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N-glycosylation profiling of porcine reproductive and respiratory syndrome virus envelope glycoprotein 5

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense ssRNA virus whose envelope contains four glycoproteins and three nonglycosylated proteins. Glycans of major envelope glycoprotein 5 (GP5) are proposed as important for virus assembly and entry into permissive cells. Structural characterization of GP5 glycans would facilitate the mechanistic understanding of these processes. Thus, we purified the PRRSV type 2 prototype strain, VR2332, 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 carbohydrate moieties 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 in addition to sialic acids. GlcNAc and LacNAc accessibility to ligands was confirmed by lectin co-precipitation. Our findings help to explain PRRSV infection of cells lacking sialoadhesin and provide a glycan database to facilitate molecular structural studies of PRRSV.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), first isolated as the type 1 European strain, Lelystad virus (LV), in the Netherlands (Wensvoort, 1993) and shortly after as the type 2 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, 2000; Mardassi et al., 1996). The major envelope proteins GP5 and matrix (M) form heterodimeric complexes linked by N-terminal ectodomain disulfide bonds 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). 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 (type 2 PRRSV) 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

Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; N-glycan, asparagine-linked glycan; PNGase F, peptide-N-glycosidase F; Endo H_f, endoglycosidase H_f; GlcNAc, N-acetylglucosamine; LacNAc, N-acetyllactosamine; Gal, galactose; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid; Kdn, 2-keto-3-deoxynononic acid; Fuc, fucose; Sia, sialic acid; LEA, *Lycopersicon esculentum* agglutinin; DSA, *Datura stramonium* agglutinin; WGA, wheat germ agglutinin; ConA, concanavalin A

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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, detailed knowledge of glycan structural information and distribution in viral envelope glycoproteins is essential to further evaluate the contributions of viral glycans to PRRSV pathogenesis and immune protection.

Therefore, we digested highly purified PRRSV 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 that the characteristic glycan structures contain N-acetylglucosamine (GlcNAc) and N-acetyllactosamine (LacNAc) oligomers and terminal sialic acids, whose accessibility was confirmed by lectin co-precipitation.

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 exclusively 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, GP5 (~25 kD), M (19 kD) and N (14 kD), the three major structural proteins of PRRSV (Fig. 1A). The minor envelope glycoproteins, GP2a, GP3 and GP4, were not visible due to low abundance. Incubation of purified virus with increasing amounts of PNGase F (36 kD, Fig. 1A arrow) caused a disappearance of GP5 at 25 kD and the appearance with increasing intensity of a new band between M and N (Fig. 1A). Mass spectrometric analysis identified this new band to be GP5 (arrow labeled 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. 1B). Thus, enzymatic digestion predicted that GP5 is predominantly composed of Endo H_f-resistant complex-type N-glycans with small amounts of high-mannose and/or hybrid-type N-glycans.

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 compositions and

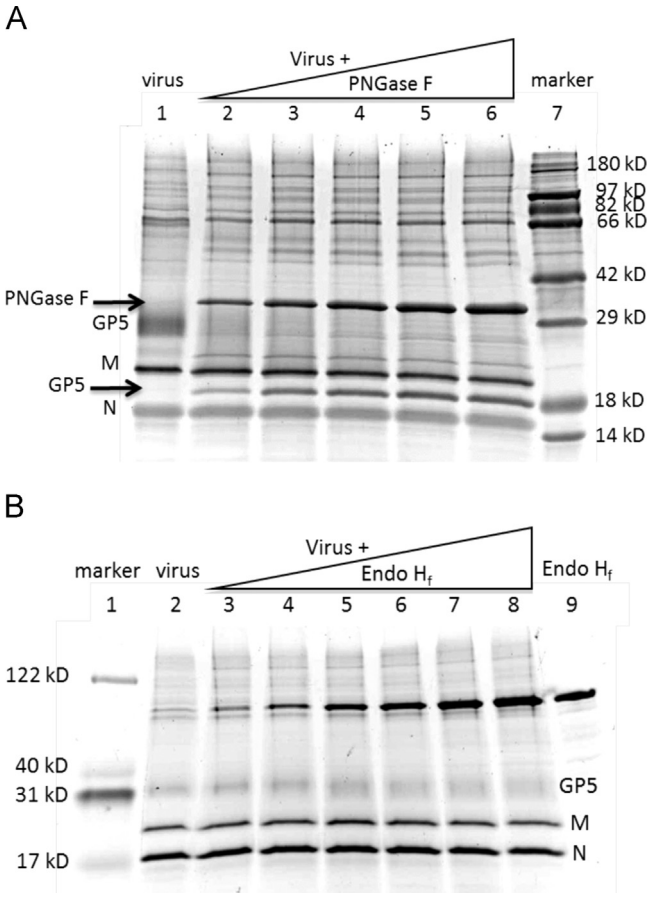

Fig. 1. Endoglycosidase digestion of purified PRRSV. (A) Purified virus was incubated with PNGase F, electrophoresed in reducing SDS-PAGE, and the gel was stained with Ruby Protein Gel Stain. Lane 1, virus only. Lanes 2–6, virus treated with 100–500 units of PNGase F at 37 °C for 1 h. Lane 7, protein markers. (B) Purified virus was incubated with Endo H_f, samples were electrophoresed as above, and the gel was stained with Deep Purple. Lane 1, protein markers. Lane 2, virus only. Lanes 3–8, virus treated with 100–400 units of Endo H_f at 37 °C for 1 h. Lane 9, Endo H_f only. Bands containing GP5, M, and N are indicated to the right.

structures. Fig. 2 shows the positive ion electrospray spectra with 12 mass/charge (m/z) peaks identified as candidate N-linked glycans. 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 Da. MS/MS fragmentation spectra were used to further reduce the candidate glycan structures by comparing the masses of the computed fragment ions to those observed experimentally. Glycan structures were considered to be reasonable candidate glycans on GP5 if their calculated precursor mass-to-charge ratios and fragmentation mass values were consistent with the experimental data. As an example of structural elucidation, the most abundant glycan (m/z 825.82) was assigned a structure with the monosaccharide composition $Hex_3HexNAc_6Fuc_2$ (Fig. 2A). The MS/MS fragmentation spectrum showed ions in agreement with computed fragments of the proposed structure (Fig. 3). This approach was followed to determine the compositions and expected structures that would correspond to all 12 observed glycan peaks. The findings are summarized in Table 1. Thus, the major m/z 825.82 peak, for example, would result from any of 5 possible glycan compositions with 7 possible structures. Five of the seven possible structures do not contain sialic acid.

Candidate GP5-linked N-glycan structures were a mixture of bi-, tri-, and tetra-antennary complex ($n=63$), high-mannose ($n=5$), and hybrid ($n=11$) carbohydrates with or without core