATGGATCCTCAAATTGCCAGTAAGATG
GP4-FLAG-BamHI-Rev	TATATGGATCCTCACTTGTCATCGTCGTCCTTGTAGTCAATTGCCAGTAAGATGGCAAAAAAACAGGC
GP4-N37A-Rev	CGGTAGT**GGC**GGTTTTGATGTC
GP4-N84A-For	CCCGTGTACATCACTGTCACAGCC**GCT**GTCACAGATG
GP4-N120A-Rev	GATGCCTGACAC**AGC**GCCAAATATC
GP4-N130A-Rev	CGTAGCTGGTAAA**GGC**GACACAC
GP5-EcoRI-For	GCCGGAATTCGGAGCCGCCGCCACCATGTTGGGGAGATGCTTGAC
GP5-Myc-XhoI-Rev	ATATATCTCGAGTTACAGATCTTCTTCAGAAATAAGTTTTTGTTCAAGACGACCCCATTGTTC
pBR131-For	CTGGATGCTGTAGGCATAGGCTTG
FL12-12816-For	GGGGCAATTTTTCCTTCGAACTCA CGG
FL12-13177-For	CTACCAACATCAGGTCGATGGCGG
FL12-13465-Rev	CTGTGATGGTGATATACACGGGTG
FL12-14473-Rev	GTCGGCCGCGACTTACCTTTAGAG

Primer sequences are in the 5' to 3' direction. Restriction enzyme sites are underlined. The nucleotides for mutation of asparagine to alanine are shown in bold.

(Ansari et al., 2006; Truong et al., 2004). Following electroporation, roughly 10% of the electroporated cells were separately plated in a 24-well plate for indirect immunofluorescence to check for the biological activity of the transfected RNA. The rest of the electroporated cells were plated in six-well plates for virus recovery.

Viral growth kinetics and plaque assay

Multi-step growth kinetics of wt or various mutant viruses was performed by infecting MARC-145 cells seeded in a six-well plate with 0.1 MOI of FL12 and glycosylation defective mutant viruses. Due to significantly lower titer of some of the recovered GP3 glycosylation mutant viruses, an MOI of 0.001 was used. The MARC-145 cells were infected with the virus following the standard procedure. The cells were incubated at 37 °C for up to 5 days. The supernatant was collected at 12 h intervals and virus titers were determined by plaque assay as described before (Ansari et al., 2006).

Metabolic labeling, immunoprecipitation, and co-immunoprecipitation (co-IP) assays

Metabolic labeling, immunoprecipitation, and co-IP assays were performed as reported previously (Ansari et al., 2006; Das et al., 2010). Appropriate antibodies were used to immunoprecipitate the proteins. The proteins were resolved by electrophoresis in reducing and denaturing polyacrylamide gels and the proteins were detected by fluorography as described previously (Ansari et al., 2006; Das et al., 2010).

Animal experiments and serum neutralization assay

Second or third passage mutant viruses were used in these studies. These studies were conducted as described previously (Ansari et al., 2006). Twenty-one-day-old, recently weaned pigs were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. All animals were negative for anti-PRRSV antibodies as tested by ELISA (IDEXX Labs, Portland, ME). Groups of four pigs were used for each mutant virus. Each group of animal was housed separately and away from the other groups. The pigs were intramuscularly injected with wt or mutant viruses with a viral load of 10^5 $TCID_{50}$/animal. The serum samples were collected on 0, 7, 14, 21, 35, and 46 days post-inoculation. Serum samples of 7, 14, and 21 days were used to examine viremia by isolation on MARC-145 cells. All the serum samples collected on different time points were used to check neutralizing antibody titer by serum neutralization assay.

The serum neutralization assay was performed in 96-well cell culture plates. Serum from each animal was diluted by two-fold serial dilution. Fixed amount of virus was incubated with diluted serum samples for 1 h at 37 C and then used to infect MARC-145 cells. The infected MARC-145 cells were then incubated for 36 h at 37 °C in cell culture incubator, after which the cells were fixed and stained for nucleocapsid protein by SDOW-17 primary antibody. Alexa fluor-488 conjugated goat anti-mouse IgG antibody was used as secondary antibody.

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