Structural and Functional Characterization of Porcine Reproductive and Respiratory Syndrome Virus N-Glycans

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Juan Li

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Dedication

This dissertation is dedicated to my family, my mentors and all the people that have helped and supported me.

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most severe infectious diseases facing the swine industry worldwide. The etiologic agent, PRRS virus (PRRSV), belongs to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*. It is a positive-sense ssRNA virus and has a smooth spherical envelope embedded with seven proteins, four of which are glycoproteins (GP). Since the major envelope proteins GP5 and matrix (M) have short ectodomains, the broadly distributed viral glycans likely cover the virion surface and stretch out as antennae, thus interacting with host cells and contributing to viral infection. Previous studies suggest potential roles of PRRSV envelope protein-linked glycans in virus assembly, virus attachment to target cells, virus neutralization and antigenicity. In particular, sialic acids on GP5 have been shown to bind sialoadhesin on porcine macrophages, mediating virus attachment and internalization. Nevertheless, the complete profile of GP5-linked glycan compositions and structures, and the role of specific glycan moieties in virus infection have yet to be determined. Herein, we purified the North American prototype PRRSV, VR-2332, and analyzed viral glycans in the aspects of composition, structures and functions in virus infection. Endoglycosidase digestion of virus showed that GP5 was the primary protein substrate among all the four envelope glycoproteins, and that the glycans were primarily complex-type N-glycans. Mass spectrometric analysis (HPLC-ESI-MS/MS) of GP5 N-glycans revealed an abundance of N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers and terminal sialic acids, which was also confirmed by lectin co-precipitation. Based on the structural information, we further demonstrated that GlcNAc and LacNAc oligomer-specific lectins bound to PRRSV and blocked virus attachment, resulting in reduced infection. However, GlcNAc oligomers and LacNAc did not compete with virus to block infection, suggesting that GlcNAc and LacNAc oligomers are not directly involved in virus entry. Finally, removal or alteration of N-glycans from PRRSV

envelope proteins did not affect infection, indicating that envelope protein-linked N-glycans are not required for PRRSV infection. In conclusion, GP5 contains most of the PRRSV glycans, which are primarily complex-type N-glycans. GlcNAc and LacNAc oligomers and sialic acids on the PRRSV envelope are accessible for specific recognition that may reduce infection by steric hindrance. Envelope protein-linked N-glycans are not required for PRRSV infection. Our findings provide a glycan database for molecular structural studies of PRRSV and facilitate a better understanding of molecular host-virus interactions.

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CHAPTER I

Introduction and Literature Review

1. General introduction of PRRSV

1.1. Historical perspective

Porcine reproductive and respiratory syndrome (PRRS) emerged as "Mystery Swine Disease" in the late 1980s due to an unknown causative agent. In U.S. swine herds, the disease outbreaks were reported to show clinical syndromes of reproductive losses (characterized by late-term abortions, increased numbers of stillborns, mummified and weak-born piglets), extensive post-weaning pneumonia, reduction of growth performance, and increased mortality (Hill, 1990; Keffaber, 1989). In Europe, similar outbreaks occurred in Germany in 1990 and spread rapidly across Europe in the following several years (Zimmerman et al., 2006). This disease soon earned another name as "blue-ear pig disease", referring to the syndrome of cyanosis or blue-reddish coloration of pig ears. In Asia, outbreaks were first reported in Japan in 1988 (Hirose et al., 1995) and in Taiwan in 1991 (Chang et al., 1993).

The etiologic agent PRRSV was first isolated as the type 1 European strain, Lelystad virus (LV), in the Netherlands (Wensvoort et al., 1991) and shortly after as the type 2 North American strain, VR-2332, in the USA (Benfield et al., 1992; Benfield et al., 1992). Based on similar genomic organization and transcription strategy, PRRSV, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV), belongs to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Cavanagh, 1997; Conzelmann et al., 1993).

PRRS has now become the most infectious and economically important disease in the swine industry worldwide. China is the biggest country of pig and pork production. Since 2006, "swine high fever disease", which was primarily caused by PRRSV in combination with other pathogens, has spread throughout the country and has affected millions of pigs with high morbidity and mortality (20%) (An et al., 2010; Tong et al., 2007).

1.2. Pathogenesis

PRRSV viremia usually peaks at 4 to 7 days after primary infection (Greiner et al., 2000), and decreases rapidly thereafter. Most pigs are no longer viremic by 28 days after infection (Duan, Nauwynck, and Pensaert, 1997b). However, PRRSV can persist in tonsil and lymph nodes up to 132 days (Christopher-Hennings et al., 2001; Rowland et al., 2003), especially in inguinal and sternal lymph nodes and tonsil for extended periods of time (Bierk et al., 2001; Xiao et al., 2004).

In vivo, the virus replicates primarily in porcine alveolar macrophages (PAM) of the lung and other well-differentiated cells of the monocyte/macrophage lineage including pulmonary intravascular macrophages (PIM), subsets of macrophages of lymph nodes and spleen and intravascular macrophages of the placenta and umbilical cord (Duan, Nauwynck, and Pensaert, 1997a; Lawson et al., 1997; Thanawongnuwech et al., 2000). Thus, the highest titers of PRRSV and most significant lesions are found in these tissues during acute infection. In vitro, the virus infects both primary cultures of PAM and a green monkey kidney epithelial-like cell line, MA-104 cells, and its subclone, MARC-145 (Kim et al., 1993).

PRRSV induces apoptosis in virus-infected lung macrophages and lymphoid tissue macrophages in pigs, and in infected MARC-145 (Kim et al., 2002; Labarque et al., 2003; Lee and Kleiboeker, 2007; Sur et al., 1998). In pregnant sows, the virus replicates in the fetal implantation sites and causes apoptosis in infected macrophages and surrounding cells at the last stage of gestation, probably contributing to the reproductive disorders (Karniychuk et al., 2011). It was shown that the virus stimulates anti-apoptotic pathways in macrophages in early infection to maintain cell survival for progeny virus production, and that infected cells die by apoptosis late in infection, which is characterized by morphological changes, chromatin condensation, DNA fragmentation and specific caspase activation (caspase-3, 8, 9) (Costers et al., 2008). The

mechanisms of PRRSV-induced apoptosis were shown to involve Fas/FasL interaction and through a mitochondria-mediated pathway (Chang et al., 2007; Lee and Kleiboeker, 2007). The substances released from infected macrophages, e.g., p25, apoptogenic cytokines, reactive oxygen species, and nitric oxide, are potential causes of PRRSV-induced apoptosis (Palzer et al., 2007; Shi et al., 2008). Nonetheless, PRRSV also induces apoptosis in higher numbers of bystander cells that are not infected but in proximity to infected cells with unknown mechanisms (Choi and Chae, 2002; Labarque et al., 2003; Sirinarumitr et al., 1998; Sur et al., 1998).

1.3. Virus genome and heterogeneity

PRRSV has a positive single-stranded RNA genome of approximately 15 kb, which is capped at the 5' end and polyadenylated at the 3' terminus (Dea et al., 2000). There are 10 open reading frames (ORF). ORF 1a and ORF 1b comprise 75% of the viral genome and are translated into 2 polyproteins, which are then cleaved into14 non-structural proteins (nsp) that have replicase, protease and polymerase activities required for virus replication (Fang and Snijder, 2010; Meulenberg et al., 1993; Music and Gagnon, 2010; Snijder and Meulenberg, 1998). The 3' end of the viral genome contains 8 ORFs (ORF 2a, 2b, 3, 4, 5, 5a, 6 and 7) and encodes 5 minor and 3 major structural proteins via the generation of a 3'-coterminal nested set of subgenomic mRNAs (Conzelmann et al., 1993; Meng et al., 1994; Meng et al., 1996; Yuan et al., 2000).

PRRSV isolates are divided into two genotypes that are genetically as well as biologically distinct, represented by Lelystad virus (LV) of the type 1 European strains (Wensvoort et al., 1991) and VR-2332 of the type 2 North American strains (Benfield et al., 1992; Collins et al., 1992). But now both genotypes circulate globally (Kimman et al., 2009; Lunney et al., 2010). Phylogenetic analysis has revealed approximately 40% genomic difference between type 1 and type 2 PRRSV, and considerable genetic variation within each genotype (Andreyev et al., 1997; Fang et al., 2007; Gagnon and Dea, 1998; Kapur et al., 1996; Madsen et

al., 1998; Meng et al., 1995; Nelsen et al., 1999; Nielsen et al., 2000; Pirzadeh et al., 1998). Type 1 virus appeared to change slowly, but showed greater genetic diversity (~30% maximum difference in pairwise comparisons) than type 2 virus (~21% maximum) that periodically gave rise to more virulent forms (Murtaugh et al., 2010). The highly pathogenic PRRSV emerged in China in 2006 is a type 2 virus, which was introduced from North America and followed by local diversification leading to increased virulence (An et al., 2010; Hu et al., 2009; Shi et al., 2010; Zhou et al., 2009).

PRRSV phylogenetic studies are primarily based on the sequence analysis of GP5, which is the most variable structural protein with 50-55% amino acid homology between type 1 and type 2 (Mardassi et al., 1996; Meng et al., 1995; Nielsen et al., 2000). ORF3 and ORF7 have also been used in phylogenetic studies (Forsberg et al., 2001; Forsberg, 2005; Stadejek et al., 2006). In contrast, the sequences of the RNA-dependent RNA polymerase that are universally present in RNA virus has not been extensively analyzed (Murtaugh et al., 2010). The rate of nucleotide substitution in PRRSV RNA polymerase is lower than in ORF5 or OR7 (Chang et al., 2002).

1.4. PRRSV structure and structural proteins

The PRRSV virion consists of a lipid envelope that contains several envelope proteins surrounding a nucleocapsid core that encapsidates the RNA genome. By cryo-electron microscopy, the virions appear as round to egg-shaped particles ranging in diameter from 50 nm to 74 nm with a median value of 54 nm (Spilman et al., 2009). The outer surface has a very smooth outline with only a few protruding features, consistent with the small ectodomains of the major envelope proteins, GP5 and M. The inner double-layered and hollow core is 39 nm in average diameter and separated from the envelope by a 2-3 nm gap (Spilman et al., 2009).

The most abundant protein of PRRSV is nucleocapsid (N) protein, which represents 20-40% of the total protein content of the virion (Bautista et al., 1996; Dea et al., 2000). The ORF7-

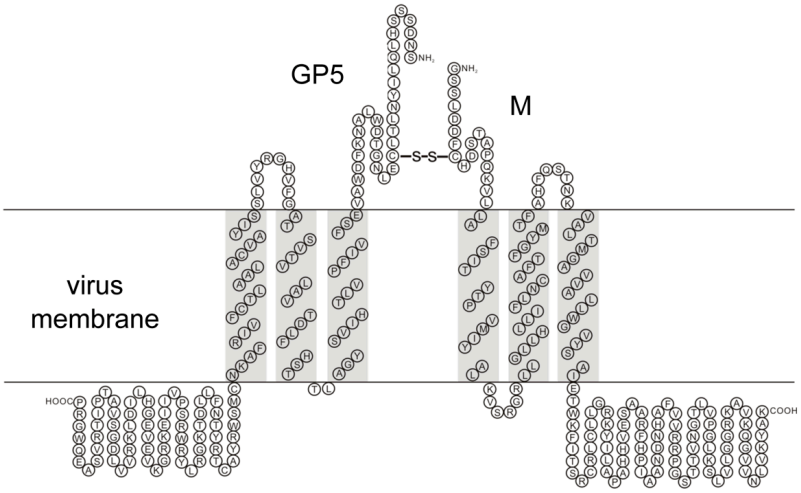
encoded N protein has 123-128 amino acids (14-15 kD) and contains an N-terminal RNA-binding domain and a C-terminal dimerization domain (Dokland, 2010). The N-terminal sequence contains a large number of positive charges, facilitating the interaction with viral RNA in the assembly of infectious particles (Mardassi et al., 1995; Meulenberg et al., 1995; Yoo et al., 2003). It also contains sequences that target N to the nucleolus of infected cells by nuclear and nucleolar localization and may influence nuclear processes during replication (Rowland and Yoo, 2003; Yoo et al., 2003). N is incorporated into virions as disulfide-linked dimers in combination with non-covalent dimerization of the C-terminal domain (Mardassi et al., 1996; Spilman et al., 2009; Wootton and Yoo, 2003). Due to its abundant expression and high immunogenicity, N protein is generally used in diagnostic assays. But none of the N-specific monoclonal antibodies (mAbs) have been associated with virus neutralization.

Glycoprotein 5 (GP5) and matrix (M) protein are encoded by ORF 5 and ORF 6, respectively. They are major envelope proteins that together comprise at least half of the viral protein content and form disulfide-bonded heterodimeric complexes that are crucial for infectious virus production (Dea et al., 2000; Delputte et al., 2002; Mardassi et al., 1996; Meulenberg et al., 1995; Snijder et al., 2003; Wissink et al., 2005). The 200-amino acid GP5 is 24.5-26 kD in molecular weight and is the most variable protein among PRRSV isolates, with only 51-55% amino acid sequence identity between European and North American isolates (Indik et al., 2000; Kapur et al., 1996; Mardassi et al., 1995). It has a predicted signal peptide of about 25 (American strain) or 32 (European strain) residues in the N-terminus (Dea et al., 2000). But for VR-2332 (GenBank ID: U87392), the prototype type 2 PRRSV strains, the signal peptide cleavage site is predicted to be between amino acids 31 and 32 by the program SignalP 4.0 (Petersen et al., 2011). N-terminal amino acid sequencing of purified VR-2332 GP5 confirmed this prediction (Johnson et al. unpublished data). The signal peptide is followed by a hydrophilic stretch of about 33 amino

acids which contains 3 putative N-linked glycosylation sites at residues 33, 44 and 51 for VR-2332 or 2 glycosylation sites at residues 46 and 53 for LV (Indik et al., 2000; Mardassi et al., 1995; Mardassi et al., 1996; Meulenberg et al., 1995). The 173 or 174-amino acid M protein is 18-19 kD and is not glycosylated (Mardassi et al., 1995; Mardassi et al., 1996). It is the most highly conserved structural protein of PRRSV (Mardassi et al., 1995; Meng et al., 1995; Meulenberg et al., 1993). The 3 highly hydrophobic regions in M protein and the characteristic hydropathy profile of GP5 indicate the potential membrane-spanning domains in both proteins (Dea et al., 2000). Predicted from three transmembrane protein topology programs: SOSUI, TMHMM and Phobius, GP5 and M both contain two short putative ectodomains, amino acids S32 to V64 and G100 to S109 for GP5, and G2 to L19 and A63 to K70 for M (Fig. 1.1), consistent with the structural analysis of GP5/M (Dokland, 2010). The GP5/M heterodimeric complexes are linked by disulfide bonds of cysteine residues in the N-terminal ectodomains of both proteins (Mardassi et al., 1995; Mardassi et al., 1996).

Because of their abundance in the PRRSV envelope, GP5/M are considered to have a primarily structural role of imposing curvature on the viral membrane during budding (Dokland, 2010). They have also been extensively studied in roles of virus entry, immune response targets, and vaccine candidates. PRRSV attachment to heparin-like and sialoadhesin receptors on porcine macrophages has been associated with GP5/M (Delputte et al., 2002; Delputte et al., 2007; Van Breedam et al., 2010; Van Gorp et al., 2008). However, a genetic study showed that PRRSV with the M ectodomain swapped with other arterivirus M ectodomains survived and did not change cell tropism for other arteriviruses (Verheije et al., 2002). Several neutralization epitopes have been identified in GP5 ectodomains (Ostrowski et al., 2002; Pirzadeh and Dea, 1997; Plagemann et al., 2002; Weiland et al., 1999; Zhang et al., 1998), but recent studies suggest that porcine antibodies specific to GP5/M peptides do not neutralize PRRSV (Li and Murtaugh,

Fig. 1.1. Schematic representation of the GP5/M heterodimeric complex in the viral envelope. The proposed GP5/M topology in the viral envelope was summarized though sequence analysis using three transmembrane protein topology prediction programs: SOSUI, TMHMM and Phobius. GP5 and M contain two short ectodomains, amino acids S32 to V64 and G100 to S109 for GP5, and G2 to L19 and A63 to K70 for M. The N terminal ectodomains are linked by a disulfide bond to form a heterodimeric complex.



manuscript in preparation; Vanhee et al., 2011).

GP5 is postulated to induce apoptosis in PRRSV-infected cells (Gagnon et al., 2003; Suarez et al., 1996). The first 119 amino acids of GP5 were shown to constitute a region capable of fully inducing apoptosis (Fernandez et al., 2002). In contrast, Lee et al. (2004) did not observe any indications of apoptosis, such as reduction of cell number, DNA fragmentation, and strand-breaks in a stable GP5-expressing HeLa subline. Microarray analysis of GP5-transfected HeLa cells did not show any expression of genes involved in cell apoptosis (Lee et al., 2004).

Minor envelope proteins GP2a, E, GP3 and GP4 are incorporated as multimeric complexes in the PRRSV envelope (Wissink et al., 2005). Immunoprecipitation studies also suggest that this complex interacts with GP5 via GP4 (Das et al., 2010). The 256-residue GP2a in the type 2 virus strain VR-2332 is encoded by ORF2a and is 29-30 kD (Meulenberg et al., 1995). It contains a predicted 40-amino acid signal sequence in the N-terminus followed by a 169-residue ectodomain, a single transmembrane (TM) helix and a 24-residue endodomain as predicted by TMHMM 2.0. There are two conserved N-glycosylation sites at N178 and N184 in GP2a (Murtaugh et al., 2010; Wissink et al., 2004). The 73-residue E protein (10 kD) is an unglycosylated small hydrophobic protein expressed from ORF2b, which initiates only 6 nucleotides downstream of ORF2a and is completely embedded within ORF2a (Wu et al., 2001). ORF2b is in a better context for translation and thus may be expressed preferentially over ORF2a (Wu et al., 2001). This region is conserved in all arteriviruses (Snijder and Meulenberg, 1998). Indeed, PRRSV E protein is so-named due to the perinuclear localization pattern similar to EAV E protein (Wu et al., 2001; Wu et al., 2005).

The 254-residue GP3, encoded by ORF3, is the most heavily glycosylated envelope protein of PRRSV. Glycosidase digestion reduces its molecular weight from 45-50 kD to 27-29 kD (Gonin et al., 1998; Mardassi et al., 1998). The topology of GP3 is unclear. It may not have an

N-terminal signal peptide, since the predicted signal sequence (residues 1-23) is primarily within the putative TM helix (residues 7-29). Consistently, the predicted signal peptide of EAV GP3 is not cleaved off (Wieringa et al., 2002). According to TMHMM 2.0 and NetNGlyc 1.0 (Gupta et al., manuscript in preparation) predictions, GP3 has a short 6-amino acid N-terminal endodomain followed by a single TM helix and a long 225-amino acid C-terminal ectodomain, which contains 7 putative N-glycosylation sites at N29, 42, 50, 131, 152, 160 and 195. N29 is within the TM helix, thus presumably is not used. Six glycosylation sites provide ~16 kD glycans, consistent with EAV (Gonin et al., 1998; Wieringa et al., 2002). Type 1 and type 2 PRRSV share 58% identity in GP3 sequence, being highly divergent in the C-terminal 30-50 residues, whereas type 1 virus has an additional 11 amino acids (Dokland, 2010). GP3 was previously reported to be secreted into the cell medium during PRRSV infection (Gonin et al., 1998; Mardassi et al., 1998). However, later studies demonstrated that GP3 is exposed on the viral surface and is a minor envelope protein (de Lima et al., 2009; van Nieuwstadt et al., 1996; Wissink et al., 2005). Mass spectrometric analysis of purified VR-2332 also confirmed the presence of GP3 in PRRSV virions (Johnson and Murtaugh, data not published).

The 178-residue GP4, encoded by ORF4, is 31-35 kD in molecular weight that can be reduced to 16 kD by N-glycosidase F digestion (van Nieuwstadt et al., 1996). GP4 is a putative class I integral membrane protein with an N-terminal signal peptide of residues 1-22, a C-terminal membrane anchor at residues 156-177, and 4 potential N-glycosylation sites at N37, 84, 120 and 130 (Dokland, 2010; Music and Gagnon, 2010). The expression of GP4 protein in GP4-transfected MARC-145 cells has been shown to be associated with the expression of 16 genes, including those involved in cell adhesion, cell growth, replication, transcription, and protein degradation, and other unknown functional pathways (Lee et al., 2004), suggesting that GP4 may utilize or alter host cell machinery to transport viral or cellular components to the cell surface.

GP2a, E, GP3 and GP4 are expressed together in the endoplasmic reticulum and collectively transported through the Golgi complex to the plasma membrane. The absence of any one of the minor proteins impedes the incorporation of the multimeric complex and viral infectivity, although it does not affect viral particle formation (Lee and Yoo, 2006; Wissink et al., 2005), indicating that these proteins have key roles in virus entry by mediating receptor binding but do not contribute to the PRRSV structural scaffold. In addition, the E-deleted non-infectious PRRSV particles were able to enter cells but unable to continue further steps of replication (Lee and Yoo, 2006). It was then postulated that E protein likely acted as an ion-channel protein embedded in the viral envelope to facilitate uncoating of virus and release of the genome into the cytoplasm. Furthermore, myristoylation of E protein in the N-terminus is non-essential for virus infectivity but promotes its growth (Du et al., 2010).

ORF5a protein was recently discovered by two research groups at the same time (Firth et al., 2011; Johnson et al., 2011). It is expressed from an alternative ORF of subgenomic mRNA 5, which precedes the ORF5 initiation codon by 10 nucleotides, via a translation initiation mechanism involving leaky ribosomal scanning (Firth et al., 2011). ORF5a protein is universally present in all arteriviruses and is predicted to be a type III membrane protein with a short N-terminal luminal domain, a central signal-anchor/transmembrane sequence, and a cytosolic C-terminal domain. For PRRSV VR-2332 strain, ORF5a protein is a 51-amino acid structural protein that is found in purified virions (Johnson et al., 2011). ORF5a knock-out mutants of EAV appear to grow poorly and are less pathogenic than the wildtype virus (Firth et al., 2011). Nonetheless, the biological relevance of PRRSV ORF5a protein remains to be investigated.

1.5. PRRSV Immunology

It was reported that PRRSV infection compromises host innate anti-viral immunity by downregulating the production of inflammatory cytokines such as type 1 interferons (IFN-α and

IFN-β), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and inducing the expression of immunosuppressive cytokines including IL-10, IL-4 and transforming growth factor β (TGFβ) (Aasted et al., 2002; Albina et al., 1998; Buddaert et al., 1998; Royaee et al., 2004; Suradhat and Thanawongnuwech, 2003; Thanawongnuwech et al., 2001; Thanawongnuwech et al., 2004; Van Reeth et al., 1999). However, it appears that inflammatory responses in the lung are an indicator of disease, rather than an indicator of immunity (Van Reeth et al., 1999). Recent data suggest that the interferon production upon PRRSV infection varies greatly depending on strain types and virulence (Lee et al., 2004; Liu et al., 2010).

PRRSV-specific humoral immune responses have been extensively studied. Humoral immunity initiates with IgM antibodies, which appear in serum by 5-7 days post infection (PI) and decline rapidly to undetectable levels after 2-3 weeks (Loemba et al., 1996; Mulupuri et al., 2008; Murtaugh et al., 2002). Anti-PRRSV IgG antibodies are first detected 7-10 days PI, peak at 2-4 weeks PI, remain constant for a period of months, and then decline to low levels by 300 days PI (Loemba et al., 1996; Mulupuri et al., 2008; Murtaugh et al., 2002; Nelson et al., 1994). Most antibodies that appear shortly after infection are non-neutralizing and are principally targeted to N and nsp2 proteins, followed by GP5 and then M proteins (Loemba et al., 1996; Lunney et al., 2010; Mulupuri et al., 2008; Nelson et al., 1994).

Anti-PRRSV antibodies are also present in boar semen and sow colostrum (Eichhorn and Frost, 1997; Oleksiewicz et al., 2001). Maternal immunity in piglets appears to be of relatively short duration, until 3-10 weeks of age, and then PRRSV infection increases as maternal antibodies decline (Albina et al., 1994; Chung et al., 1997; Houben et al., 1995; Zimmerman et al., 2006).

PRRSV-specific serum neutralizing antibodies (NA) that inhibit viral infectivity usually appear at about 3 weeks PI and are maintained for long periods, but at low levels (Loemba et al.,

1996; Yoon et al., 1995). Substantial variation has been observed in the NA responses of individual pigs, including the lack of response, the kinetics of their appearance, and relative titers (Loemba et al., 1996; Nelson et al., 1994). Despite substantial variation among animals, NA has been considered as an indicator of protection against PRRSV infection. Clearance of PRRSV viremia and prevention of repeat infection is attributed to the host NA response (Batista et al., 2004; Labarque et al., 2000; Lopez and Osorio, 2004; Lopez et al., 2007; Osorio et al., 2002). Thus, identification of viral neutralization epitopes might greatly facilitate the rational design of PRRSV vaccines.

Many reports implicate the major envelope proteins GP5 and M as key targets for host NA response (Ostrowski et al., 2002; Pirzadeh and Dea, 1997; Plagemann et al., 2002; Plagemann, 2004; Weiland et al., 1999; Wissink et al., 2003; Yang et al., 2000). Neutralization epitopes in GP5 were identified as linear peptides containing amino acids 29-35 (WSFADGN) of the type 1 Lelystad virus, and amino acids 37-45 (SHLQLIYNL) and 36-52 (SSHLQLIYNLTLCELNG) of the type 2 strain VR-2332, via peptide-based epitope mapping (Pepscan) (Ostrowski et al., 2002; Wissink et al., 2003) and generation of GP5 peptide-specific neutralizing mAbs (Ostrowski et al., 2002; Pirzadeh and Dea, 1997; Plagemann et al., 2002; Wissink et al., 2003). Neutralizing mAbs to M protein were also described, but specific epitopes were not identified (Cancel-Tirado et al., 2004; Yang et al., 2000). In contrast, immunization of pigs with recombinant GP5/M ectodomain polypeptides that contained the reported neutralization epitopes did not elicit detectable NA response, and porcine antibodies purified against these polypeptides from PRRSV-neutralizing serum did not neutralize the homologous type 2 virus (Li and Murtaugh, manuscript in preparation). Consistently, in the case of type 1 PRRSV, porcine antibodies specific to GP5 or M peptides from LV-infected pigs did not neutralize virus (Vanhee et al., 2011).

Besides GP5 and M, mAbs specific for the minor envelope protein GP4 have been shown to neutralize PRRSV, though less effectively than anti-GP5 mAbs (Meulenberg et al., 1997; van Nieuwstadt et al., 1996; Weiland et al., 1999). The neutralization domain in LV GP4 spans amino acids 40-79, which is the most variable region of GP4 and is susceptible to immunoselection in vitro (Costers et al., 2010; Meulenberg et al., 1997; Vanhee et al., 2010). In vivo, GP4-specific NA also appear to cause amino acid substitution in the GP4 neutralization epitope, thus becoming a driving force in PRRSV evolution and immune evasion (Costers et al., 2010). GP3 and GP2a also are reported to contain peptide epitopes that induce NA (Cancel-Tirado et al., 2004; Vanhee et al., 2011).

The T-cell responses to PRRSV are generally classified as weak, transient and highly variable (Lunney et al., 2010). Paralleling the NA responses, the lymphocyte proliferative responses were not detected until ~4 weeks after PRRSV infection and were primarily CD8+ T cells (Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). The T-cell targets were found in all structural proteins encoded by ORFs 2-7 (Bautista et al., 1999). Cytokine responses were mainly IFN-λ, which was secreted from CD4+CD8+ cells with a small proportion of CD4-/CD8αβ+ cytotoxic T cells, and to a less extent, IL-2 (Lopez Fuertes et al., 1999; Mateu and Diaz, 2008). However, the T-cell response following infection did not correlate with the level of virus replication in lymphoid tissues (Xiao et al., 2004), and the number of PRRSV-specific IFN-λ producing T cells was low in infected pigs (Meier et al., 2003; Xiao et al., 2004). In addition, porcine regulatory T cells (Tregs), characterized by CD4+CD25+Foxp3+ phenotype, were induced in vitro with increasing expression of TGF-β by co-culturing with porcine bone marrow-derived dendritic cells that were exposed to type 2 PRRSV, but not to type 1 virus (Silva-Campa et al., 2009; Wongyanin et al., 2010), suggesting that the PRRSV-specific T helper 3 response

may be strain-dependent. Therefore, the significance of cellular immunity against PRRSV infection still remains to be investigated.

1.6. Anti-PRRSV vaccines

There are two major types of commercial vaccines currently available against PRRSV; modified live-attenuated vaccines (MLV) and inactivated vaccines. Substantial reviews about PRRSV vaccines have all agreed that MLV are generally effective against homologous strains, but variable in success against heterologous strains (Huang and Meng, 2010; Lunney et al., 2010; Murtaugh and Genzow, 2011; Thanawongnuwech and Suradhat, 2010). Thus, the ability to induce cross protection is always a major concern in the development of PRRSV vaccines due to the heterogeneity of field isolates. Besides incomplete protection, current MLV have also been associated with other problems such as shedding of vaccine virus, persistent infection and reversion to virulence (Huang and Meng, 2010). Inactivated PRRSV vaccines do not have promising outcomes, only providing partial or no protection against challenge, though PRRSV-specific NA may be induced ((Vanhee et al., 2009; Zuckermann et al., 2007).

2. PRRSV entry into permissive cells

PRRSV has a restricted cell tropism, preferentially infecting the porcine monocyte/macrophage lineage in vivo and primary cultures of porcine alveolar macrophages (PAM) and MA-104 cells, a monkey kidney cell line, and its subclone MARC-145 cells in vitro (Benfield et al., 1992; Duan, Nauwynck, and Pensaert, 1997a; Kim et al., 1993). Several reports suggested that PRRSV attaches to specific receptors on both permissive cell types and enters cells through clathrin-mediated endocytosis in which low pH, an aspartic protease cathepsin E and an unidentified trypsin-like serine protease are involved in proper virus uncoating (Duan, Nauwynck, and Pensaert, 1997b; Duan et al., 1998; Kreutz and Ackermann, 1996; Misinzo et al.,

2008; Nauwynck et al., 1999). Cell membrane cholesterol is also critical for PRRSV entry by a lipid raft-dependent pathway (Huang et al., 2011).

Further analysis revealed that different receptors have been utilized in PRRSV entry of macrophages and MARC-145 cells. In macrophages, a heparinlike/heparan sulphate receptor was shown to interact with the viral GP5/M complex though binding to M protein (Delputte et al., 2002). PRRSV was affinity purified against heparin beads (Hu et al., 2010). Heparin or heparan sulphate, a type of proteoglycan, is present in various animal tissues and is not macrophage specific, which explains why it only binds PRRSV but does not lead to virus internalization or infection.

Sialoadhesin is a macrophage-restricted type I transmembrane glycoprotein and is the prototype member of a sialic acid-binding Ig-like lectins (siglecs) family (Van Breedam et al., 2010). Sialoadhesin seems to bind sialic acids on GP5 and internalize PRRSV virions via clathrin-mediated endocytosis (Delputte and Nauwynck, 2004; Delputte et al., 2007; Van Breedam, Van Gorp et al., 2010; Vanderheijden et al., 2003). Kinetic analysis of PRRSV attachment to macrophages showed that early attachment is mediated mainly by an interaction with heparan sulphate, followed by a gradual increase in interaction with sialoadhesin (Delputte et al., 2005). However, PRRSV interaction with sialoadhesin only allows virus attachment and internalization but not virus uncoating, and thus, is not necessary for PRRSV infection (Van Gorp et al., 2008; Wang et al., 2011).

The essential PRRSV receptor is CD163 (Welch and Calvert, 2010), a scavenger receptor mainly expressed on cells of the monocyte/macrophage lineage and also on MARC-145 cells. CD163 on macrophages binds and internalizes haemoglobin-haptoglobin complexes, protecting tissues from free-haemoglobin-mediated oxidative damage. Calvert el al. found that expression of CD163 renders non-permissive cells susceptible to PRRSV infection and yields high titers of

progeny virus (Calvert et al., 2007). After expressing CD163 but not sialoadhesin, cells became productively infected, although virus internalization was not observed (Van Gorp et al., 2008). Moreover, increase of CD163 but not sialoadhesin on cultured peripheral blood monocytes is coordinated with enhanced susceptibility to PRRSV infection (Wang et al., 2011). Finally it was shown that PRRSV interacts with CD163 via GP2a and GP4 (Das et al., 2010), and then targets CD163+ early endosomes, but does not continue to late endosomes, escaping from the dead-end of degradative lysosomes (Van Gorp et al., 2009). Therefore, CD163 is the essential receptor for PRRSV infection in permissive cells.

MARC-145 cells share CD163 and possibly heparan sulphate as PRRSV receptors (Calvert et al., 2007; Jusa et al., 1997). In addition, it was reported that anti-vimentin antibodies blocked infection in MARC-145 cells and free vimentin in cell culture competed with the virus for infection (Kim et al., 2006; Wang et al., 2011). Thus, vimentin, a type III intermediate filament protein, is expressed on the surface of MARC-145 cells and may have a role in PRRSV binding with cytoskeletal filaments that mediate transportation of the virus in the cytosol. Finally, another host protein, CD151, was shown to interact with the PRRSV 3' untranslated region (UTR) RNA and have a role in virus infection via unknown mechanisms (Shanmukhappa et al., 2007).

3. Biological roles of PRRSV-associated glycans

PRRSV has four glycoproteins, GP2a, GP3, GP4 and GP5, all present on the virus envelope. For type 2 PRRSV strain VR-2332, GP2a contains two conserved N-glycosylation sites at N178 and 184; GP3 has seven putative N-glycosylation sites at N29, 42, 50, 131, 152, 160 and 195; GP4 has four potential N-glycosylation sites at N37, 84, 120 and 130; and GP5 has three N-glycosylation sites at N33, 44 and 51. These glycans constitute a substantial portion of the mass of the envelope glycoproteins, and play various roles in the PRRSV life cycle.

Glycoprotein-associated glycans, in general, help maintain proper protein folding and thus are important for virus assembly. Glycan addition at N42, 50 and 131 of GP3, multiple locations (N37, 84, 120 and 130) of GP4, and N44 or N46 (the LV strain homologue of N44) of GP5 is critical for PRRSV virion assembly and recovery of infectious virus (Ansari et al., 2006; Das et al., 2011; Wissink et al., 2004). Glycans linked to N184 of GP2a are important for type 2 virus particle formation (Das et al., 2011), whereas they are not essential for type 1 virus (Wissink et al., 2004).

PRRSV-linked glycans are also suggested to interact with host cells and thus are important for virus infection. Glycan addition to certain sites seems to be important for PRRSV infectivity. Double mutation of both glycosylation sites in GP2a and double or quadruple mutation of 2 or 3 glycosylation sites in GP4 abrogated GP2a and GP4 interactions with the PRRSV receptor CD163 (Das et al., 2010). Mutant virus with glycans deleted at N34, N51, and N34/51 of GP5 grew to lower titers than the wildtype virus (Ansari et al., 2006), although mutation of N53 (the LV strain homologue of N51) of GP5 did not affect type 1 virus infectivity (Wissink et al., 2004). Certain types of glycans seem to have a role in PRRSV infection. Removal of complex-type N-glycans from PRRSV reduced infectivity in porcine macrophages (Delputte and Nauwynck, 2004). In particular, sialic acids on GP5 bind sialoadhesin on macrophages, mediating virus attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). Ficolin, a collagenous lectin specific for N-acetylglucosamine (GlcNAc), bound and reduced PRRSV infectivity in MARC-145 cells (Keirstead et al., 2008). Thus, multiple viral glycan moieties may be involved in PRRSV interaction of permissive cells.

Finally, PRRSV-associated glycans appear to shield antigenic epitopes in viral proteins and assist virus escape from neutralizing antibody recognition to achieve viral immune evasion. Hypoglycosylation of GP5 significantly increased virus sensitivity to in vitro neutralization by the

same porcine antisera and enhanced the immunogenicity of the nearby neutralization epitopes to induce neutralizing antibodies in host pigs (Ansari et al., 2006). In consistence, Vu et al. found that a natural mutant PRRSV without glycans at N131 of GP3 and N51 of GP5 was highly susceptible to neutralization and induced an atypically rapid and robust NA response in vivo (Vu et al., 2011). Furthermore, reintroduction of glycosylation at both sites reduced the susceptibility of the virus to antibody neutralization and induced lower NA response (Vu et al., 2011). Thus, viral glycans at these sites shield PRRSV interference by host immunity. In contrast, hypoglycosylation of minor envelope glycoproteins, including GP3, did not enhance NA response in pigs (Das et al., 2011). The glycans of minor proteins in immune evasion remains to be investigated.

4. General introduction to N-glycans

4.1. Composition, structure and classification

The glycan-relevant concepts here follow the definitions in Essentials of Glycobiology, 2nd edition (Varki et al., 2009). Glycan is a generic term for any sugar or assembly of sugars, in free form or in covalent attachment to another macromolecule such as a protein or lipid to form a glycoconjugate, that is, a glycoprotein or glycolipid. Glycan refers to any form of mono-, oligo-, or polysaccharide. The basic structural units of glycans are monosaccharides, which cannot be hydrolyzed into a simpler form, including polyhydroxyaldehydes and polyhydroxyketones with three or more carbon atoms. Although hundreds of distinct monosaccharides occur in nature, only a small number are commonly found in animal glycans, as listed in Table 1.1. Oligosaccharide is a linear or branched chain of monosaccharides attached to one another via α or β glycosidic linkages. Polysaccharide is usually used for large glycans with repeating units, generally greater than ten monosaccharide units in length.

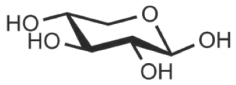
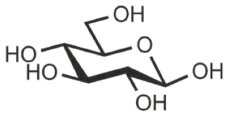
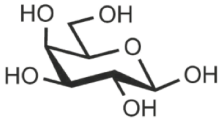
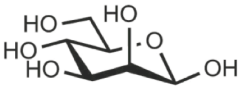
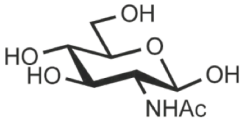
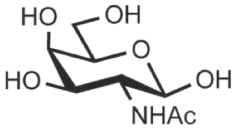
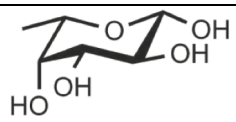
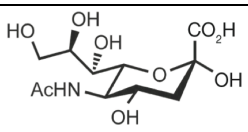
A glycoprotein carries one or more glycans covalently attached to a polypeptide, usually via N or O linkages. An O-glycan is attached to a serine (Ser or S) or threonine (Thr or T) residue in a polypeptide. An N-glycan is an oligosaccharide chain covalently linked to an asparagine (Asn or N) residue of a polypeptide chain in the consensus sequon Asn-X-Ser/Thr, where X is any amino acid except proline. The efficiency of N-glycosylation at the sequon may also be reduced when X is acidic such as aspartate or glutamate. Nonetheless, this sequon has been used to predict the potential N-glycosylation sites in a protein sequence, such as the software NetNGlyc 1.0 (Gupta et al., 2004, in preparation). N-glycans share a common pentasaccharide core (Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn) and are generally classified into three main types: high-mannose, hybrid and complex types, according to their branches. N-glycans can have two, three and four antennae or branches attached to the core α-mannoses via GlcNAc to form bi-, tri- and tetra-antennary complex N-glycans or carry a GlcNAc residue at the core β-mannose to form bisecting complex N-glycans.

4.2. Biosynthetic pathway of N-glycans

The biosynthesis of N-glycans is highly conserved in all eukaryotic cells and is performed in the endoplasmic reticulum (ER) and Golgi compartments. Monosaccharides are the building blocks of glycans, and are either synthesized within a cell or salvaged from the environment. Biosynthesis initiates by transferring GlcNAc-P to a lipid-like precursor, dolichol phosphate (Dol-P), on the cytoplasmic face of the ER membrane to form Dol-P-P-GlcNAc. Fourteen sugars are then sequentially added to Dol-P to form a complete precursor structure, $Glc_3Man_9GlcNAc_2$-P-P-Dol, which flips into the lumen of the ER during this process. It is of note that each sugar addition is catalyzed by a specific glycosyltransferase.

The 14-sugar glycan is transferred entirely to an Asn-X-Ser/Thr sequon in a nascent protein that is synthesized and translocated through the ER membrane. The peptide-linked N-

Table 1.1. Common monosaccharides found in vertebrates.

Class	Monosaccharide	Abbreviation	*Symbol	**Structure
pentose	xylose	Xyl		
hexose	glucose	Glc		
	galactose	Gal		
	mannose	Man		
hexosamine	N-acetylglucosamine	GlcNAc		
	N-acetylgalactosamine	GalNAc		
deoxyhexose	fucose	Fuc		
sialic acid	N-acetylneuraminic acid	NeuAc		

* Symbols follow the standard Consortium for Functional Glycomics (CFG) nomenclature for monosaccharides.

** Chair structures were adapted from Essentials of Glycobiology, 2nd edition (Varki et al., 2009). All monosaccharides are shown in D-stereoisomers, except that fucose is shown in L-stereoisomer.

glycan is then trimmed with sequential removal of terminal glucose residues by specific glucosidases. After the glycoprotein transfers to the *cis*-Golgi, terminal mannose residues are sequentially removed by specific mannosidases to form the structure $Man_5GlcNAc_2$. Inhibition of glucose or mannose removal during the above process results in a high-mannose structure on mature glycoproteins.

Biosynthesis of hybrid and complex N-glycans begins in the *medial*-Golgi with the addition of a GlcNAc residue to the core α-mannose to form a hybrid structure. Once this step has occurred, the majority of N-glycans are trimmed by α-mannosidase II, which removes two terminal mannoses to form $GlcNAcMan_3GlcNAc_2$, being the precursor of complex N-glycans. Inhibition of α-mannosidase II will block the formation of complex N-glycans and result in hybrid or high-mannose N-glycans. One GlcNAc is added to another core α-mannose to form $GlcNAc_2Man_3GlcNAc_2$, a complex N-glycan with two branches. More residues or branches can be extended by different enzymes to form N-glycans with much more complexity. After elongation and branching, a mature hybrid or complex N-glycan is usually decorated with the addition of one or more of sialic acid, fucose, galactose, GlaNAc and sulfate.

In summary, glycan structures are not encoded directly in the genome, instead, they are generated by a variety of competing and sequentially acting glycosyltransferases and glycosidases. Many of these enzymes are sensitive to the biochemical and physiological state of the cell in which the glycoprotein is expressed. Therefore, any alteration of the cell status may change the outcome of the N-glycan biosynthesis.

4.3. Glycan diversity

In general, the totally expressed glycan repertoire (glycome) of a given cell type or organism is much more structurally complex than the genome or proteome. First, glycans have much greater inherent structural complexity than nucleotides or proteins that each can only

contain one basic type of linkage between monomers. The building blocks of glycans, monosaccharides, can theoretically be linked to each other at any one of several positions on a sugar unit by either α- or β-linkages. The two types of linkages confer very different structural properties and biological functions, although the glycan compositions are identical. In addition, glycan chains can be linear or branched, and modified by a variety of different substituents, such as acetylation and sulfation. Moreover, glycans show microheterogeneity, a phenomenon that at any given glycosylation site on a given protein synthesized by a particular cell type, a range of variations can be found in the attached glycan structures (Varki et al., 2009). Glycan microheterogeneity accounts for the existence of numerous glycoforms for a given protein and explains the observation of multiple or diffuse bands of a single protein in SDS-PAGE. Finally, added diversity arises from intraspecies and interspecies variations in glycosylation.

5. Structural analysis of glycans

The substantial complexity of glycans makes it very difficult to profile the complete glycan composition and structure of a glycoconjugate. Many chemical, enzymatic and mass spectrometric methods have been utilized in combination to determine the complex glycan structures.

5.1. Lectin array

Lectin is a protein that specifically recognizes and binds to glycans without catalyzing a modification of the glycan. For example, Pokeweed mitogen (PWM) and *Lycopersicon esculentum* agglutinin (LEA) specifically recognize GlcNAc and LacNAc oligomers (Irimura and Nicolson, 1983; Yokoyama et al., 1978). Wheat germ agglutinin (WGA) binds a broad range of sialic acids and *Maackia amurensis* agglutinin (MAA) specifically binds α2-3-linked sialic acids. *Dolichos biflorus* agglutinin (DBA) has an affinity for terminal N-acetyl-α-D-galactosaminyl residues (Etzler and Kabat, 1970). ConA is specific for terminal α-D-mannose and α-D-glucose

(Maupin et al., 2011). Thus, the specific lectin-binding to a glycoconjugate provides very straightforward structural information of un-characterized glycans. In combination with "throughput" technologies, lectin microarray has been developed recently and has attracted increasing attention from researchers in various fields (Hirabayashi et al., 2011; Katrlik et al., 2010; Kiessling and Splain, 2010). However, there are several limitations with this technique. First, the single-site-binding affinities in many lectins appear to be low, thus multivalent binding is often required to reach high avidity. Otherwise, it is difficult to monitor low-affinity binding. Second, the structures of glycans recognized by some lectins have not been defined clearly. For example, WGA is usually considered to be specific for sialic acids, whereas it actually has a higher affinity for GlcNAc oligomers. Third, the recognition specificities of known lectins are restricted and only about 100 lectins are commercially available, while more than 300 lectins have been discovered (Katrlik et al., 2010; Kiessling and Splain, 2010). Therefore, lectin array can provide some structural information of glycans but may be constrained in application.

5.2. Enzymatic release of glycans from glycoconjugates

Enzymatic release of glycans by different endoglycosidases helps roughly to classify the types of the digested glycans. For example, peptide-N-glycosidase F (PNGase F) cleaves all the three high-mannose, hybrid and complex types of N-glycans from asparagine. Thus, a successful PNGase F digestion differentiates N-glycans from other linkage glycans such as O-glycans. PNGase F is an amidase that releases N-glycans attached to the nitrogen of asparagine, thereby converting asparagine to aspartate and increasing the molecular weight of the digested peptide by 1 Dalton. In combination with an ^{18}O water environment, PNGase F release of N-glycans increases the mass of the digested peptide by 3 Daltons. Thus, this method is developed to determine the trully occupied N-glycosylation sites in a glycoprotein (Angel et al., 2007; Dalpathado and Desaire, 2008; Kuno et al., 2005; Tateno et al., 2007).

Endoglycosidase H (Endo H) selectively releases high-mannose and hybrid N-glycans, but not complex N-glycans. The cleavage site is between the two core GlcNAc residues, leaving one GlcNAc attached to asparagine. A complete Endo H digestion thus excludes the presence of complex N-glycans.

An exoglycosidase is a glycoside hydrolase enzyme that specifically breaks the glycosidic bond at the terminal residue. Thus, sequential digestion of glycans by different exoglycosidases is often utilized in combination with mass spectrometry to structurally define the terminal residues of glycans (Li et al., 2011; Montesino et al., 2009; Yu et al., 2011). However, for an unknown glycan structure, many efforts are needed to test exoglycosidases with different specificities to identify the correct enzymes with a proper digestion sequence.

5.3. Mass spectrometric analysis

As mentioned above, chemical and biochemical analyses are useful for providing glycan structural information. However, they are either incomplete or require large amounts of samples. Thus, a more sensitive, efficient and accurate technology with high information output, mass spectrometry (MS), has been widely adopted in the analysis of glycans and other biomolecules such as proteins (Azadi and Heiss, 2009; Dell and Morris, 2001; Harvey, 2005; Harvey et al., 2008; Montesino et al., 2009; Pabst and Altmann, 2011).

In order to perform MS analysis of N-glycans, the glycans typically are removed from glycoproteins by an endoglycosidase (e.g. PNGase-F) and purified by C18 reversed-phase chromatography. Purified glycans then undergo derivatization by permethylation, which increases the volatility of the relatively high molecular weight N-glycans and improves sensitivity in MS. Processed glycan samples are ready for MS analysis that generally includes ionization, detection and mass analysis of ions. Soft ionization methods such as electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI) and fast atom bombardment (FAB) have been

developed to obtain molecular ions with minimal fragmentation. Conversely, controlled fragmentation of ions by MS/MS allows sequence analysis of glycans and provides more structural information.

In ESI-MS, a stream of liquid containing the purified and processed glycan sample is introduced into the atmospheric-pressure ion source of a mass spectrometer through a capillary (Dell and Morris, 2001; Harvey, 2005). Proper voltage application to the capillary then generates an aerosol of highly charged microdroplets. After encountering a drying gas, the microdroplets, devoid of solvent, generate gaseous ions, whose charge distribution is proportional to the number of ionizable groups in the molecule. ESI produces primarily double- or multiple-charged ions, which can be selected by the first MS analyzer and fragmented by collision-induced dissociation (CID) to produce fragment ions that are further screened by the second MS analyzer, so-called MS/MS experiments. The quadrupole orthogonal acceleration time of flight (Q-TOF) mass spectrometer is the most powerful technology for low-energy MS/MS (Dell 2001).

MALDI-MS predominantly generates singly charged ions and is considered as the most sensitive ionization technology for simple mass analysis (Dell and Morris, 2001; Harvey, 2005). In MALDI, the glycan sample is embedded in a low molecular weight ultraviolet-absorbing matrix. The matrix then absorbs energy from a pulsed laser and transfers it to the sample to produce singly charged ions. MALDI appears to be more sensitive and accurate than ESI, thus is better suited to analyze the mass of simple molecules, whereas ESI provides more structural information.

In summary, a full structural characterization of a glycoprotein is not only about glycan composition, but also includes identification of glycan branching, linkages, configurations, sugar isomers with the same mass, and the glycosylation sites (Dell and Morris, 2001). Therefore, a complete analysis may need to combine various methods to complement each other's limitations.

6. The biological roles of glycans

Glycans are ubiquitously present on the outer surface of many cellular and secreted macromolecules, e.g. glycoproteins. Thus, they are naturally in a position to modulate or mediate a wide range of events in cell–cell, cell–matrix, and cell–molecule interactions either non-essential or critical to the development and function of a complex, multicellular organism. In general, glycan functions are categorized as structural and modulatory. Terminal sequences, unusual structures and modifications are more likely to mediate specific biological roles.

6.1. Structural functions

In addition to steric effects on protein conformation and binding, glycans play an active role in facilitating proper protein folding in the endoplasmic reticulum (ER). During protein synthesis, nascent polypeptides begin to acquire their correct conformations by passing through folding intermediates in the ER. Proper folding involves formation of secondary structures (α-helices and β-strands), burying of hydrophobic residues in the interior of the protein, formation of disulfide bonds, and quaternary association via oligomerization or multimerization (Varki et al., 2009). Proteins that are not properly folded are prevented from exit to the Golgi and, if permanently misfolded, are transported to the cytosol, where they are degraded in the proteasome.

Most membrane and secreted proteins undergo co-translational modification with N-glycans. N-glycosylation here in the ER is involved in the glycoprotein quality control process, helping identify and exclude misfolded proteins and ensure that others fold properly (Braakman and van Anken, 2000; Parodi, 2000). There are two lectin-like chaperones in the ER, membrane-bound calnexin (CNX) and its soluble homologue calreticulin (CRT), which specifically bind to monoglucosylated N-glycans (i.e. containing a single α-linked glucose residue) of a glycoprotein with low affinity and retain the protein in the ER until proper and complete folding occurs (Varki et al., 2009). Binding of CNX or CRT to monoglucosylated N-glycans also maintains

glycoproteins in solution and prevents aggregation, thus allowing a functional interaction between protein moieties and classical ER chaperones, such as other folding-assisting proteins (Parodi, 2000).

6.2. Modulatory functions

It has been extensively appreciated that glycans and the enzymes responsible for glycosylation (e.g. glycosyltransferases and glycosidases) are essential in the development and physiology of living organisms (Ohtsubo and Marth, 2006). Complete elimination of major glycan classes generally causes early developmental lethality. Glycans also participate in many key biological processes including cell adhesion, molecular trafficking and clearance, signal transduction, endocytosis, immune cell migration, pathogen recognition, inflammation, cancer immunity, and modulation of the innate and adaptive immune responses (Marth and Grewal, 2008; Ohtsubo and Marth, 2006; Sperandio et al., 2009; van Kooyk and Rabinovich, 2008).

6.3. The roles of glycans in viral infections

Cellular glycans can be involved in viral infections by regulating host cell tropism. Hemagglutinin on influenza virus binds to specific sialic acid structures on the host cell surface, determining the tissue and species tropism of the virus. For example, human influenza virus targets N-acetylneuraminic acids (Neu5Ac), whereas swine influenza virus can use N-glycolylneuraminic acids (Neu5Gc). Both Neu5Ac and Neu5Gc belong to the family of sialic acids. In addition, human influenza virus preferentially binds to α2-6-linked sialic acids, while bird influenza virus favors binding to α2-3-linked sialic acids. Pigs can be infected with both human and bird strains because sialic acids with both linkages naturally occur in pigs (Varki et al., 2009).

In many enveloped viruses, the envelope proteins are usually modified by the addition of glycan moieties, which have a key role in receptor binding, membrane fusion, penetration into cells, and virus budding (Braakman and van Anken, 2000; Doms et al., 1993). For example, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) recognizes mannose residues and serves as an entry protal for multiple viral pathogens, including human immunodeficiency virus (HIV), ebolaviruses and arthropod-borne viruses (arboviruses) within the *Flaviviridae* and *Togaviridae* (Baribaud et al., 2002; Klimstra et al., 2003; Rogers and Heise, 2009). Viral glycans of arboviruses can be recognized by C-type lectin receptors on immature dendritic cells to facilitate productive infection by increasing the efficiency of virus binding (Baribaud et al., 2002; Rogers and Heise, 2009).

7. Hypothesis and specific aims

PRRSV envelope protein-associated N-glycans have been shown to exhibit multiple biological functions, such as virus assembly, virus attachment to host cells, virus antigenicity and neutralization. So far, the only known PRRSV glycan with a defined function is sialic acid. Viral sialic acid binds to sialoadhesin on porcine macrophages, the naturally PRRSV-permissive cells, to mediate virus attachment and internalization. Since sialoadhesin is not expressed on MARC-145 cells, a simian cell type also permissive for PRRSV infection, sialic acid is not involved in viral infection of MARC-145 cells. Interestingly, ficolin, which specifically recognizes GlcNAc, reduces PRRSV infection in MARC-145 cells, suggesting a role of viral GlcNAc in viral entry and infection of MARC-145 cells. But the role of viral GlcNAc in infection of porcine macrophages has not been investigated yet.

In summary, the current knowledge of PRRSV glycans and their interactions with host cells is very limited. Considering the huge diversity of glycans and their general biological roles, there may be other glycan moieties, besides sialic acid, which are present on envelope

glycoproteins and contribute to virus infection in similar or different ways. To fulfill this knowledge gap, a profile of the composition and structures of PRRSV glycans is needed. Potential functional properties may then be assigned to defined structural features using biochemical and cell biologic methods. Herein, I hypothesize that specific N-glycan structures on the PRRSV envelope proteins mediate infection of permissive cells.

Specific aims:

1. Characterize the glycan composition and structures on PRRSV envelope proteins.
2. Determine the function of envelope protein-linked N-glycans in PRRSV infection of permissive cells.

CHAPTER II

N-Glycosylation Profiling of Porcine Reproductive and Respiratory Syndrome Virus Envelope Glycoproteins

Porcine reproductive and respiratory syndrome (PRRS) is one of the most severe infectious diseases facing the swine industry worldwide. The etiologic agent PRRS virus is a positive-sense ssRNA virus and has a smooth spherical envelope embedded with proteins, of which four of seven are glycoproteins. Since the major envelope proteins GP5 and matrix (M) have short ectodomains, the broadly distributed viral glycans likely cover the virion surface and stretch out as antennae, thus interacting with host cells and contributing to viral biology. Previous studies suggest that sialic acids on GP5 bind sialoadhesin on porcine macrophages, mediating virus attachment and internalization. In addition, ficolin, which recognizes N-acetylglucosamine, binds and reduces viral infectivity of type 2 PRRSV isolates in MARC-145 cells. Nevertheless, the complete profile of PRRSV glycan composition and structure has yet to be determined. To better understand the glycan structures of PRRSV, we purified the type 2 prototype, VR-2332, and analyzed the virion-associated glycans by both biochemical and mass spectrometric methods. Endoglycosidase digestion showed that GP5 was the primary protein substrate, and that the glycans were primarily complex-type N-glycans. Mass spectrometric analysis (HPLC-ESI-MS/MS) of GP5 N-glycans revealed an abundance of N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers, which was further confirmed by lectin co-precipitation. Finally, our findings, for the first time, directly demonstrate the presence of terminal sialic acids in PRRSV, and provide a glycan database for molecular structural studies of PRRSV.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), first isolated as the European strain Lelystad virus (LV), in Netherlands (Wensvoort, 1993) and shortly after as the North American strain, VR-2332, in the USA (Benfield et al., 1992; Collins et al., 1992), is the etiologic agent of "mystery swine disease" that emerged in 1980s and spread worldwide thereafter. The representative syndromes of the disease include reproductive failure in sows and respiratory distress in growing pigs. Based on similar genomic organization and transcription strategy, PRRSV, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV), belongs to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Cavanagh, 1997).

Mature PRRS virions are composed of a nucleocapsid core enclosing a positive-sense, single-stranded RNA genome of ~15 kb, and an envelope harboring critical transmembrane proteins (Conzelmann et al., 1993; Dea et al., 1995; Dea et al., 2000; Mardassi et al., 1996). The major envelope proteins GP5 and matrix (M) form disulfide-bonded heterodimeric complexes in N-terminal ectodomains and together comprise at least half of the viral proteins (Dea et al., 2000; Mardassi et al., 1996; Meulenberg et al., 1995; Wissink et al., 2005). By cryo-electron microscopy, PRRSV particles display a smooth outline of the envelope with few protruding features, consistent with predicted small ectodomains of GP5 and M (30 residues for GP5 and 16 for M) (Dokland, 2010; Spilman et al., 2009). GP5 contains 3 putative N-glycosylation sites at residues 33, 44 and 51 in VR-2332 and 2 putative N-glycosylation sites at residues 46 and 53 in LV. Lack of the oligosaccharides linked to N44 (the North American strain) and N46 (LV) in GP5 impairs the production of infectious progeny virus and significantly reduces viral infectivity (Ansari et al., 2006; Wissink et al., 2004). Minor proteins GP2a, E, GP3 and GP4 are incorporated as multimeric complexes in the envelope, with the glycoproteins containing

conserved N-glycosylation sites in both strains (Wissink et al., 2005). Therefore, the broadly distributed viral glycans likely cover the virion surface and stretch out as antennae, thus interacting with host cells and contributing to viral biology. Removal of complex-type N-glycans from PRRSV reduced infectivity in porcine macrophages, suggesting an important role of viral glycans in infection (Delputte and Nauwynck, 2004). In particular, sialic acids on GP5 bind sialoadhesin on macrophages, mediating virus attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). An N-acetylglucosamine (GlcNAc)-specific ligand also binds and reduces viral infectivity in MARC-145 cells (Keirstead et al., 2008).

Significant roles for PRRSV-associated glycans have been postulated in virus assembly, virus attachment to target cells, virus neutralization and immunological protection (Ansari et al., 2006; Das et al., 2011; Delputte and Nauwynck, 2004; Wissink et al., 2004). However, to further evaluate the contributions of viral glycans to PRRSV pathogenesis and immune protection, detailed knowledge of glycan structural information and distribution in viral envelope glycoproteins is essential.

Therefore, we purified PRRSV, digested virus with endoglycosidases and showed that GP5 is the major source of predominantly complex-type N-glycans. Mass spectrometric analysis confirmed this finding, and further revealed the characteristic glycan structures containing N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers and terminal sialic acids, whose presence in PRRSV was confirmed by lectin co-precipitation.

Materials and Methods

Virus and cells

The North American prototype PRRSV, ATCC VR-2332, GenBank ID: PRU87392 (American Type Culture Collection, Manassas VA) (Benfield et al., 1992; Nelsen et al., 1999), was propagated in MARC-145 cells (Kim et al., 1993) using MEM (Mediatech, Herndon VA) containing 10% FBS (Mediatech), 1 mg/ml sodium bicarbonate (Sigma-Aldrich, St. Louis MO), 1% (v/v) 100X nonessential amino acid solution (Mediatech), 10 mM HEPES solution (Mediatech) and 20 µg/ml gentamicin (Invitrogen, Carlsbad CA) at 37°C in a 5% CO_2 incubator. After 2 days of infection, virus was collected from culture supernatant, clarified by centrifugation, titered on MARC-145 cells, and stored in aliquots at -80°C.

Virus purification

VR-2332 was propagated in MARC-145 cells for two days. Debris was removed by centrifugation at 17,000 x g for 1 h, and supernatant was mixed with 10% polyethylene glycol-8000 (Fisher Scientific, Fair Lawn NJ) overnight at 4°C. The virus was pelleted at 22,000 x g for 2 h, resuspended in Tris-buffered saline (TBS), and pelleted twice through a 0.5 M sucrose (Fisher Scientific) cushion at 110,000 x g for 3 h. The final pellet was re-suspended in 20% iodixanol (Sigma) in TBS and banded twice in a self-generating gradient by ultracentrifugation at 250,000 x g for 9 h. The purified virus band was removed with a sterile needle and stored in aliquots at -80°C.

Endoglycosidase digestion and SDS-PAGE

In non-reducing conditions, 2 µg of purified PRRSV was incubated with 100~500 units of peptide-N-glycosidase F (PNGase F, New England Biolabs, Ipswich MA) in 50 mM sodium phosphate, pH 7.5, or 100~400 units of endoglycosidase H_f (Endo H_f, New England Biolabs) in

50 mM sodium citrate, pH 5.5, at 37°C for 1 h. The mixture of virus and endoglycosidase was added to gel loading buffer with 5% β-mercaptoethanol, boiled for 10 min and electrophoresed in 10-20% gradient Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules CA). For PNGase F samples, CandyCane Glycoprotein Molecular Weight Standards (Invitrogen) were used to estimate protein size and protein bands were visualized with RubyProtein Gel Stain (Invitrogen). For Endo H_f samples, Kaleidoscope Prestained Standards (Bio-Rad) were used to estimate protein size and protein bands were visualized with Deep Purple Total Protein Stain (GE Healthcare, Buckinghamshire, UK). Finally, proteins were analyzed in EpiChemi^3 Darkroom (UVP, Upland CA) using software LabWorks 4.5 (UVP).

N-glycan release from GP5

Approximately 150 μg of purified VR-2332 was run with 5% β-mercaptoethanol in SDS-PAGE as above and visualized by Imperial Protein Stain (Thermo Scientific, Rockford IL). In-gel trypsin digestion was performed to extract GP5 glycopeptides (adapted from Shevchenko, 1996). Briefly, GP5 bands were excised and Coomassie blue dye was washed off with a mixture of 50% acetonitrile (Sigma) and 50% 100 mM ammonium bicarbonate (Sigma). The protein bands were then treated with 10 mM dithiothreitol to reduce cysteines, alkylated in 55 mM iodoacetamide and thereafter incubated with 12.5 ng/ul trypsin (Invitrogen) at 37°C overnight. GP5 glycopeptides were then collected and lyophilized in a Speed-Vac refrigerated concentrator (Thermo Scientific). To deactivate trypsin, sample digests were reconstituted in 50 mM ammonium bicarbonate and heated at 100°C for 10 min. After cooling, 1500 units of PNGase F was added and incubated at 37°C overnight to release N-glycans.

N-glycan purification and modification

Lyophilized PNGase F digests were reconstituted in 5% acetic acid and passed through a Sep-Pak C18 cartridge (J.T. Baker, Phillipsburg NJ) to separate peptides and glycans. Glycans

were eluted with 5% acetic acid and lyophilized overnight. Purified glycans were resuspended in a slurry of NaOH in DMSO and permethylated with iodomethane. After chloroform extraction, permethylated glycans were dried in a stream of N_2. Permethylated N-glycans were dissolved in 50% methanol and applied to a C18 column to remove salts and other contaminants before MS analysis. The column was washed with Milli-Q water and followed by 15% acetonitrile. N-glycans were eluted with 85% acetonitrile, and dried in a stream of N_2.

Liquid chromatography and electrospray mass spectrometry

Reversed phase HPLC-ESI-MS/MS analysis of permethylated N-glycans was performed on a hybrid linear ion-trap Fourier Transform ion cyclotron resonance (FTICR) mass spectrometer (LTQ-FT, Thermo Finnigan). The separation column was a C18 reversed phase capillary column (Magic C18 AQ, 0.2x50mm). Mobile phases consisted of solvent A, 99.9% deionized H_2O and 0.1% formic acid, and solvent B, 99.9% acetonitrile and 0.1% formic acid, which were pumped at a flow rate of 5 µl/min. MS/MS analysis was operated in a data-dependent scanning mode: a full MS scan within the m/z range 600-2000 followed by data-dependent MS/MS scans of the nine most intense glycan ions from the full MS scan. Glycan precursor ions were isolated for MS/MS using an isolation width of 3.0 m/z, and a normalized collision energy of 35% was applied for fragmentation. Manual interpretation of all glycan structures was carried out with in-house fragmentation rules and GlycoWorkbench (http://www.glycoworkbench.org/). Cartoons were constructed using standard Consortium for Functional Glycomics (CFG) nomenclature for monosaccharides.

Lectin-virus co-precipitation

Lycopersicon esculentum agglutinin (LEA), *Datura stramonium* agglutinin (DSA), wheat germ agglutinin (WGA) and concanavalin A (ConA) were purchased from Sigma. Approximately 15 nmoles of lectin was coupled to CNBr-activated Sepharose^{TM} 4B beads. Coupled and blank

beads were then incubated with virus solution at 4°C for 3 h. After washing with PBS three times, the amount of virus bound to beads was determined by quantitative RT-PCR.

PRRSV quantitative reverse transcriptase (RT)-PCR

Total RNA isolation was performed using a Viral RNA Mini Kit following the manufacturer's protocol (Qiagen, Valencia CA). RNA was eluted in 50 µl water and 10 µl was used to prepare cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA). Quantitative real time PCR was carried out with 5 µl of 1:10 diluted cDNA, 10 µl of PerfeCta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg MD), and 5 µl of 1 µM primers with forward primer GATAACCACGCATTTGTCGTC, and reverse primer TGCCGTTGTTATTTGGCATA. To quantify viral RNA copies, RNA extracted from a titered VR-2332 stock was used as standards. The final results were expressed as total RNA copies according to the standard curve.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego CA). Lectin co-precipitation data were analyzed by one-way ANOVA with Tukey's multiple comparison test. A p-value <0.05 was considered statistically significant.

Results

GP5 contains complex-type N-glycans

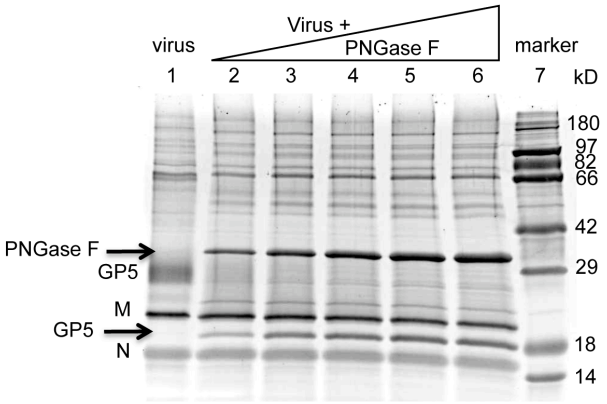
There are four glycoproteins in the PRRSV envelope, the major protein GP5 and minor proteins GP2a, GP3 and GP4. According to the glycosylation prediction programs NetNGlyc 1.0 and NetOGlyc 3.1 (Center for Biological Sequence Analysis, Technical University of Denmark), all the envelope glycoproteins have exclusive N-linked glycosylation sites, but no O-linked glycosylation sites. Thus we focused our study on N-linked glycans.

In reducing SDS-PAGE, purified PRRSV showed 3 major protein bands, which were identified to be GP5 (~25 kD), M (19 kD) and N (14 kD), the three major structural proteins of PRRSV (Fig. 2.1A). The minor envelope glycoproteins, GP2a, GP3 and GP4, were undetectable due to low abundance. Incubation of purified virus with increasing amounts of PNGase F (36 kD) caused a disappearance of GP5 at 25 kD and the appearance with increasing intensity of a new band between M and N (Fig. 2.1A). Mass spectrometric analysis identified this new band to be GP5. Deglycosylated GP5 is about 19 kD, similar to M, but has a lower isoelectric point (pI 8.87) than M (pI 10.03), accounting for its appearance below M in the gel. Therefore, GP5 from VR-2332 contains exclusively PNGase F-sensitive N-glycans. No other bands shifted in the gel after PNGase F treatment, showing that GP5 contains the vast majority of viral N-glycans. A faint contaminating band between GP5 and M in purified virus was identified as trypsin by mass spectrometry, and stayed at the same position after PNGase F treatment.

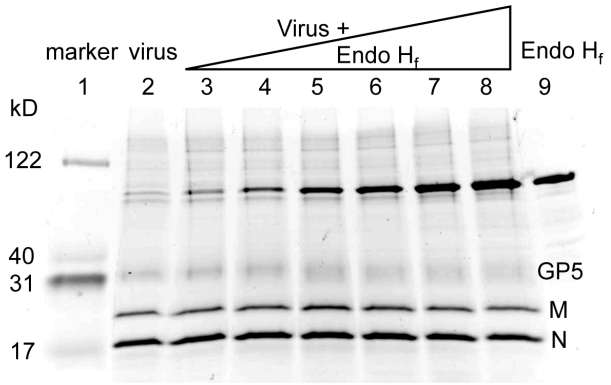
Treatment of purified virus with Endo H_f (70 kD), which cleaves high-mannose and hybrid-type N-glycans, caused the GP5 band to broaden but did not shift the protein to a new location (Fig. 2.1B). Thus, GP5 is predominantly composed of Endo H_f-resistant complex-type N-glycans with small amounts of high-mannose and hybrid-type N-glycans.

Fig. 2.1. Endoglycosidase digestion of purified PRRSV. (A) Purified virus was incubated with increasing amounts (100~500 units) of PNGase F (36 kD) at 37°C for 1 h. Samples were run in reducing SDS-PAGE, and the gel was stained with Ruby Protein Gel Stain. Lane 1, virus only. Lane 2-6, virus treated with PNGase F. Lane 7, protein marker. (B) Purified virus was incubated with increasing amounts (100~400 units) of Endo H_f (70 kD) at 37°C for 1 h and samples were electrophoresed as above. The gel was stained with Deep Purple. Lane 1, protein marker. Lane 2, virus only. Lane 3-8, virus treated with Endo H_f. Lane 9, Endo H_f only.

A



B



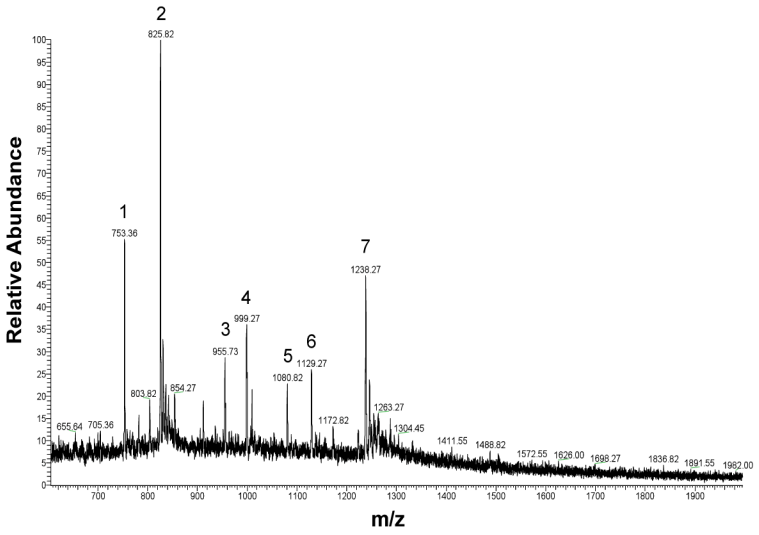
GP5-linked N-glycans contain GlcNAc and LacNAc oligomers and terminal sialic acids

Mass spectrometric analysis of GP5-derived N-glycans was performed to characterize specific glycan composition and structures. Figure 2.2 showed the positive ion electrospray spectra, which were potentially complicated by multiple charging. Candidate glycan structures from Carbbank (Doubet and Albersheim, 1992) were assigned by GlycoWorkbench (Ceroni et al., 2008) according to the m/z values with a tolerance of ±1. Furthermore, MS/MS fragmentation spectra were used to calculate potentially represented masses, which were then matched to the masses of computed fragments of the assigned glycan structures. As an example of structural elucidation, the most abundant glycan (m/z 825.82) in Fig. 2.2A was assigned a structure with the monosaccharide composition $Hex_3HexNAc_6Fuc_2$ (Fig. 2.3A). The MS/MS spectrum showed ions in agreement with the computed fragments of a proposed structure (Fig. 2.3B). Glycan composition was then summarized based on candidate glycan structures.

Candidate GP5-linked N-glycan structures were a mixture of bi-, tri-, and tetra-antennary complex (n=75), high-mannose (n=4), and hybrid (n=10) carbohydrates with or without core fucose (Table 2.1). Consistent with the endoglycosidase digestion results, GP5 contained primarily complex type N-glycans. Antennae were mainly terminated with galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetyllactosamine (LacNAc) and various forms of sialic acid (NeuAc, NeuGc and Kdn). N-acetylneuraminic acid (NeuAc) was the major sialic acid terminating the candidate glycan structures with even distribution of α2-3 and α2-6 linkages. In particular, GlcNAc oligomers were found in all the candidate hybrid and complex-type structures. Bisecting GlcNAc was detected in 1 hybrid and 8 complex candidate structures. Type 2 LacNAc (Galβ1-4GlcNAc) was present in 3 hybrid and 66 complex glycan structures, with 9 containing 2-4 tandem repeats of LacNAc.

Fig. 2.2. Electrospray spectra of putative N-glycans. There were 12 marked peaks, which were predicted to be N-glycans. (A) LC Retention time 53:47-54:28. (B) LC Retention time 55:52-56:14.

A



B

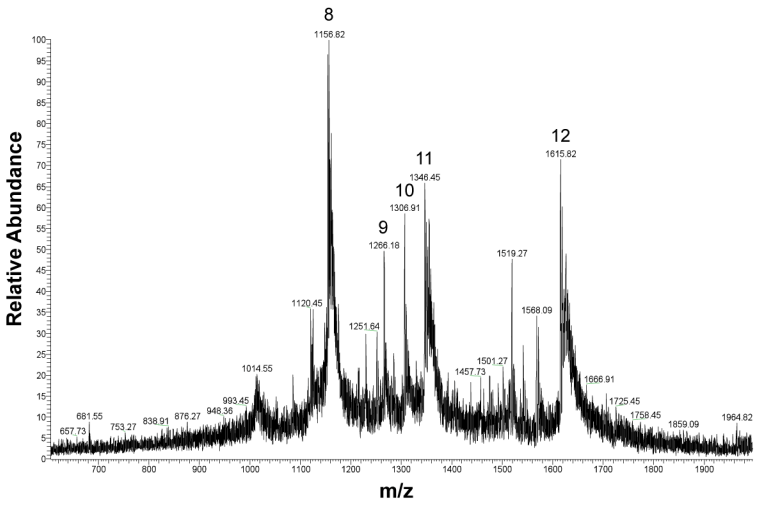
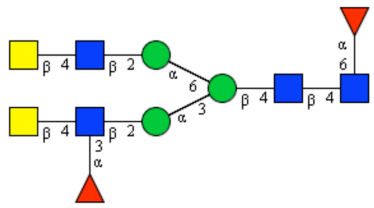


Fig 2.3. A candidate glycan structure of peak 2 (m/z 825.82) and its corresponding MS/MS spectrum. (A) A candidate glycan structure of peak 2. (B) Fragmentation of structure A matches the MS/MS spectrum of peak 2.

A



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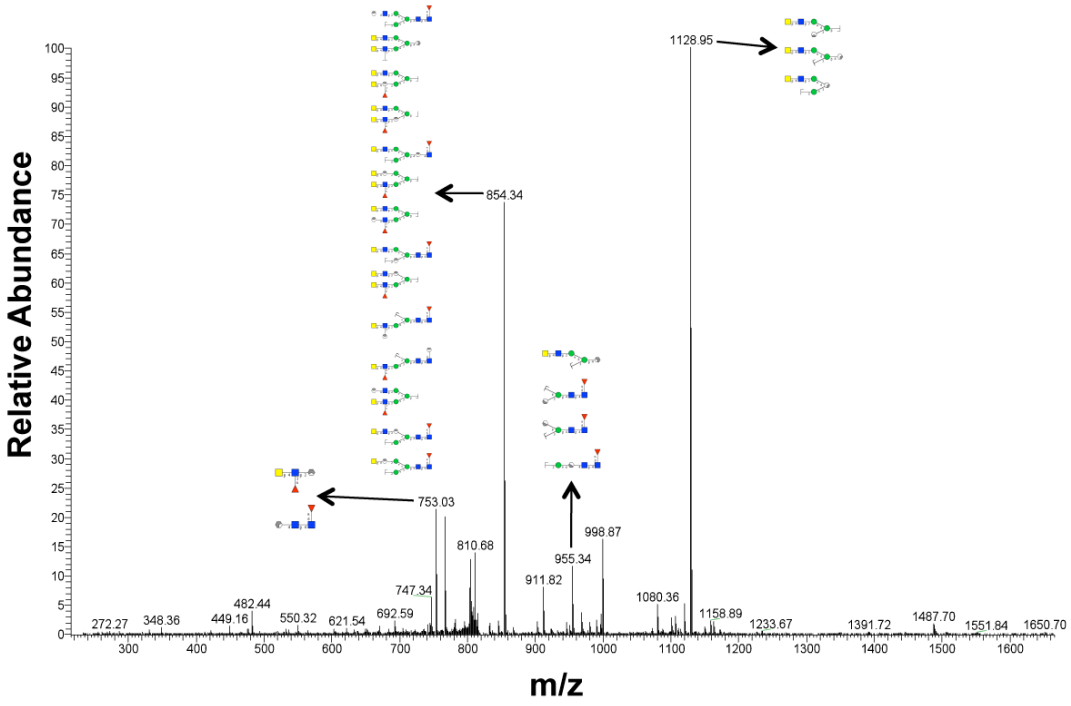
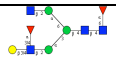
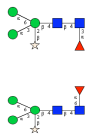
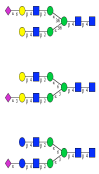
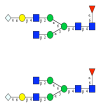
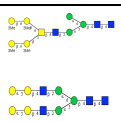
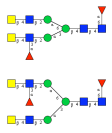
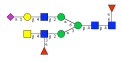
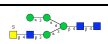
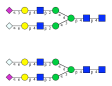
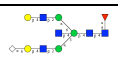
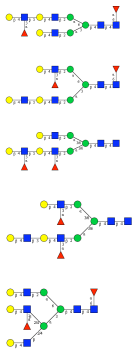
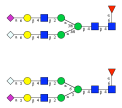
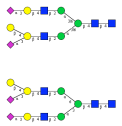
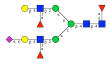
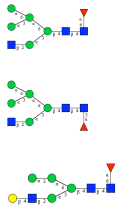
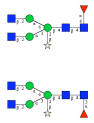
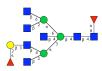
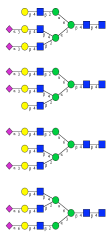
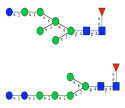


Table 2.1. Monoisotopic masses, compositions and putative structures of GP5-linked N-glycans.

Peak	m/z	Theory m/z	Mass	Charge	Composition							Proposed structure
					Hex	HexNAc	Fuc	Xyl	NeuAc	NeuGc	Kdn	
1	753.36	753.36	2191.12	3	4	4	2	0	0	0	0	
		753.38	1482.75	2	3	2	1	1	0	0	0	
		752.82	1459.66	2	3	3	0	0	0	0	0	
		752.37	2210.12	3	7	3	0	0	0	0	0	
2	825.82	825.73	2408.22	3	5	4	0	0	1	0	0	
		825.73	2408.22	3	4	4	1	0	0	1	0	
		826.73	2411.22	3	6	4	1	1	0	0	0	
		826.75	2455.24	3	7	4	0	0	0	0	0	
		826.77	2477.27	3	3	6	2	0	0	0	0	
3	955.73	955.83	2864.47	3	3	9	0	0	0	0	0	
		955.46	2797.42	3	4	5	2	0	1	0	0	
		955.45	1908.88	2	4	4	0	0	0	0	0	
		956.12	2799.40	3	5	4	0	0	1	1	0	
		956.14	2799.44	3	5	5	1	0	0	1	0	

		956.49	2844.45	3	6	5	2	0	0	0	0	
4	999.27	999.50	2973.49	3	5	4	1	0	1	1	0	
		999.50	2973.49	3	6	4	0	0	2	0	0	
		999.82	2930.49	3	5	4	3	0	1	0	0	
		1000.00	1976.01	2	5	3	1	0	0	0	0	
		998.50	1973.01	2	3	4	1	1	0	0	0	
		998.49	2926.50	2	4	7	2	0	0	0	0	
5	1080.82	1081.21	3218.61	3	6	5	0	0	2	0	0	
		1081.54	2139.08	2	7	2	1	0	0	0	0	

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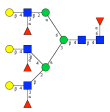
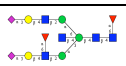
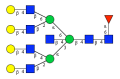
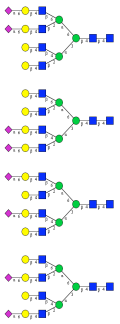
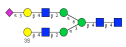
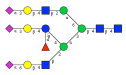
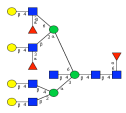
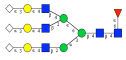
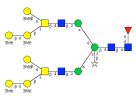
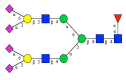
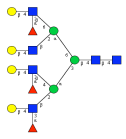
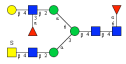
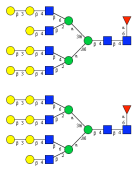
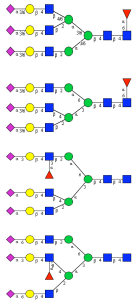
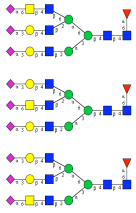
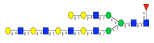
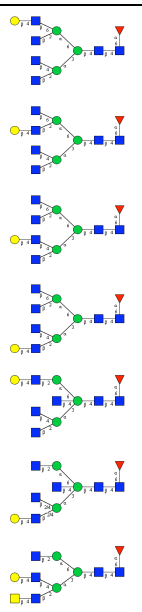
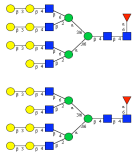
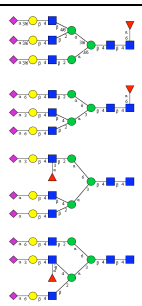
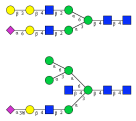
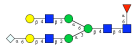
		1079.87	3214.61	3	6	5	4	0	0	0	0	
6	1129.27	1129.23	3362.69	3	5	5	2	0	2	0	0	
		1129.91	3364.71	3	7	7	1	0	0	0	0	
7	1238.27	1238.28	3667.84	3	7	6	0	0	2	0	0	
		1238.09	2474.16	2	5	4	0	0	1	0	0	
		1238.63	3712.85	3	7	4	0	0	3	0	0	
		1238.64	3712.88	3	7	7	3	0	0	0	0	
		1238.96	3669.89	3	6	5	1	0	0	3	0	
		1237.62	3687.86	3	9	6	1	1	0	0	0	
		1237.61	3665.83	3	5	4	1	0	4	0	0	
8	1156.82	1156.93	3467.76	3	7	6	3	0	0	0	0	
9	1251.64	1252.10	2502.19	2	4	5	2	0	0	0	0	

		1252.30	3731.88	3	10	6	1	0	0	0	0	
		1252.30	3753.88	3	6	5	1	0	3	0	0	
10	1266.18	1265.98	3794.90	3	5	6	1	0	3	0	0	
		1265.97	3772.91	3	9	7	1	0	0	0	0	
		1265.64	2507.29	2	4	6	1	0	0	0	0	
		1266.95	3731.89	3	10	6	1	0	0	0	0	

		1266.96	3753.88	3	6	5	1	0	3	0	0	
11	1306.91	1307.17	2612.31	2	6	4	0	0	1	0	0	
		1307.17	2612.31	2	5	4	1	0	0	1	0	
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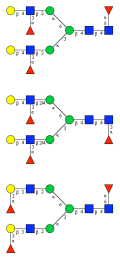
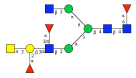
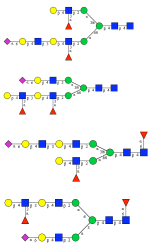
		1307.65	2569.31	2	5	4	3	0	0	0	0	
		1306.18	2610.34	2	4	5	3	0	0	0	0	
12	1615.82	1614.81	1590.81	2	6	5	2	0	1	0	0	

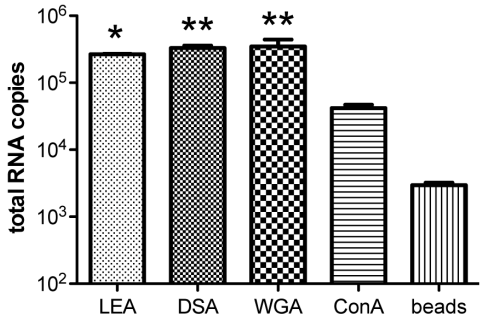
Lectins specific for GlcNAc and LacNAc oligomers and sialic acids bind PRRSV

To verify the presence of GlcNAc and LacNAc oligomers and terminal sialic acids in the viral glycan structures, lectins of varied specificities were coupled to Sepharose beads and incubated with PRRSV to evaluate their virus-binding capabilities. Based on the amount of precipitated virus, the order of the bead treatments was WGA ≈ DSA > LEA > ConA > blank beads (Fig. 2.4A). WGA, DSA and LEA significantly depleted PRRSV, and significantly reduced cell-culture infectivity of the resulting solution, compared to ConA-beads, blank beads, and untreated virus preparation (Fig. 2.4B). Also, viral growth, as measured by progeny virus in culture supernatant, was significantly reduced in the groups treated with WGA, DSA and LEA (Fig. 2.4C). These results demonstrated the binding of PRRSV virions to lectins specific for GlcNAc and LacNAc oligomers and terminal sialic acid, but not for mannose.

Although the lectins, WGA, DSA and LEA, all recognize GlcNAc oligomers, there are subtle differences in carbohydrate specificity. WGA reacts strongly with the chitobiose core of bisected hybrid-type N-glycans, specifically with the oligosaccharide GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc (Yamamoto et al., 1981; Yodoshi et al., 2011), which was proposed in the candidate glycan structure of peak 11 (Fig. 2.2B, m/z 1306.91). To a lesser extent, WGA has an affinity for broadly linked sialic acids (Neu5Ac), which were found in the termini of 1 hybrid and 34 complex-type candidate structures. DSA and LEA recognize GlcNAc oligomers that may not be consecutive (Kawashima et al., 1990) and were found in most candidate hybrid and complex-type N-glycan structures. In addition, DSA reacts with the complex-type N-glycans containing the structure Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Man or at least one LacNAc (Galβ1-4GlcNAc) unit in an outer chain, and the interaction is not affected by the presence of a bisecting GlcNAc residue (Yamashita et al., 1987). There were 66 candidate glycan structures that satisfy the LacNAc binding properties of DSA. In contrast, LEA requires 2 consecutive LacNAc

Fig. 2.4. Lectin co-precipitation of PRRSV. LEA, DSA, WGA and ConA-coupled beads and control beads were incubated with PRRSV, then pelleted, and washed. Effect of treatment was assessed by quantitation of virus amount bound to beads, by qRT-PCR (A), depletion of infectious virus from supernatant determined by inoculation of supernatant onto MARC-145 cells and assessment of infection 1 day later by flow cytometry (B), and reduction in progeny virus from MARC-145 culture. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test. Significant differences shown were compared to blank beads. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

A



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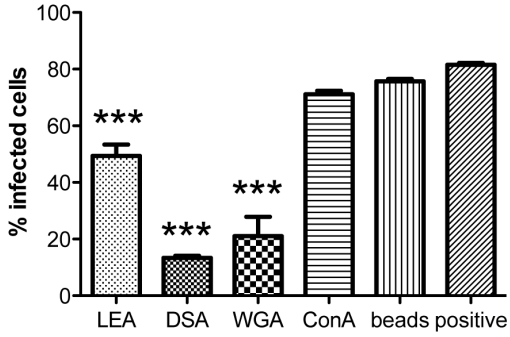

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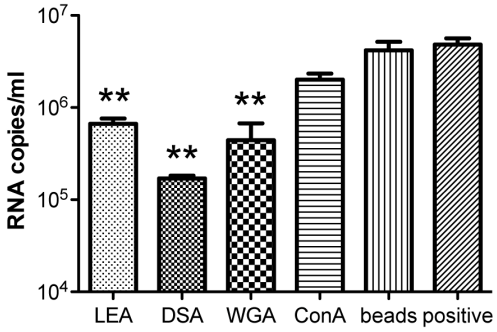

residues (Kawashima et al., 1990), which were found in only 9 candidate structures. The difference in LacNAc-binding properties explains why DSA bound more virus than LEA. ConA is specific for terminal α-D-mannose and α-D-glucose (Maupin et al., 2011), which were present in all 4 high-mannose and 10 hybrid-type candidate structures. The lack of significant ConA-binding is consistent with endoglycosidase digestion results showing that high-mannose and hybrid-type N-glycans are minor components of the PRRSV virion.

Discussion

PNGase F sensitivity and Endo H resistance, indicating the predominance of complex-type N-glycans, is a general characteristic of PRRSV grown on MA-104 cells, including type 1 Lelystad virus and type 2 IAF-Klop strain (Mardassi et al., 1996; Meulenberg et al., 1995). These glycans reside primarily on GP5 and, as we now show, have characteristic features of GlcNAc and LacNAc oligomers and terminal sialic acids. Viral protein glycans are obtained from host cells, and the glycan patterns are dependent on the specific modifying enzymes (Dalpathado et al., 2006; Liedtke et al., 1994).The main pathway of N-glycan biosynthesis is conserved among eukaryotic species, beginning as a dolichol-linked precursor that is transferred to an Asn-X-Ser/Thr sequon in a protein and modified to a high-mannose type in the endoplasmic reticulum (Varki et al., 2009). The protein-bound N-glycan is then translocated to the Golgi and further processed to hybrid and complex-type N-glycans. Thus, high-mannose glycans imply minimal processing and a more protected local conformational structure, as would occur if buried within a folded protein. In contrast, fully processed (hybrid and complex) glycans in PRRSV implies that the glycosylation site is readily accessible, and thus reflects a more flexible and exposed protein structure.

PRRSV has a restricted cell tropism, infecting primarily porcine macrophages, the natural host cell, and a green monkey kidney epithelial-like cell line (MA-104, also known as CL2621, and a subclone, MARC-145) that has been routinely used for in vitro propagation of PRRSV isolates and modified live vaccines. Structural glycan characterization of virus grown in simian cells will facilitate a better understanding of PRRSV molecular and cellular biology, and provide a reference for interpretation of glycan features of PRRSV grown in macrophages. Interestingly, the external envelope glycoprotein of human immunodeficiency virus type 2 (HIV-2) grown in macrophages displays a complex-type N-glycan pattern with LacNAc repeats (Liedtke et al., 1994).

Among the characteristic features of PRRSV glycans, sialic acid binding to sialoadhesin on porcine macrophages mediates virus attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). It is not known whether GlcNAc and LacNAc oligomers interact with host cells of PRRSV. GlcNAc and LacNAc oligomer-specific lectins bound and precipitated PRRSV, showing that these glycans are present and accessible for recognition on the PRRSV envelope. Macrophages express galectin-3, which binds the poly-LacNAc-containing N-glycans, thus providing a mechanism for binding (Dumic et al., 2006).

The mass spectrometric tool used here is positive ion mode electrospray tandem MS. The electrospray spectrum is complicated by the presence of ions with multiple charges. In positive ion mode, the MS/MS spectra contain ambiguous information because fragment ions of the same mass and composition can arise from different regions of a molecule. The situation is even more difficult with the concern of isomers. Future studies of PRRSV glycans may narrow down the candidate structure list by more powerful tools, including sequential exoglycosidase digestion, to reveal more glycan structural information.

The VR-2332 strain GP5 contains 3 N-glycosylation sites but it is not certain if all are occupied by oligosaccharides or if they are fully glycosylated. The total mass of glycans on GP5 is estimated to be 6-7 kD. It was postulated that all 3 sites contain oligosaccharides of ~2.5 kD (Ansari et al., 2006). Since the glycan structures identified here are in the range of 2 to 4 kD, either 2 or 3 glycosylation sites may be utilized. VR-2332 GP5 N_{33} in the sequon NDS, especially, may be hypoglycosylated since the site is not highly conserved (Murtaugh et al., 2010), it contains D_{34}, which is linked to poor glycosylation efficiency (Kasturi et al., 1997), and it contains S_{35}, which is less preferred in the third position than T that is present in the N_{44} and N_{51} sequons (Kasturi et al., 1997; Rao and Bernd, 2010). We attempted to determine the efficiency of glycosylation at N_{33}, N_{44} and N_{51} by PNGase F digestion of glycopeptides in ^{18}O-labeled or normal water. Product peptides with an "occupied" glycosylation site, when digested in ^{18}O water, would show a 3-Dalton increase compared to the peptides digested in normal water. Since all the three GP5 glycosylation sites reside in the same tryptic peptide, endoproteinases Glu-C and Asp-N were used to isolate each glycosylation site on an individual peptide. Unfortunately, in-gel digestion of GP5 with these enzymes was not successful for unknown reasons (data not shown).

The discovery that GlcNAc and LacNAc oligomers as well as terminal sialic acids are on the PRRSV envelope and readily accessible for specific binding will facilitate the generation of testable hypotheses to elucidate the mechanistic basis for variation in efficiency of cell culture of PRRSV isolates and potential glycan differences in porcine macrophage versus simian cell glycosylation that might contribute to attenuation of vaccine strains or other biological characteristics.

CHAPTER III

Functional Analysis of Porcine Reproductive and Respiratory Syndrome Virus N-Glycans in Infection of Permissive Cells

The arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense ssRNA virus. It has a smooth spherical envelope embedded with seven proteins of which four are glycoproteins. Previous studies suggested potential roles of PRRSV-associated glycans in virus assembly, virus attachment to target cells, virus neutralization and antigenicity. For example, sialic acids on the major envelope glycoprotein 5 (GP5) bind sialoadhesin on porcine alveolar macrophages (PAM), mediating virus attachment and internalization. Recently, we identified N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers in GP5 and showed that they were accessible for specific lectin binding. Here, we demonstrate that GlcNAc and LacNAc oligomer-specific lectins bind to PRRSV and block virus attachment, resulting in reduced viral infection. However, addition of GlcNAc oligomers and LacNAc to cell culture together with PRRSV did not block infection. Removal or alteration of envelope protein-linked N-glycans also did not affect virus infection, indicating that PRRSV N-glycans are not required for virus infection. These findings show that GlcNAc and LacNAc oligomers on the PRRSV envelope are accessible for ligand recognition, which can cause steric hindrance that prevents virus attachment to host cells, leading to reduced infection. Thus, steric hindrance of glycans on the PRRSV virion by lectins or, presumably, other space-filling molecules may interfere nonspecifically with infection by blocking protein interactions with cell surface receptors. Glycans themselves appear not to be required for infection of permissive cells, but may have important roles in avoidance of host immunity and in intracellular growth and assembly of virions.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), which is characterized by reproductive failure in sows and respiratory distress in growing pigs, emerged in the 1980s and soon became an economically important infectious disease worldwide. The etiologic agent PRRSV was first isolated as the type 1 European strain Lelystad virus (LV) in the Netherlands (Wensvoort et al., 1991) and shortly after as the type 2 North American strain VR-2332 in the USA (Benfield et al., 1992; Collins et al., 1992). Based on similar genomic organization and transcription strategy, PRRSV, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV), belongs to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Cavanagh, 1997).

PRRSV has a positive single-stranded RNA genome of approximately 15 kb and contains ten open reading frames (ORFs). ORF1a and ORF1b encode nonstructural proteins (nsps) responsible for virus replication and transcription (Kroese et al., 2008; Meulenberg, 2000; Snijder and Meulenberg, 1998). ORF2a, ORF2b, ORF5a and ORFs3-7 encode 8 structural proteins, four of which are envelope glycoproteins (GPs), GP2a, GP3, GP4 and GP5 (Dea et al., 2000). The E protein (encoded by ORF2b), matrix (M) protein (encoded by ORF6) and recently discovered ORF5a protein are also present on virus envelope, but are not glycosylated (Johnson et al., 2011; Mardassi et al., 1996; Wu et al., 2001). GP5 and M are the major envelope proteins that form disulfide-bonded heterodimeric complexes and together comprise at least half of the viral protein mass (Dea et al., 2000; Mardassi et al., 1996; Meulenberg et al., 1995; Wissink et al., 2005). GP5 contains 3 putative N-glycosylation sites at residues 33, 44 and 51 in VR-2332 and 2 putative N-glycosylation sites at residues 46 and 53 in LV. Lack of the oligosaccharides linked to N44 (the North American strain) and N46 (LV) in GP5 impairs the production of infectious progeny virus and significantly reduces viral infectivity (Ansari et al., 2006; Wissink et al., 2004). Minor

proteins GP2a, E, GP3 and GP4 are incorporated as multimeric complexes in the envelope, with the glycoproteins containing conserved N-glycosylation sites in both types (Wissink et al., 2005). Glycan addition at N184 of GP2a, N42, N50 and N131 of GP3, and multiple locations (N37, 84, 120 and 130) of GP4 is also necessary for infectious virus production (Das et al., 2011).

While PRRSV-linked glycans are critical structural components in virus assembly, possibly due to proper protein folding, they are also suggested to interact with host cells and thus are important for virus infection. Removal of complex-type N-glycans from PRRSV reduced infectivity in porcine macrophages (Delputte and Nauwynck, 2004). In particular, sialic acids on GP5 bind sialoadhesin on macrophages, mediating virus attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Van Gorp et al., 2008). In addition to sialic acid, we recently identified some characteristic N-glycan moieties, N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers in GP5 of type 2 PRRSV and showed that they were accessible for specific lectin binding (Chapter II). A previous study also showed that ficolin, specific for GlcNAc, bound and reduced PRRSV infectivity in MARC-145 cells (Keirstead et al., 2008), a simian cell line routinely used for in vitro PRRSV propagation.

We show here that GlcNAc and LacNAc oligomer-specific lectins bind to type 2 PRRSV and block virus attachment, resulting in reduced viral infection. However, addition of soluble GlcNAc oligomers and LacNAc to permissive cell cultures does not block infection. Moreover, removal or alteration of N-glycans from PRRSV does not affect virus infection. These observations suggest that N-glycans are not required for efficient PRRSV infection, but their binding to soluble lectins can reduce infection via steric hindrance.

Materials and Methods

Virus and cells

The type 2 prototype PRRSV, ATCC VR-2332, GenBank ID: PRU87392 (American Type Culture Collection, Manassas VA) (Benfield et al., 1992; Nelsen et al., 1999), was propagated in MARC-145 cells (H. S. Kim et al., 1993) using MEM (Mediatech, Herndon VA) containing 10% FBS (Mediatech), 1 mg/ml sodium bicarbonate (Sigma-Aldrich, St. Louis MO), 1% (v/v) 100X nonessential amino acids (Mediatech), 10 mM HEPES solution (Mediatech) and 20 μg/ml gentamicin (Invitrogen, Carlsbad CA) at 37°C in a 5% CO_2 incubator. After 2 days of infection, virus was collected from culture supernatant, clarified by centrifugation, titered on MARC-145 cells and porcine alveolar macrophages (PAM), and stored in aliquots at -80°C.

Porcine alveolar macrophages (PAM) were harvested by lung lavage of 4-6-week old pigs as previously described (Lin et al., 1994). Three to four 300-ml washings were centrifuged at 500 x g and resuspended at 10^7 cells/ml in 90% FBS with 10% (v/v) dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh PA) for cryopreservation in liquid nitrogen. Prior to use, PAMs were thawed in a 37°C water bath, washed and maintained in RPMI 1640 (Invitrogen) containing 5% FBS, 1% (v/v) sodium pyruvate (Mediatech), 1% (v/v) 100X nonessential amino acids, 10 mM HEPES solution and 20 μg/ml gentamicin at 37°C in 5% CO_2.

Virus purification

VR-2332 was propagated in MARC-145 cells with or without swainsonine for two days. Debris was removed by centrifugation at 17,000 x g for 1 h, and supernatant was mixed with 10% polyethylene glycol-8000 (Fisher Scientific, Fair Lawn NJ) at 4°C overnight. Virus was pelleted at 22,000 x g for 2 h, resuspended in Tris-buffered saline (TBS), and pelleted twice through a 0.5 M sucrose (Fisher Scientific) cushion at 110,000 x g for 3 h. The final pellet was resuspended in

20% iodixanol (Sigma) in TBS and banded twice in a self-generating gradient by ultracentrifugation at 250,000 x g for 9 h. The purified virus band was removed with a sterile needle and stored in aliquots at -80°C.

Endoglycosidase digestion and SDS-PAGE

In non-reducing conditions, 2 μg of purified PRRSV was incubated with 100, 200 or 400 units of peptide-N-glycosidase F (PNGase F, New England Biolabs, Ipswich, MA) in 50 mM sodium phosphate solution, pH 7.5, or endoglycosidase H_f (Endo H_f, New England Biolabs) in 50 mM sodium citrate solution, pH 5.5, at 37°C for 1 h. The mixtures of virus and enzyme were added to gel loading buffer with 5% β-mercaptoethanol, boiled for 10 min and electrophoresed in 4-20% Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, Hercules CA). CandyCane Glycoprotein Molecular Weight Standards (Invitrogen) were used to estimate protein size and protein bands were visualized with Ruby Protein Gel Stain (Invitrogen). Finally, proteins were analyzed in EpiChemi^3 Darkroom (UVP, Upland CA) using software LabWorks 4.5 (UVP).

Inhibition of PRRSV binding and infection

Pokeweed mitogen (PWM), *Lycopersicon esculentum* agglutinin (LEA), wheat germ agglutinin (WGA), *Maackia amurensis* agglutinin (MAA) and *Dolichos biflorus* agglutinin (DBA) were purchased from Sigma. For virus attachment analysis, PRRSV was incubated with increasing concentrations of PWM in medium at 37°C for 30 min. The virus-PWM mixtures were cooled and added to pre-cooled MARC-145 cells at 4°C for 30 min. For virus infection analysis, PRRSV was incubated with 100 or 500 nM lectin at 37°C for 1 h. MARC-145 cells were inoculated with the virus-lectin mixture at 37°C for 1 h, washed to remove unbound virus, and further incubated for 19 h. Cells pretreated with lectin prior to virus infection were used to control a lectin effect on cells. Virus without lectin treatment served as a positive control for infection.

GlcNAc monomer, LacNAc monomer and mannose were purchased from Sigma. GlcNAc dimer (chitobiose), trimer (chitotriose) and tetramer (chitotetraose) were purchased from Northstar BioProducts, East Falmouth, MA. MARC-145 cells were infected with PRRSV in the presence of different concentrations of glycans at 37°C for 1 h. After washing, cells were further incubated for 19 h.

PRRSV was treated with various concentrations of PNGase F or Endo H_f at 37°C for 1 h. Mixtures were inoculated into MARC-145 cells or PAM at 37°C for 1 h. After washing, cells were further incubated for 19 h. To control for an enzyme effect on cells, cells were pretreated with PNGase F or Endo H_f at 37°C for 1 h and washed extensively prior to virus infection. Virus without enzyme treatment was incubated at 37°C for 1 h to serve as a temperature stability control.

To change the N-glycosylation pattern of PRRSV, MARC-145 cells were pre-cultured with 1 or 5 μg/ml of swainsonine at 37°C for 1 h. After removing the medium, cells were infected with PRRSV in the presence of swainsonine at 37°C for 1 h. Cells were then washed and further incubated with swainsonine for 3 days for growth curve analysis.

PRRSV was collected from the culture supernatant of MARC-145 that were infected and cultured with/without 1 or 5 μg/ml of swainsonine for two days. The three virus preparations were normalized to contain the same viral RNA concentration by quantitative RT-PCR, and were inoculated in PAM at MOI 0.5 or 2.0 at 37°C for 1 h. After washing, PAM were further cultured at 37°C for 9 h. The percentage of virus-infected cells was analyzed by flow cytometry.

Flow cytometry

For virus binding analysis, after washing with PBS three times, MARC-145 cells were treated with 1 mM EDTA (Invitrogen) for cell dissociation; for virus infection analysis, MARC-

145 cells were treated with 0.25% trypsin-EDTA (Invitrogen) at 37°C for 5 min. PAM were directly resuspended by flushing. Then cells were collected, fixed and permeabilized with BD Cytofix/Cytoperm solution (Becton-Dickinson Biosciences, San Jose CA) at 4°C for 20 min, washed with 1:10 diluted Perm/Wash buffer (BD Biosciences), and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-nucleocapsid monoclonal antibody SR30-F (Rural Technologies, Brookings SD) at 4°C for 30 min to detect PRRSV. Finally, cells were washed and analyzed with a BD FACSCanto flow cytometer. Ten thousand cells were analyzed for each sample. Cells without virus infection were used as a negative control. Data were analyzed with FlowJo software (Tree Star, Ashland OR). The mean fluorescence intensity (MFI) was used to measure the amount of virus bound to cells. The infected cell percentage was used to measure the level of virus infection.

PRRSV quantitative reverse transcriptase (RT)-PCR

Total RNA isolation was performed using a Viral RNA Mini Kit following the manufacturer's protocol (Qiagen, Valencia CA). RNA was eluted in 50 µl water and 10 µl was used to prepare cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA). Quantitative real time PCR was carried out with 5 µl of 1:10 diluted cDNA, 10 µl of PerfeCta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg MD), and 5 µl of 1 µM primers with forward primer GATAACCACGCATTTGTCGTC, and reverse primer TGCCGTTGTTATTTGGCATA. To quantify viral RNA copies, RNA extracted from a titered VR-2332 stock was used as standards. The final results were expressed as RNA copies/ml according to the standard curve.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego CA). Data were analyzed either by one-way ANOVA with Tukey' multiple comparison

test or two-way ANOVA with Bonferroni posttest. A p-value <0.05 was considered statistically significant.

Results

GlcNAc and LacNAc oligomer-specific lectins reduce PRRSV binding and infection of MARC-145 cells

Previously, we showed that GlcNAc and LacNAc oligomer-specific lectins bound PRRSV. To understand how this binding would affect virus entry and infection, PRRSV was treated with different concentrations of lectins before inoculation into MARC-145 cells. Pokeweed mitogen (PWM), a lectin specific for GlcNAc and LacNAc oligomers (Irimura and Nicolson, 1983; Yokoyama et al., 1978), significantly blocked virus binding to cells in a dose-dependent manner (Fig. 3.1A). *Lycopersicon esculentum* agglutinin (LEA) shares similar carbohydrate specificity with PWM (Kawashima et al., 1990). Both lectins at 100 and 500 nM significantly reduced PRRSV infection at 20 hours post infection when compared to the "cell + lectin" control (Fig. 3.1B). Pretreatment of cells with PWM or LEA did not reduce virus infection (Fig. 3.1B). Consistently, the progeny virus in culture supernatant from the first-cycle replication at 10 hours after infection was significantly decreased by PWM treatment of virus compared to PWM treatment to cells (Fig. 3.1C). Progeny virus at 20 hours post infection was also decreased, but the difference was not statistically significant (data not shown).

Wheat germ agglutinin (WGA) binds a broad range of sialic acids and *Maackia amurensis* agglutinin (MAA) specifically binds α2-3-linked sialic acids. Pretreatment of MARC-145 cells with WGA and MAA resulted in stronger inhibition of virus infection than did lectin treatment of virus (Fig. 3.1B), suggesting that these two lectins interact with MARC-145 cells

Fig. 3.1. Effect of lectins on PRRSV binding and infection of MARC-145 cells. (A) To test virus binding, PRRSV was incubated for PWM at 37°C for 30 min. The virus-PWM mixtures were inoculated to MARC-145 cells at 4°C for 30 min and immediately processed for flow cytometric analysis. The mean fluorescence intensity (MFI) was used to measure the amount of virus bound to cells. PWM treatment groups were compared to the positive virus control using one-way ANOVA. (B) To test virus infection, PRRSV was incubated with lectins at 37°C for 1 h. MARC-145 cells were incubated with virus-lectin mixtures at 37°C for 1 h, washed, and further cultured for 19 h. Control cells were pretreated with lectins prior to infection. The infected cell percentage was measured by flow cytometry. (C) Progeny virus in culture supernatant at 10 hours post infection was measured by quantitative RT-PCR. "Virus + lectin" groups were compared to "cell + lectin" groups using two-way ANOVA. Data represent means ± standard errors of replicate or triplicate samples. * indicates statistical significance at $p \leq 0.05$; **, $p \leq 0.01$; $p \leq 0.001$.

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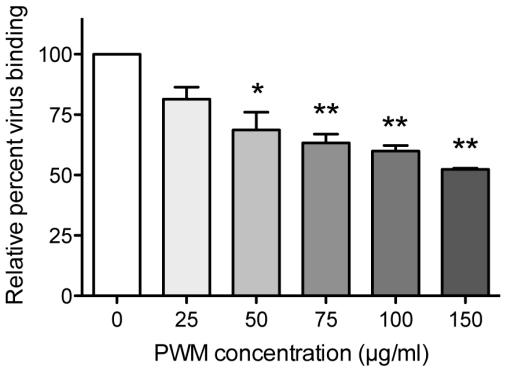

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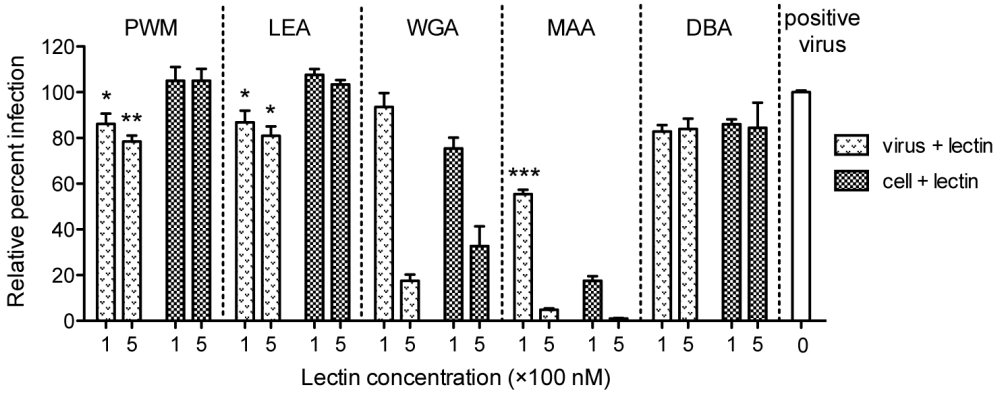
PWM
LEA
WGA
MAA
DBA
positive virus
Relative percent infection
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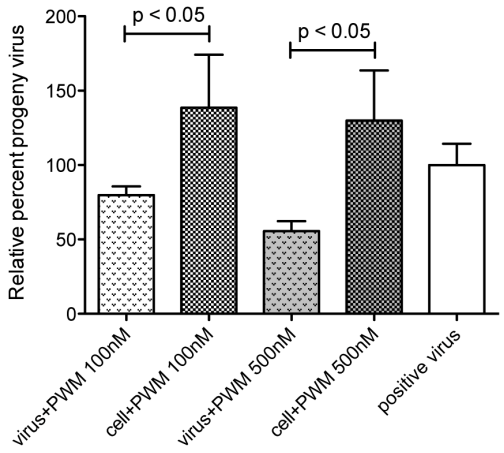
100
80
60
40
20
0
*
**
*
*
***
1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 1 5 0
Lectin concentration (×100 nM)
virus + lectin
cell + lectin

C


Relative percent progeny virus
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150
100
50
0
p < 0.05
p < 0.05
virus+PWM 100nM
cell+PWM 100nM
virus+PWM 500nM
cell+PWM 500nM
positive virus

and confound the analysis of their effect on virus infection. *Dolichos biflorus* agglutinin (DBA), which has an affinity for terminal N-acetyl-α-D-galactosaminyl residues (Etzler and Kabat, 1970), did not significantly affect virus infection either by lectin treatment of virus or cells.

These lectins were also tested in porcine alveolar macrophages (PAM), the naturally permissive cells for PRRSV infection. However, except for DBA, the lectins had a stronger inhibitory effect on virus infection when PAM were treated with lectins than when virus was treated with lectins prior to infection (data not shown). Thus PAM did not serve as a good cell model for lectin inhibition experiments due to potentially complex interactions between PAM, PRRSV and lectins.

GlcNAc oligomers and LacNAc do not affect PRRSV infection of MARC-145 cells

The observation that GlcNAc and LacNAc oligomer-specific lectins bound PRRSV and reduced infection suggested that these glycan moieties on the PRRSV envelope might be involved in virus entry of host cells. To directly test this hypothesis, MARC-145 cells were infected in the presence of increasing concentrations of GlcNAc monomer, dimer, trimer, tetramer, LacNAc monomer or mannose to block virus attachment. Surprisingly, none of the tested glycan moieties affected virus infection at concentrations from 10 μM to 5 mM (data not shown), suggesting that they were not directly involved in PRRSV entry and that specific-lectin binding to virus might interfere with virus attachment to host cells via steric hindrance. LacNAc oligomers were not tested due to commercial unavailability.

Removal of N-glycans from PRRSV does not affect virus infection of MARC-145 or PAM

To further investigate the role of PRRSV glycans in infection, we examined the effect of enzymatic glycan removal on virus infectivity. Digestion conditions were established by monitoring reactions for molecular weight changes in GP5, the most abundant envelope

glycoprotein. The minor envelope glycoproteins, GP2a, GP3 and GP4, were not discernible in the gel due to low abundance. In reducing SDS-PAGE, PRRSV showed 3 major protein bands, GP5 (Fig. 3.2A, band 1, 25-29 kD), M (19 kD) and N (14 kD). Intact PRRSV treatment with increasing amounts of PNGase F (36 kD), which cleaves all types of N-glycans, resulted in the disappearance of GP5 and the appearance of a new band (Fig. 3.2A, band 2) between M and N. Mass spectrometric analysis identified this new band as GP5. Deglycosylated GP5 is approximately 19 kD, similar to M, but has a lower isoelectric point (pI 8.87) than M (pI 10.03), accounting for its appearance below M in the gel. Thus, PNGase F treatment quantitatively removed N-glycans from PRRSV envelope proteins. After Endo H_f treatment, which cleaves high-mannose and hybrid-type N-glycans, the GP5 band (Fig. 3.2A, band 3) migrated only ~2 kD lower in the gel. Thus, the complex-type N-glycans, which are resistant to Endo H_f digestion, are the dominant glycan type and are still present in the PRRSV envelope after Endo H_f treatment.

Although PRRSV N-glycans were differentially sensitive to PNGase F and Endo H_f, treatment with either enzyme did not alter infection in MARC-145 cells (Fig. 3.2B). In PAM, virus infection was not reduced by PNGase F treatment of virus at 100 and 200 U/ml (Fig. 3.2C), concentrations that removed N-glycans from GP5 (Fig. 3.2A). Treatment of PRRSV with PNGase F at 400 U/ml and Endo H_f at all concentrations significantly reduced infection in PAM compared to the untreated virus control (Fig. 3.2C). However, the inhibitory effect was not specific to the virus, since endoglycosidase treatment of PAM before addition of virus also significantly reduced infection (Fig. 3.2C), a result consistent with the nonspecific effect of lectin treatment observed previously. Heat treatment of PRRSV at 37°C for 1 h did not significantly impair virus infectivity (Fig. 3.2B and 3.2C). These results further indicate that N-glycans are not required for PRRSV infection.

Fig. 3.2. Effect of endoglycosidase digestion on PRRSV infection of MARC-145 cells and PAM. PRRSV was treated with PNGase F or Endo H_f (each at 100, 200 and 400 U/ml) at 37°C for 1 h. (A) The virus-enzyme mixtures were run in SDS-PAGE. Band 1, GP5; band 2, GP5 without N-glycans; band 3, GP5 without high-mannose or hybrid-type N-glycans. The virus-enzyme mixtures were also inoculated into MARC-145 cells (B) or PAM (C) at 37°C for 1 h. After infection, cells were washed to remove enzyme and unbound virus, and further incubated for 19 h. To control the enzyme effect on cells, cells were pretreated with PNGase F or Endo H_f at 37°C for 1 h and washed extensively prior to virus infection. Virus without enzyme treatment was treated at 37°C for 1 h to serve as a control for temperature treatment. Data represent means ± standard errors of triplicate samples. Treatment groups were compared to the positive virus control. * indicates statistical significance at $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

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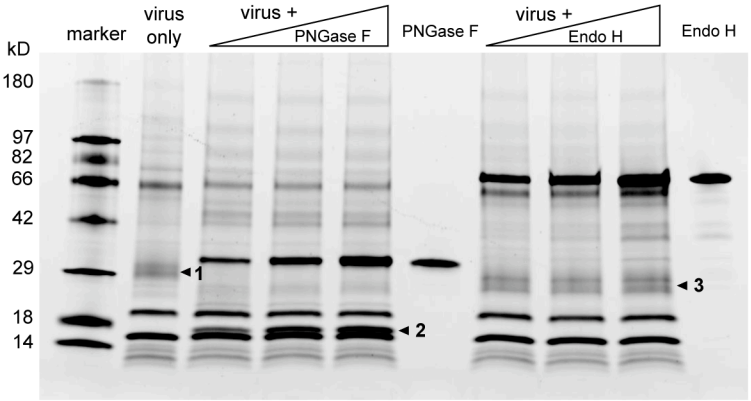

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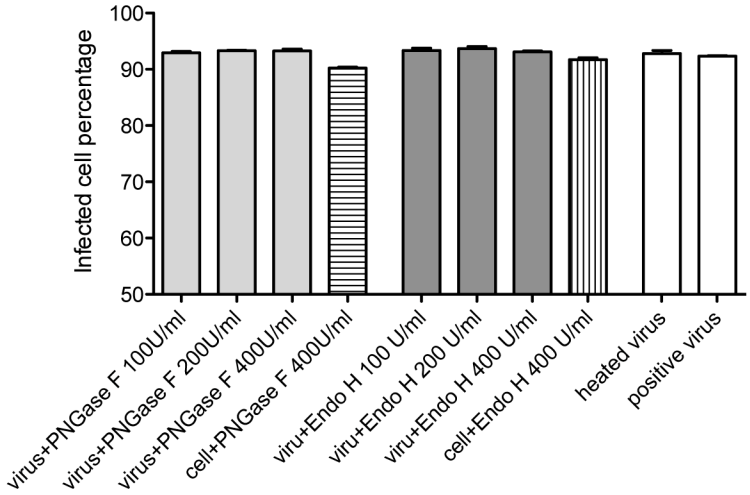
Infected cell percentage
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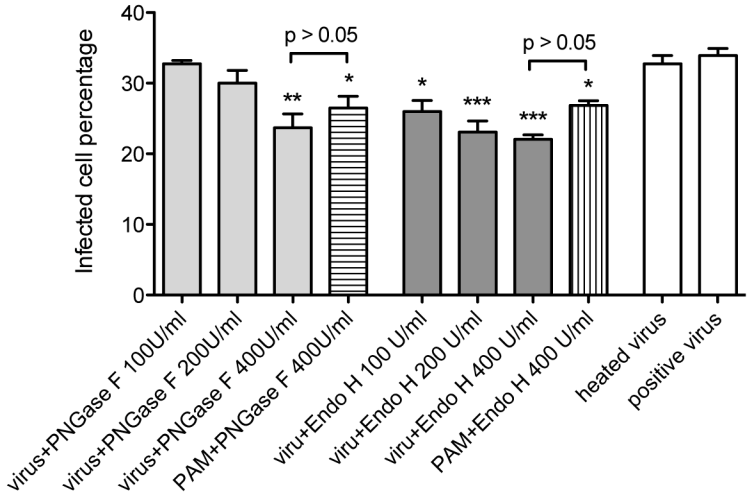
90
80
70
60
50
virus+PNGase F 100U/ml
virus+PNGase F 200U/ml
virus+PNGase F 400U/ml
cell+PNGase F 400U/ml
viru+Endo H 100 U/ml
viru+Endo H 200 U/ml
viru+Endo H 400 U/ml
cell+Endo H 400 U/ml
heated virus
positive virus

C


Infected cell percentage
40
30
20
10
0
p > 0.05
p > 0.05
**
*
*
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*
virus+PNGase F 100U/ml
virus+PNGase F 200U/ml
virus+PNGase F 400U/ml
PAM+PNGase F 400U/ml
viru+Endo H 100 U/ml
viru+Endo H 200 U/ml
viru+Endo H 400 U/ml
PAM+Endo H 400 U/ml
heated virus
positive virus

Alteration of the N-glycosylation pattern does not affect virus infection

The observation that PNGase F treatment of PRRSV did not reduce infection is in contrast to a previous report that complex-type N-glycans are important for PRRSV infection of PAM (Delputte and Nauwynck, 2004). Therefore, we repeated the experiment with an independent approach to alter viral envelope protein glycosylation without enzymatic digestion. The viral N-glycosylation pattern was modified by pharmacological inhibition of Golgi α-mannosidase II, which catalyzes the first committed step in the biosynthesis of complex-type N-glycans, with swainsonine (SW) (Tulsiani et al., 1982). Inhibition of the formation of complex-type N-glycans increases the portion of high-mannose and hybrid-type N-glycans (Tulsiani and Touster, 1983). Thus, PRRSV was propagated in MARC-145 cells in the presence of swainsonine at 1.5 μg/ml and purified.

Endoglycosidase treatment of purified virus confirmed that swainsonine altered glycan types on PRRSV. The virus became highly sensitive to Endo H_f digestion, showing multiple GP5 bands of reduced molecular weight on SDS-PAGE while retaining complete sensitivity to PNGase F (Fig. 3.3A). The identities of the new bands as GP5 glycoforms were confirmed by mass spectrometric analysis (data not shown). Since PRRSV GP5 from cells not treated with swainsonine was primarily resistant to Endo H_f (Fig. 3.2A), the increased sensitivity to Endo H_f was due to a shift in glycan composition from Endo H_f-resistant complex-type N-glycans to Endo H_f-sensitive high-mannose and hybrid-type N-glycans. Although swainsonine treatment altered the glycan pattern on PRRSV envelope proteins, there was no difference in virus growth curves in MARC-145 at swainsonine concentrations up to 5 μg/ml (Fig. 3.3B); the infectivity of the virus in PAM did not have significant changes (Fig. 3.3C), indicating that the glycan type of PRRSV does not affect the virus infection and replication cycle, including virus entry and assembly.

Fig. 3.3. Effect of swainsonine on PRRSV infection of permissive cells. (A) PRRSV was propagated in MARC-145 cells with swainsonine at 1.5 μg/ml. Purified virus was treated with PNGase F or Endo H_f at 37°C for 1 h and tested in SDS-PAGE. Band 1, GP5; band 2-5, GP5 without high-mannose or hybrid-type N-glycans; band 6, GP5 without N-glycans. (B) PRRSV was cultured in MARC-145 cells with/without swainsonine at different concentrations for 3 days. The amount of progeny virus in culture supernatant was measured by quantitative RT-PCR. Data represent means ± standard errors of replicate samples. (C) PRRSV was collected from the culture supernatant of MARC-145 that were infected and cultured with/without 1 or 5 μg/ml of swainsonine for two days. PAM were infected with the three virus preparations at MOI 0.5 or 2.0 at 37°C for 1 h, and were further cultured at 37°C for 9 h. The percentage of virus-infected cells was analyzed by flow cytometry. Data represent means ± standard errors of quadruple samples.

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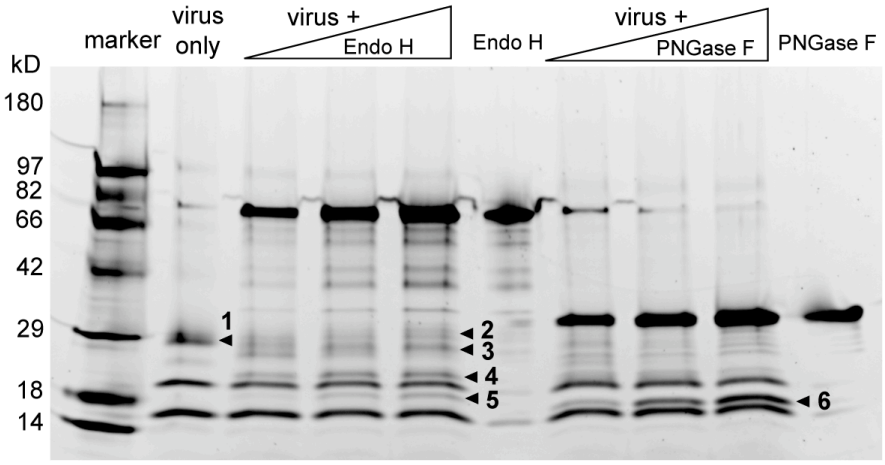

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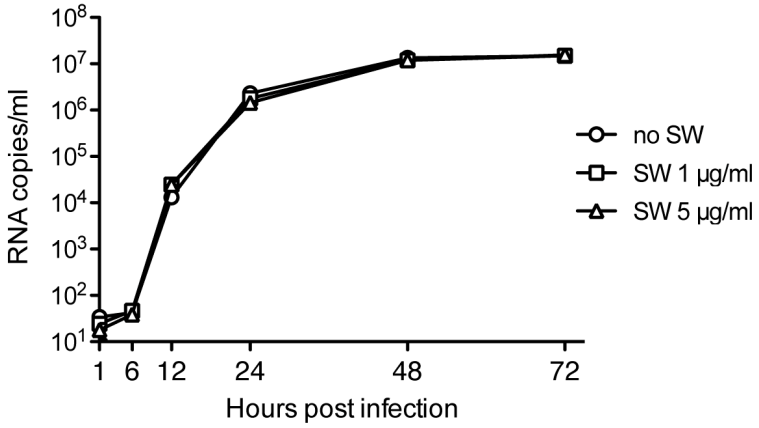
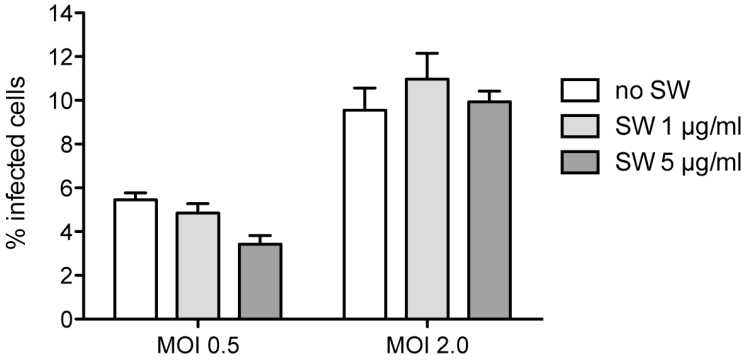

RNA copies/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
1 6 12 24 48 72
Hours post infection
no SW
SW 1 µg/ml
SW 5 µg/ml

C


% infected cells
14
12
10
8
6
4
2
0
MOI 0.5
MOI 2.0
no SW
SW 1 µg/ml
SW 5 µg/ml

Discussion

PRRSV envelope protein glycans play important roles in virus assembly, virus neutralization and antigenicity (Ansari et al., 2006; Das et al., 2011; Wissink et al., 2004). In addition to sialic acid that binds sialoadhesin (Delputte and Nauwynck, 2004), we recently identified GlcNAc and LacNAc oligomers in GP5-linked N-glycans and showed that lectins specific for these glycan moieties bound PRRSV (Chapter II). Therefore, we investigated the effect of N-glycan perturbations to understand the role of PRRSV N-glycans in permissive cell infection.

Lectins specific for GlcNAc and LacNAc oligomers, when pre-incubated with PRRSV, reduced virus binding and infection of MARC-145 cells. However, GlcNAc and LacNAc oligomers themselves did not compete with virus to block infection. Thus, the inhibitory effect of these specific lectins appeared to be due to a nonspecific mechanism such as steric hindrance. Consistent with this observation, removal or alteration of envelope glycoprotein N-glycans did not alter infection, further demonstrating that the presence of glycans is not required for PRRSV infection.

This conclusion is in contrast to previous reports that GP5-linked sialic acids bind sialoadhesin on PAM to mediate virus attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010). Delputte et al. showed that lectins specific for sialic acids reduced type I PRRSV infection in PAM and had no effect when PAM were pre-incubated with lectins prior to infection (Delputte and Nauwynck, 2004). However, we found that pre-incubation of PAM with lectins specific for sialic acids, GlcNAc and LacNAc oligomers inhibited type 2 PRRSV infection. The same result was observed when sialic acid-specific lectins were tested in MARC-145 cells. A specific binding interaction between cells and lectins was not surprising since terminal sialic acids, GlcNAc and LacNAc oligomers are widely distributed in tissues and

cells (Varki et al., 2009). In particular, the essential PRRSV receptor CD163, which exists in both PAM and MARC-145 cells, has 6 or 7 predicted N-glycosylation sites in monkey (GenBank ID: AEF30542.1) and pig (GenBank ID: ABV80230.1), respectively. Thus lectin binding to CD163-linked glycans directly or to glycoconjugates in the local vicinity could sterically hinder PRRSV interaction with CD163.

We previously identified terminal sialic acids in GP5-linked N-glycan structures and showed that a sialic acid-specific lectin bound to PRRSV (Chapter II). Since sialoadhesin, a sialic acid receptor, is exclusively expressed on macrophages (Crocker and Gordon, 1989), it likely binds sialic acid residues on PRRSV envelope proteins to facilitate virus attachment. However, this interaction may not be required for PRRSV infection of porcine macrophages, and sialoadhesin is not expressed in MARC-145 cells (Van Gorp et al., 2008; Wang et al., 2011; Welch and Calvert, 2010). In addition, in an experiment to evaluate how sialic acids would compete with PRRSV for binding to sialoadhesin on PAM, Delputte et al. showed that a low molecular weight trisaccharide, sialyllactose (~634 Dalton), at a concentration of 1 mM only reduced infection by 20%, whereas sialic acid-containing glycoproteins (50~66 kD) reduced infection by 53% at ~0.5 μM and 77% at ~2 μM (Delputte and Nauwynck, 2004). The increased inhibitory efficacy of space-filling glycoproteins compared to small molecules suggests that the inhibitory effect was due to steric hindrance.

A remarkable observation in our experiments was that removal or alteration of the Endo H_f-resistant complex-type N-glycans did not affect infection. It was previously shown that PNGase F treatment of type 1 PRRSV reduced infection in PAM by 71%, whereas treatment of PAM had no effect (Delputte and Nauwynck, 2004). However, our results showed that PAM treatment with either PNGase F or Endo H_f significantly reduced type 2 PRRSV infection equivalently to the reduction caused by endoglycosidase treatment of the virus. This observation

is consistent with modification of glycan moieties on cell surface membrane proteins such as CD163.

Inhibition of terminal glycan maturation in the Golgi, which occurs before viral assembly, also did not alter viral infection. When the N-glycan biosynthesis pathway was blocked by swainsonine, there was no effect on virus infection, demonstrating that PRRSV complex N-glycans are not important for infection. Alteration of the N-glycosylation pattern in PRRSV envelope proteins did not affect protein folding or virus assembly since the growth curve was not affected.

A variety of genetic ablation experiments have been performed to examine the role of envelope protein N-linked glycosylation in PRRSV biology. Lack of glycans linked to N184 of GP2a, N42, N50 and N131 of GP3, multiple locations (N37, 84, 120 and 130) of GP4, and N44/46 of GP5 is lethal for infectious PRRSV production (Ansari et al., 2006; Das et al., 2011; Wissink et al., 2004), suggesting glycans in these sites are important for proper protein folding and stability, and thus for virus assembly. A multimeric structure containing GP2a, E, GP3 and GP4 is required for PRRSV infectivity, and GP2a and GP4 are important for the interaction of PRRSV with CD163, its receptor on permissive cells (Calvert et al., 2007; Das et al., 2010; Wissink et al., 2005). The importance of minor envelope protein glycosylation for viral infectivity, however, is not clear. In one study, mutations in the two predicted GP2a sites had no effect on viral infectivity (Wissink et al., 2004), but in another, viral interaction with CD163 was reduced, although single mutations in either site had no effect (Das et al., 2011). Overall, it can be concluded that envelope protein glycosylation plays important roles in PRRSV envelope glycoprotein stability, virion assembly and maturation, but the evidence that envelope protein glycans play a direct role in infection of permissive cells is not compelling. In conclusion, steric hindrance of glycans on the PRRSV virion by lectins or, presumably, other space-filling

molecules may interfere nonspecifically with infection by blocking protein interactions with cell surface receptors. Glycans themselves appear not to be required for infection of permissive cells, but may have important roles in avoidance of host immunity and in intracellular growth and assembly of virions.

In conclusion, steric hindrance of glycans on the PRRSV virion by lectins or, presumably, other space-filling molecules may interfere nonspecifically with infection by blocking protein interactions with cell surface receptors. Glycans themselves appear not to be required for infection of permissive cells, but may have important roles in avoidance of host immunity and in intracellular growth and assembly of virions.

CHAPTER IV

General Discussion

The overall goal of this study was to characterize the structures of PRRSV envelope glycans and their potential functions in infection. I focused on glycan structure and function because glycans have been widely implicated in viral infection and immune resistance mechanisms, but there is little or no structural evidence to provide a mechanistic basis for their role. First of all it was necessary to determine the distribution of PRRSV glycans in all four envelope glycoproteins, GP2a, GP3, GP4 and GP5. PNGase F digestion of purified virus showed that viral N-glycans were predominantly contained in GP5. It was not surprising since GP5 is the major envelope protein and is more abundant than minor proteins, including GP2a, GP3 and GP4, which were not visible in SDS-PAGE stained by a sensitive protein detection kit that had a minimum limit of 0.25-1 ng. The extremely low abundance of minor glycoproteins thus limited the ability to analyze their glycans on infectious virions. The structural studies presented here were largely restricted to GP5 and the use of GP5 as an indicator for evaluating the glycosylation status of the PRRSV envelope.

Both endoglycosidase digestion and mass spectrometric analysis showed that GP5-linked glycans were primarily Endo H-resistant complex N-glycans with a relatively small amount of high-mannose or hybrid N-glycans. As reviewed in CHAPTER I, the N-glycan biosynthetic pathway is well conserved in eukaryotic cells. High-mannose N-glycans exit early from this pathway, suggesting minimal processing and a more protected local conformational structure, as would occur if buried within a folded protein. By contrast, the presence of fully processed, complex N-glycans usually indicate that the glycosylation site is readily accessible for recognition, thus reflecting a more flexible and exposed protein structure. Hybrid N-glycans are intermediate. Therefore, the abundant complex N-glycans in GP5 are in a position to interact with other molecules, possibly the receptors on the surface of PRRSV-permissive cells.

GlcNAc and LacNAc oligomers and terminal sialic acids, which are characteristic for most complex N-glycans, were identified in purified GP5 and intact PRRSV virions by mass spectrometric and specific lectin-binding analysis, respectively. Sialic acid has been shown to bind sialoadhesin on porcine macrophages to mediate PRRSV attachment and internalization (Delputte and Nauwynck, 2004; Van Breedam et al., 2010; Vanderheijden et al., 2003). The role of GlcNAc in virus infection was suggested in a ficolin inhibition experiment that specifically recognized GlcNAc (Keirstead et al., 2008), but has not been fully investigated. Therefore, to pursue this opportunity, several virus infection-inhibition experiments were performed with various glycan conditions.

GlcNAc and LacNAc oligomer-specific lectins bound PRRSV and reduced virus attachment and infection in MARC-145 cells in a dose-dependent manner. Surprisingly, the lectin binding to PRRSV appeared to sterically, rather than specifically, interfere with virus attachment, in that GlcNAc oligomers and LacNAc, when added to cell culture together with the virus, did not compete with PRRSV to reduce infection. Further investigation by removal or alteration of viral N-glycans confirmed that envelope protein-linked N-glycans, including sialic acid, were not required for PRRSV infection of permissive cells, although the unknown interaction of endoglycosidases and PAM had made the experiment less conclusive. This conclusion is also consistent with previous reports that sialic acid was not necessary for PRRSV infection (Van Gorp et al., 2008; Wang et al., 2011).

The effect of lectins on virus infection could not be directly tested in porcine macrophages, because the tested lectins, including lectins specific for GlcNAc and LacNAc oligomers and terminal sialic acids, since incubation of macrophages with lectins also inhibited viral infection for unknown reasons. Thus, a specific inhibitory effect of lectins on viral infection could not be demonstrated. The inhibitory effect of lectin treatment of macrophages was even

greater than occurred when macrophages were treated with lectins and virus simultaneously. These results were not surprising because GlcNAc and LacNAc oligomers and terminal sialic acids are common characteristics of complex N-glycans, which are ubiquitously present on the cell membrane surface. Moreover, the essential PRRSV receptor, CD163, is a conserved protein with 6 or 7 predicted N-glycosylation sites in monkeys or pigs.

Although this study showed some contradictory results with previously published literature and was sometimes restricted to GP5 analysis, it had some important strengths. For example, endoglycosidase digestion in combination with SDS-PAGE clearly showed the glycosylation status of envelope glycoproteins, as indicated by GP5. Swainsonine treatment of cells not only altered the complex N-glycan type in the virus, it also changed the cellular glycosylation machinery. Thus, the observation that swainsonine treatment of cells did not change PRRSV growth further suggested that the N-glycans types on PRRSV did not affect the folding and assembly of viral proteins, as long as the glycosylation sites were occupied by some glycans. Taken together with the previous reports that mutation of certain N-glycosylation sites in viral glycoproteins was lethal for infectious progeny virus production, all the data pointed out that viral glycans play a structural, rather than modulatory, function in PRRSV life cycles. This role appears to require complex-type N-glycans, though it is possible that high-mannose and hybrid type N-glycans may also be involved.

Another strength of this study was the identification of GlcNAc and LacNAc oligomers on PRRSV envelope. Although these glycan moieties were not directly involved in virus entry, they certainly could serve as potential targets for PRRSV intervention strategies. First, they are predominantly present on the virus envelope and readily accessible for recognition. Second, GlcNAc and LacNAc oligomer-specific lectins showed very low efficiency of PRRSV inhibition, likely because the interaction between lectins and glycans is often in low affinity. Thus

biochemical engineering technologies could be utilized to increase the binding affinity and enhance the virus-inhibition efficiency, while using the same targets of GlcNAc and LacNAc oligomers.

Therefore, the advances provided by the studies presented here have opened up new research fronts in terms of probing glycans as targets in the design and development of anti-viral therapies that may provide novel treatments and preventatives to reduce and eliminate the devastation of PRRS.

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