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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

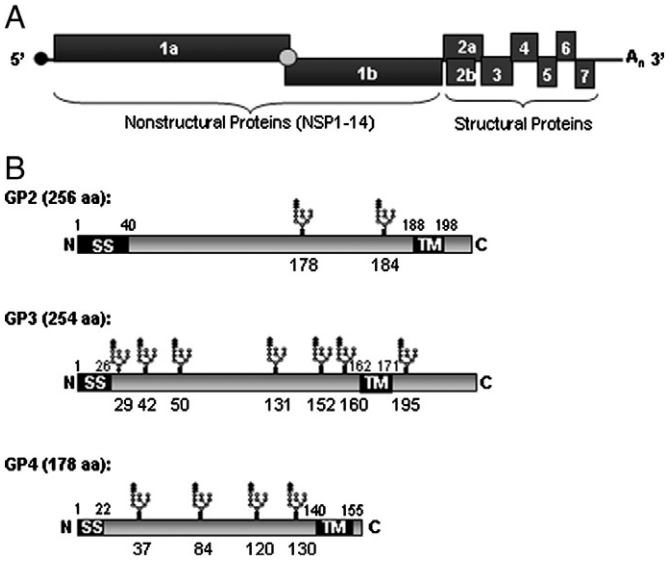

Fig. 1. PRRSV genome organization and the three minor envelope glycoproteins. (A) Schematic representation of PRRSV genome organization. The ORFs are shown as solid rectangles from 5' to 3' end with their names shown. The 5' cap is shown with a dark-filled circle; the ribosomal frame-shifting position 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.