Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP_{2a} and GP_5 for infectious virus production

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The arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) contains four glycoproteins, GP_{2a}, GP_3, GP_4 and GP_5, the functions of which are still largely unresolved. In this study, the significance of the *N*-glycosylation of the GP_{2a} and GP_5 proteins of PRRSV strain LV was investigated. Both glycoproteins contain two predicted *N*-glycosylation sites that are highly conserved between North American-type and European-type PRRSV. Using site-directed mutagenesis, single and double mutant full-length PRRSV cDNA clones were generated. After analysing the expression of the mutant proteins and the actual use of the four putative glycosylation sites in the wild-type proteins, the production of mutant virus particles and their infectivities were investigated. The results showed that the *N*-linked glycans normally present on the GP_{2a} protein are not essential for particle formation, as is the oligosaccharide attached to N53 of the GP_5 protein. In contrast, the oligosaccharide linked to N46 of the GP_5 protein is strongly required for virus particle production. The specific infectivities of the mutant viruses were investigated by comparing their infectivity-per-particle ratios with that of wild-type virus. The results showed that the lack of either one or both of the *N*-linked oligosaccharides on GP_{2a} or of the oligosaccharide attached to N53 of GP_5 did not significantly affect the infectivities of the viruses. In contrast, the two recombinant viruses lacking the oligosaccharide bound to N46 exhibited a significantly reduced specific infectivity compared with the wild-type virus. The implications of the differential requirements of the modifications of GP_{2a} and GP_5 for PRRSV assembly and infectivity are discussed.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-strand RNA virus that has been assigned to the family *Arteriviridae*. Other members of this family are lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus. The family *Arteriviridae* has been grouped within the order Nidovirales together with the *Coronaviridae* and the *Roniviridae* (Cavanagh, 1997; Cowley *et al.*, 2000). PRRSV strains have been grouped into North American- and European-type strains on the basis of antigenic and genetic differences between the isolates on the two continents (reviewed by Meng, 2000).

PRRSV virions are spherical particles with a diameter of 50–60 nm consisting of a putatively icosahedral core surrounded by an envelope (Benfield *et al.*, 1992). The core contains the 15 kb genome packaged in a capsid consisting of 14 kDa nucleocapsid protein (N) subunits. In the envelope, up to six structural proteins have presently been identified, of which the most abundant are the 17 kDa non-glycosylated membrane protein (M) and the 25 kDa glycosylated GP_5 protein, which occur as disulphide-linked heterodimers (Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995). The topologies of the GP_5 and M proteins have

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not yet been resolved, but, by analogy with LDV (Faaberg & Plagemann, 1995), the M protein is predicted to be a polytopic class III integral membrane protein with an N-terminal ectodomain of only 16 amino acids, three membrane-spanning domains and a C-terminal endodomain of about 78 amino acids (Meulenberg *et al.*, 1995). The GP_5 protein is assumed to have a cleaved N-terminal signal sequence of approximately 30 amino acids followed by an ectodomain of approximately 35 amino acids and a hydrophobic region of about 60 amino acids that may span the membrane once or thrice. The 70 C-terminal amino acids are thought to form an endodomain (Meulenberg *et al.*, 1995). The disulphide bond between GP_5 and M probably occurs between Cys^{50} and Cys^{8} of the respective proteins (Verheije, 2002).

The remaining envelope proteins are the GP_{2a}, GP_3 and GP_4 glycoproteins and the small, non-glycosylated E protein, each of which occurs only in minor quantities in the viral envelope (Meulenberg & Petersen-den Besten, 1996; van Nieuwstadt *et al.*, 1996; Wu *et al.*, 2001). The 29–30 kDa GP_{2a} and 31–35 kDa GP_4 proteins are both putative class I integral membrane proteins with a cleaved N-terminal signal sequence and a C-terminal membrane anchor. The 45–50 kDa GP_3 protein has an N-terminal hydrophobic domain (Meulenberg *et al.*, 1995). Conflicting data on the presence of the GP_3 protein in PRRSV particles have been reported. For European-type strains, the GP_3 protein was found to be incorporated into virions (van Nieuwstadt *et al.*, 1996), whereas for North American strains the protein was reported to be a soluble, secreted protein (Gonin *et al.*, 1998; Mardassi *et al.*, 1998). This discrepancy might be real or due to different analytical procedures. Recently, a study on the minor glycoproteins of EAV showed these proteins to occur in viral particles as covalently linked complexes, the incorporation of which occurs in concert with that of the E protein (Wieringa, 2003; Wieringa *et al.*, 2003).

Based on the available data, the GP_5 and M proteins of PRRSV seem to play a role in virus assembly (Verheije, 2002). Such a function has been clearly demonstrated for the GP_5 and M proteins of EAV (Wieringa, 2003). Moreover, PRRSV GP_5–M complexes were shown to interact with heparin (Delputte *et al.*, 2002), suggesting an additional role in attachment of virus to alveolar macrophages. The functions of the PRRSV minor envelope proteins, including the E protein, are still unknown, but a recent study showed that the homologous proteins of EAV are dispensable for virus particle production. Since EAV particles devoid of these proteins were non-infectious, the minor envelope proteins are thought to be involved in EAV tropism and entry (Wieringa, 2003). Correspondingly, the minor envelope proteins of PRRSV might have a similar role in PRRSV infection.

The importance of *N*-glycans for viral glycoprotein folding and functioning has become clear from many studies (reviewed by Doms *et al.*, 1993). For arteriviruses, a correlation has been demonstrated between the number of *N*-glycosylation sites on the ectodomain of the ORF5 product VP-3P of LDV and important biological properties such as neuropathogenicity and susceptibility to immune response (Chen *et al.*, 2000), whereas mutation of the single *N*-glycosylation site in the GP_5 ectodomain of EAV abolished virus particle production (Snijder *et al.*, 2003).

For PRRSV, nothing is known about the significance of any of its *N*-glycans in the viral life cycle. Of the four glycoproteins, both the GP_{2a} and GP_5 protein have two potential *N*-glycosylation sites, whereas computer analysis indicates the presence of seven and four potential *N*-glycosylation sites, respectively, in the GP_3 and GP_4 protein sequences (Meulenberg *et al.*, 1995). Endoglycosidase treatments suggested that all putative sites are occupied by complex-type *N*-glycans (Meulenberg *et al.*, 1995; van Nieuwstadt *et al.*, 1996).

In the present study, we investigated the role of *N*-glycosylation of the GP_{2a} and GP_5 proteins in the PRRSV life cycle. Comparative sequence analysis of the ORF2a and ORF5 genes of European and North American PRRSV isolates shows that the encoded proteins each share two highly conserved putative glycosylation sites, suggesting that they are functional and that the *N*-glycans are important for folding or functioning of these glycoproteins. To determine their significance for virus production and viral infectivity, we generated recombinant viruses in which the *N*-glycosylation sites were mutated. The mutant viruses were then analysed for their capacity to produce infectious viral particles.

METHODS

Cells and media. BHK-21 cells were grown in Glasgow minimal essential medium (GMEM; Gibco-BRL) supplemented with 5 % FBS, 10 % tryptose phosphate broth, 20 mM HEPES/NaOH (pH 7·4), 2 mM glutamine, 100 U penicillin ml^{-1} and 100 U streptomycin ml^{-1}. Porcine alveolar lung macrophages (PAMs) (Wensvoort *et al.*, 1991) were maintained in MCA-RPMI 1640 medium (Gibco-BRL) containing 5 % FBS, 500 U penicillin ml^{-1} and 500 U streptomycin ml^{-1}.

Monoclonal antibodies and antisera. Monoclonal antibody (mAb) P10/a46, directed against amino acids 29–35 of the GP_5 protein of the PPV strain (Wissink *et al.*, 2003), was generously provided by Dr E. Weiland, Tübingen, Germany. mAb 122.17 is directed against the PRRSV N protein (van Nieuwstadt *et al.*, 1996). Polyclonal rabbit serum p690 was raised against a Lelystad virus (LV)-specific peptide consisting of amino acids 64–78 of the GP_2 protein (Meulenberg *et al.*, 1995). LV was the first European isolate described (Wensvoort *et al.*, 1991).

Construction of mutant full-length cDNA clones. Mutations affecting the *N*-linked glycosylation sites were introduced into ORF2a and ORF5 in two different vectors, the pCIneo mammalian expression vector (Promega) and pABV437, a *Pac*I-mutant of the genome-length cDNA clone of LV (Meulenberg *et al.*, 1998). The consensus sequences for *N*-linked glycosylation sites (N-X-S/T) in the GP_{2a} and GP_5 proteins were altered by mutating the asparagine (N) codon AAC/AAT into a glutamine (Q) codon CAG by performing a fusion PCR (Dekker *et al.*, 2000). Glutamine was chosen because of its similarity to asparagine. To generate the GP_{2a} glycosylation mutants, pABV437 (referred to below as wt-LV) was used as a

Table 1. Primers and probes used for site-directed mutagenesis, sequencing and RNA quantification

Primer	Purpose	Sequence (5′–3′)*	Orientation	Position†
LV24	PCR	AATCGGATCCTCAGGAAGCGTGCACACTGATGA	−	12419
LV96	PCR	CCAACTGCAGACCGGATGTCC	+	12015
LV293	PCR; *Stu*I site	CTAGTCTAGACTAGCTAGGCCTCCC	−	14091
LV294	PCR GP_2-N173Q	GCCGTTGGCcAgGTGAGCCTACAG	+	12293
LV295	PCR GP_2-N173Q	CTGTAGGCTCACcTgGCCAACGGC	−	12293
LV296	PCR GP_2-N179Q	GCCTACAGTACcAgACCACGTTGG	+	12310
LV297	PCR GP_2-N179Q	CCAACGTGGTcTgGTACTGTAGGC	−	12310
LV298	PCR GP_5-N46Q	CCAATACATATATcAgTTGACGATATGCGAGCTG	+	13615
LV299	PCR GP_5-N46Q	CAGCTCGCATATCGTCAAcTgATATATGTATTGG	−	13615
LV300	PCR GP_5-N53Q	GACGATATGCGAGCTGcAgGGGACCGACTGG	+	13634
LV301	PCR GP_5-N53Q	CCAGTCGGTCCCcTcCAGCTCGCATATCGTC	−	13634
LV371	PCR	GCAGAACCATTCTCTTGGCATATGAGATGTTCTC	+	13490
LV466II	PCR; *Bst*XI site	CATTGCTTGTTTGTTCGCCATTCTCTTGGC	+	13479
RT-OLE	RT-PCR ORF7	TCGCCCTAAT	−	15041
L5	LightCycler ORF7	ATGKGGCTTCTCMGGSTTT	−	14744
iLC-PRRSV	LightCycler ORF7	ATGGCCAGCCAGTCAAXTC	+	14653
FL-PRRSV	LightCycler ORF7	GCACCCAGCAACTGGCACAGTTGA	−	14669

*Restriction enzyme sites are indicated by underlining. Underlined nucleotides in lower case are mutated compared with the original genome sequence of the PRRSV LV strain. K indicates G or T, M indicates A or C and S indicates C or G; X indicates the position of the label.
†Relative to the nucleotide sequence of PRRSV LV (GenBank accession no. M96262).

template, whereas pABV911 (referred to below as wt-NS, neutralization-sensitive) was used as a template to introduce an additional mutation in the GP_5 glycosylation mutants. The wt-NS virus is identical to PRRSV LV except that it has a proline residue at position 24 of the GP_5 protein, which enables recognition of the mutant GP_5 proteins by neutralizing mAb P10/a46 (Wissink *et al.*, 2003). Individual parts were amplified with the forward or the reverse primer containing the desired mutation (for a detailed description of the primers, see Table 1) and the two mutated PCR products were hybridized together and amplified with two primers outside the mutated region. The amplified fragments were then digested with the appropriate restriction enzymes (Table 1) and ligated into the similarly digested pCIneo vector. Alternatively, the fragments were ligated into the similarly digested plasmid pABV651, a cDNA clone containing the 3′ end of the LV genome. From the resulting clones, the *Aat*II–*Hpa*I fragment was excised and introduced into the unique *Aat*II/*Hpa*I sites of pABV437. The following recombinant clones were thus obtained: pABV832 (mutation N173Q in the GP_{2a} protein, referred to as GP_{2a}-N173Q), pABV833 (GP_{2a}-N179Q), pABV836 (GP_5-N46Q) and pABV837 (GP_5-N53Q) (see Fig. 1).

In order to generate constructs that contain two mutations, fusion PCRs were performed on pABV832 and pABV836, yielding pABV847 (mutations N173Q and N179Q in the GP_{2a} protein, referred to as GP_{2a}-double mutant) and pABV848 (GP_5-double mutant). Cloning procedures were as described above. All plasmids were verified by restriction enzyme analysis and by sequencing of the entire PCR-generated fragment.

In vitro* RNA transcription and transfection of BHK-21 cells.** Full-length transcripts were generated *in vitro* from *Pvu*I-linearized cDNA clones. Fifty microlitre reaction mixtures consisting of 1·5 μg plasmid DNA, 1 mM of each of the ribonucleotides ATP, CTP and UTP and 0·5 mM GTP, 10 μl Transcription Optimized 5× buffer (Promega), 1 mM capping analogue ($m^7G[5']ppp[5']G$; New England Biolabs), 3 μl RNasin (Promega), 1 mM DTT and 120 U T7 RNA polymerase in water were incubated for 2 h at 37 °C. The yield and quality of the RNAs were assessed by agarose gel electrophoresis, after which the RNAs were transfected into BHK-21 cells by electroporation as described by Liljestrom & Garoff (1993). Briefly, subconfluent monolayers of 10^7 BHK-21 cells were trypsinized, resuspended in GMEM and pelleted at 400 ***g for 5 min. The cells were washed twice in PBS and resuspended in 800 μl PBS. The cell suspension was added to the transcription mixture, which was then transferred to an electroporation cuvette with a 4-mm gap. The cells were pulsed twice at 850 V and 25 mF, resuspended in culture medium, seeded into 10 cm^2 wells and incubated at 37 °C. At 8 h after electroporation, the cells were washed three times with culture medium and subsequently incubated with fresh culture medium. The culture medium was harvested 24 h after transfection, clarified by centrifugation at 6500 ***g*** for 15 min and stored at −70 °C until further use. To compare the transfection efficiencies of the constructs, immunostaining was performed and the numbers of positive cells were quantified with the Image-Pro Plus image analysis software (Media Cybernetics). The transfection efficiencies were observed to be equivalent within one experiment.

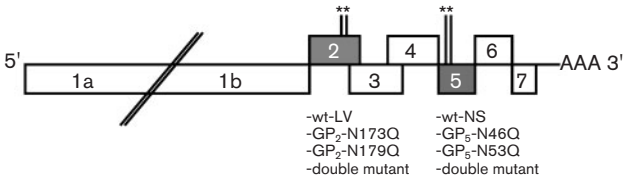

Fig. 1. Schematic representation of the 3′ part of the PRRSV genome. The positions of the consensus sequences for *N*-linked glycosylation in the translated ORF2 and ORF5 are indicated (*). The designations of the constructs are shown below the ORFs.

Inoculation of PAMs. To investigate the infectivity of the mutant viruses, 200 µl aliquots of the culture supernatants of transfected BHK-21 cells were used to inoculate PAMs. After 1 h, fresh culture medium was added. At 24 h after incubation at 37 °C, the cells were washed with PBS, dried and stored at −20 °C until immunostaining was performed.

Virus titres (expressed as $TCID_{50}$ ml^{-1}) were determined on PAMs by end-point dilution (Wensvoort *et al.*, 1986). Twenty-four hours post-infection (p.i.), infected cells were assessed in an immunostaining assay (IPMA) based on the nucleocapsid protein.

Immunoperoxidase monolayer assay (IPMA). Immunostaining of BHK-21 cells and PAMs was performed by the method described by Wensvoort *et al.* (1986). mAb 122.17 was used to detect expression of the PRRSV N protein.

Preparation of radiolabelled virions. At 15 h post-transfection (p.t.), BHK-21 cells were washed three times with starvation medium (MEM-E without L-methionine, L-cysteine and L-glutamine; ICN) and subsequently incubated with starvation medium supplemented with 1·5 µg methionine ml^{-1}, 24 µg cysteine ml^{-1}, 1 % L-glutamine, 100 U penicillin ml^{-1}, 100 U streptomycin ml^{-1} and 5 % dialysed FCS. After 1 h, 157·5 µCi Tran[^{35}S]-label (ICN) was added and the cells were further incubated for 8 h at 37 °C. After clarification of the culture supernatants at 6500 ***g*** for 10 min at 4 °C, labelled virus particles were pelleted through a 0·5 M sucrose cushion (in TNE; 20 mM Tris/HCl, pH 7·6, 100 mM NaCl, 1 mM EDTA) by ultracentrifugation (Beckman R65 rotor; 200 000 ***g*** for 3 h at 4 °C). The virus pellets were then dissolved in 450 µl PBS-TDS (1 % Triton X-100, 0·5 % sodium deoxycholate and 0·1 % SDS) supplemented with protease inhibitors (25 µg Pefabloc-Sc, 0·7 µg pepstatin, 1 µg aprotinin and 0·5 µg leupeptin ml^{-1}).

Radioimmunoprecipitation and gel electrophoresis. Solubilized virions were incubated overnight at 4 °C in the presence of the appropriate antibody. The next day, 3 mg protein A–Sepharose was added to each sample and the samples were incubated at 4 °C for another 2 h. Immune complexes were washed three times with PBS-TDS, resuspended in 30 µl 2× NuPAGE LDS sample buffer supplemented with 50 µM DTT and heated for 5 min at 100 °C. Samples were analysed by SDS-PAGE using a 12 % polyacrylamide gel. Gels were fixed for at least 10 min in 40 % (v/v) methanol and 10 % (v/v) acetic acid and dried on Whatman 3MM paper and radioactivity was visualized by phosphorimaging (STORM-840).

RNA isolation and quantification. To investigate the relative amounts of viral genomic RNA (as a measure of total virus particles), a real-time RT-PCR was performed with primers positioned at the junction of ORF6 and ORF7 using the LightCycler (Roche) as described before (Jacobs *et al.*, 2000). At 24 h p.t., BHK-21 culture supernatant was harvested and cleared by centrifugation for 15 min at 6500 ***g***. Viral RNA was isolated from 200 µl of each supernatant with the High Pure RNA isolation kit (Roche). The PCR was performed using the LightCycler RNA amplification kit hybridization probes (Roche). PCR mixtures consisted of 6·5 mM $MgCl_2$, 0·2 µM reverse primer L5, 0·2 µM of an internal LC640-labelled forward primer (iLC-PRRSV), 0·15 µM of a 3′-fluorescein-labelled Hybprobe (FL-PRRSV; see Table 1) and 10 µl RNA eluate. PCR conditions were as followed: RT step of 30 min at 50 °C, a hot start for 30 s at 95 °C, followed by 45 PCR cycles of 1 s at 95 °C, 5 s at 53 °C and 10 s at 72 °C. The relative amounts of viral genomic RNA were determined by extrapolation from a linear standard curve. For this purpose, pABV437 was transcribed *in vitro*, the RNA was purified using the RNA isolation kit and the amount of RNA was estimated on an agarose gel.

Sequence analysis of genomic RNA of recombinant viruses. Viral RNA in the culture supernatant of BHK-21 cells was isolated using the High Pure RNA isolation kit (Roche). The RNA was reverse-transcribed with antisense ORF7 RT primer (Oleksiewicz *et al.*, 1998). PCRs were performed with sense primer LV466II and antisense primer LV293 flanking ORF5 (Table 1). The amplified fragments were sequenced using both PCR primers. Sequences were determined with the PRISM Ready Dye Deoxy Terminator cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer).

Statistical analysis. Overall equality of the wild-type and mutant viruses with respect to virus production, as determined by RT-PCR, and virus infectivity was tested using the Kruskal–Wallis test. Pairwise comparisons were made using the permutation test. Significance levels were set at 5 %. Statistical analyses were performed with StatXact-5 for Windows (Cytel Software Corporation).

RESULTS

Mutagenesis and expression of GP_2 and GP_5 proteins

To study the significance of the *N*-linked oligosaccharides on the GP_{2a} and GP_5 proteins in the PRRSV life cycle, mutant full-length cDNA clones were generated in which the codons for each of the relevant asparagine residues were replaced by a codon for glutamine. Six recombinant full-length cDNA clones were thus obtained: GP_{2a}-N173Q, GP_{2a}-N179Q, GP_{2a}-double mutant, GP_5-N46Q, GP_5-N53Q and GP_5-double mutant (Fig. 1).

To confirm the use of the predicted *N*-glycosylation sites and to investigate whether the mutations affect the expression and processing of the respective proteins, the mutant and wild-type glycoproteins were assessed using SDS-PAGE (Fig. 2a). The proteins were expressed by transfection of BHK-21 cells, which are non-permissive for PRRSV, with full-length transcripts of the mutant and parental clones (wt-LV and wt-NS) and immunoprecipitated from extracellular virions that were produced by the transfected cells. Assessment of the glycoproteins in the context of extracellular virions was necessary as the proteins were hard to detect in transfected cell lysates due to the limited transfection levels. The extracellular virions were concentrated through a sucrose cushion, which resulted in less background and increased quantities of the glycoproteins. In addition, by this approach, the incorporation of the mutant proteins into virions could be verified simultaneously.

The electrophoretic mobilities of the mutant proteins were as predicted (Fig. 2a). The mobility shift corresponding to the apparent removal of about 2·5 kDa seen with every mutation demonstrated that each of the theoretical glycosylation sites is actually used and that the mutations had been effectively introduced. As is known for the GP_2 and GP_5 glycoproteins of PRRSV (Meulenberg *et al.*, 1995; Meulenberg & Petersen-den Besten, 1996), both wild-type proteins appeared in the gel as somewhat diffuse bands, indicative of heterogeneous maturation of the oligosaccharides. This was also the case, though less pronounced, for the mutant proteins lacking one of the oligosaccharide side

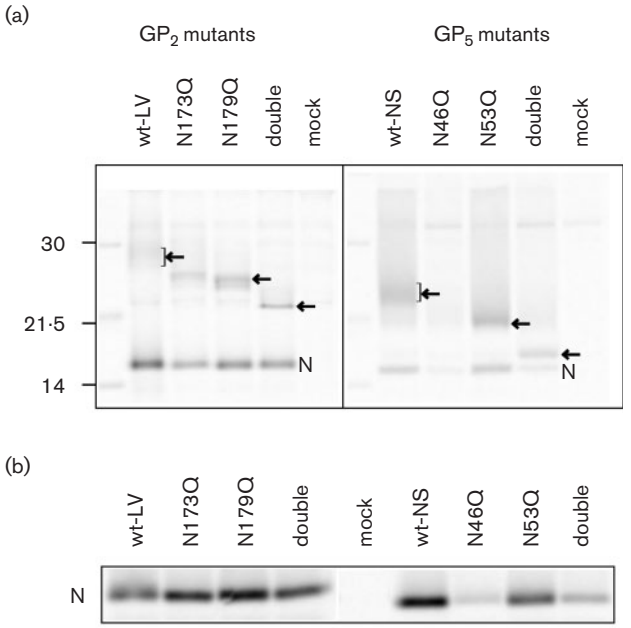

Fig. 2. Effect of the introduced mutations on expression and processing of PRRSV GP_2 and GP_5 proteins (a) and on virus particle production (b). (a) BHK-21 cells were transfected with *in vitro* transcripts of the full-length cDNA clones and labelled with Tran[^{35}S]label from 16 to 24 h p.t. After clarification of the supernatant, extracellular virions were pelleted through a 0·5 M sucrose cushion. Pellets were dissolved in PBS-TDS and subjected to immunoprecipitation with anti-GP_2 or anti-GP_5 antibodies. The supernatant of BHK-21 cells transfected with an *in vitro* transcript of pABV952, a replication-negative mutant (unpublished results), was used as a negative control. Immunoprecipitated proteins were analysed by 12% SDS-PAGE. Arrows indicate the positions of the proteins; N indicates the nucleocapsid protein that is immunoprecipitated non-specifically (Meulenberg & Petersen-den Besten, 1996). Molecular mass marker proteins are shown (kDa) in the left lane of each panel. (b) Immunoprecipitation analysis of recombinant viruses. Radiolabelled particles were generated and processed as described in (a). mAb 122.17 was used to precipitate the N protein.

chains, but not for the proteins lacking both sugars. The mutant GP_{2a} and GP_5 proteins seemed to be correctly expressed, although the intensity of the GP_5 protein bands of the N46Q mutants was greatly reduced. This reduction might be due either to reduced incorporation of these proteins in PRRSV virions or to reduced particle production.

Analysis of the effect of the mutations on the production of virus particles

To investigate whether this reduced intensity resulted from decreased production of virus particles, radioimmunoprecipitation assays were performed using an anti-N antibody (Fig. 2b). No substantial differences were observed in the intensity of the nucleocapsid protein bands immunoprecipitated from the two wild-type viruses, the GP_{2a} mutants and the GP_5-N53Q mutant. In contrast, the GP_5-N46Q mutation appeared to reduce the formation or release of particles dramatically, as judged by the small amounts of N protein precipitated from both mutants carrying this mutation. We conclude that the *N*-linked glycans normally present on the GP_{2a} protein are not essential for particle formation, as is the oligosaccharide attached to N53 of the GP_5 protein, but that the oligosaccharide linked to N46 of the GP_5 protein is strongly required.

Analysis of the effect of the mutations on the production of infectious virus

To investigate how the introduced mutations affect the production of infectious virus, equal volumes of the culture supernatants of transfected BHK-21 cells were used to inoculate PAMs, which are susceptible to PRRSV. At 24 h p.i., the cells were immunostained with an anti-N antibody (Fig. 3). Particles produced by the three GP_{2a} mutants appeared to be able to infect PAMs to the same extent as wild-type virus particles. Of the GP_5 glycosylation mutants, the GP_5-N53Q mutant showed a slight reduction in the number of positive macrophages (approx. 70% of wt-NS). The GP_5-N46Q mutant and the GP_5 double mutant, however, showed a dramatically different phenotype. These two mutants were strongly affected in their ability to produce infectious progeny virus, as could be concluded from the marginal number of positive macrophages (approx. 1–2% of wt-NS).

Characterization of the GP_5-N46Q mutants

Since the elimination of the glycosylation site at position 46 was highly detrimental for infectious virus production, we were interested in determining whether the introduced mutations were maintained in the GP_5-N46Q and GP_5-double mutants. Genomic RNA was isolated from progeny virus present in the culture supernatant of the transfected BHK-21 cells. The 3′ end of the genome encompassing the ORF5 gene was reverse-transcribed and the ORF5 genes were amplified and sequenced. It appeared that the introduced mutations were still present in both cases and that no additional mutations had been introduced in ORF5.

Analysis of the effect of the mutations on specific infectivity

To investigate the impact of the mutations on the specific infectivities of the mutant virus particles, i.e. their infectivity-to-particle ratio, we determined both the amount of genomic viral RNA (as a measure of viral particles) and the virus titre in the culture supernatant of transfected cells for each virus. For this purpose, BHK-21 cells were transfected in triplicate with full-length wild-type and mutant RNA transcripts. After 24 h, cell culture supernatants were harvested and cleared. Equal volumes from each cleared supernatant were taken to quantify comparatively the amount of viral RNA released into the medium. This was done by performing a real-time RT-PCR

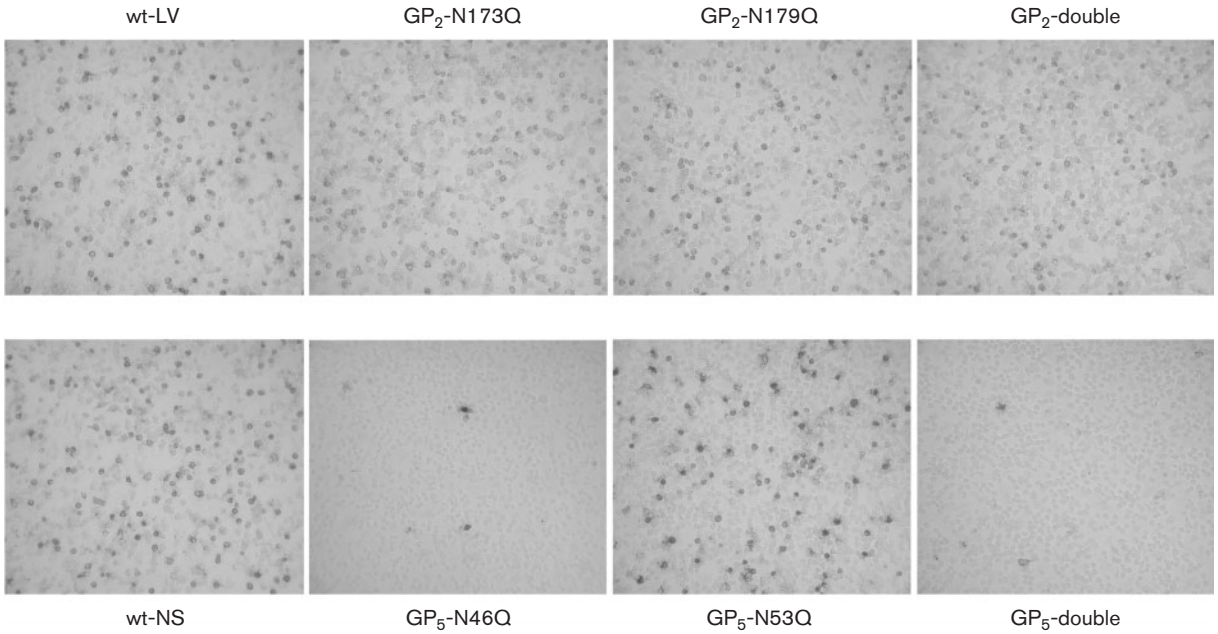

Fig. 3. Analysis of the production of infectious virus by BHK-21 cells transfected with mutant full-length RNA transcripts. Culture supernatants of transfected BHK-21 cells were used to inoculate PAMs. At 24 h p.i., the macrophages were immunostained with mAb 122.17 directed against the N protein.

on the RNA isolated from each sample with reference to an RNA standard. The results did not reveal statistically significant differences in RNA yields for the GP_{2a} mutant viruses compared with the parental virus (wt-LV) (Fig. 4a). The same held true for the GP_5-N53Q mutant when compared with its corresponding parental virus (wt-NS). However, for the two GP_5 mutants carrying the N46Q mutation, viral RNA levels were significantly reduced to a level that was 10 to 20 times lower than that of wt-NS and the GP_5-N53Q mutant. It is worth noting that the reduction in RNA yields is consistent with the observed reduction in the amounts of N protein immunoprecipitated from the GP_5-N46Q mutant viruses (see Fig. 2b).

Assuming that the amount of viral RNA in the culture medium, as measured by RT-PCR, reflects the number of viral particles, we normalized the culture supernatants to an equal number of viral particles. We then determined the infectivity titres of these supernatants by performing end-point dilution titrations on PAMs in triplicate (Fig. 4b). Obviously, the intrinsic infectivities of the GP_{2a} mutant particles are not much affected by the absence of the oligosaccharides. On the other hand, the absence of the *N*-glycan at position 53 of GP_5 reduced the infectivity of the mutant virus, though not to a statistically significant extent. Remarkably, the absence of the oligosaccharide linked to N46 of GP_5 significantly reduced the infectivity of the mutant viruses 10- to 20-fold in addition to the adverse effect on virus production.

DISCUSSION

In this study, we investigated the significance of the *N*-linked glycans on PRRSV GP_{2a} and GP_5 for virus production and viral infectivity. The results indicate that the importance of the *N*-linked glycans present on the envelope proteins of PRRSV can vary significantly. On the one hand, we observed that the *N*-glycans normally present on the GP_{2a} protein and the *N*-glycan attached to N53 of the GP_5 protein are essential for neither virus particle formation nor viral infectivity. On the other hand, the glycan linked to N46 of the GP_5 protein was found to be important both for the production of extracellular virions and for PRRSV infectivity. The observed reduction in specific infectivity of the GP_5-N46Q mutants was statistically significant and about 10- to 20-fold. Together with the similar reduction in the production of virus particles, the combined effects account for the observed overall reduction in infectious virus yields (approx. 100-fold) of the GP_5-N46Q mutants (see Fig. 3).

The GP_{2a} protein of PRRSV LV has two putative glycosylation sites at asparagine residues 173 and 179. Both sites are extremely well conserved. In fact, the only strain known to contain one site, the one corresponding to N173 in LV, is the live attenuated vaccine strain Primepac (GenBank accession no. AF066384). As both sites appeared to be actually used, the oligosaccharides are likely to serve important biological functions. It may therefore seem surprising that mutations preventing the attachment of an oligosaccharide at either one or both sites had no obvious

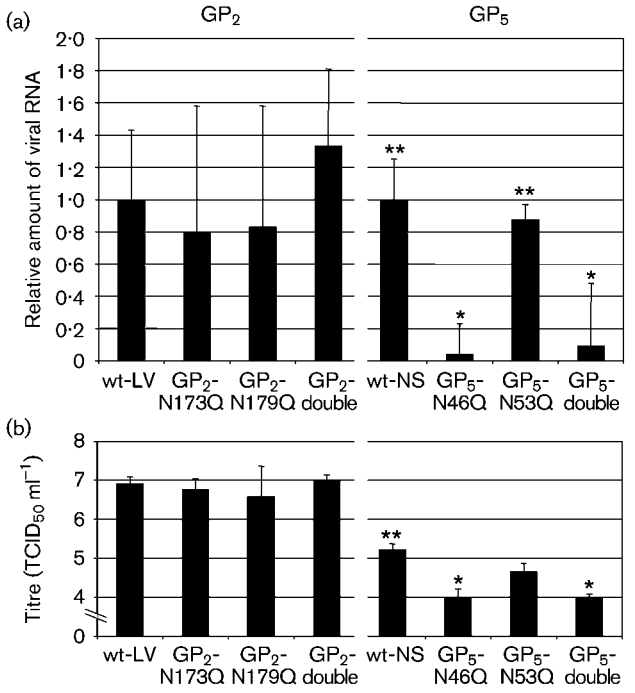

Fig. 4. (a) Quantification of relative amounts of viral RNA in transfection supernatants. For each series of viruses, BHK-21 cells were transfected in triplicate with full-length RNA transcripts. At 24 h p.t., culture supernatants were harvested and cleared by low-speed centrifugation. Subsequently, viral RNA was isolated from equal volumes of the supernatants and a real-time RT-PCR was performed. * vs ** indicates a statistically significant difference ($P \leqslant 0{\cdot}05$) between wt-NS and the GP_5-N53Q mutant on the one hand and the GP_5-N46Q and GP_5-double mutants on the other hand. (b) Specific infectivities of the mutant viruses. For each series of viruses, culture supernatants were normalized to an equal number of viral particles (i.e. the amount of RNA). Subsequently, the infectivity titres of these supernatants were determined by performing end-point dilution titrations on PAMs. * vs ** indicates a statistically significant difference ($P \leqslant 0{\cdot}05$) between wt-NS on the one hand and GP_5-N46Q and GP_5-double jointly on the other hand.

consequences, neither on the formation nor on the infectivity of virions. The observations are, however, consistent with results showing that the GP_2 protein is dispensable for particle assembly. By disrupting expression of the GP_2 gene using an infectious PRRSV cDNA clone, we found that particles are still produced but that these particles are non-infectious (E. H. J. Wissink, M. V. Kroese, H. A. R. van Wijk, F. A. M. Rijsewijk, J. J. M. Meulenberg and P. J. M. Rottier, unpublished results). These studies also showed that the GP_{2a} protein is assembled into virions as a heteromultimeric complex together with the GP_3, GP_4 and E proteins and that this complex is required to render particles infectious. The lack of *N*-linked sugars on GP_{2a} apparently does not affect the formation of the complex or its functioning in the cell culture infection system.

These observations certainly do not imply that the oligosaccharides attached to GP_{2a} are irrelevant. Rather, their conservation suggests them to endow the virus with a distinct advantage in the natural host. How the virus may benefit from having the GP_{2a} glycans is unclear, as the oligosaccharides can act at many different levels. One is the level of the receptor. As we (E. H. J. Wissink, M. V. Kroese, H. A. R. van Wijk, F. A. M. Rijsewijk, J. J. M. Meulenberg and P. J. M. Rottier, unpublished results) and others (Wieringa, 2003) have hypothesized, the multimeric complex composed of the minor proteins functions in targeting and cell entry. As has been shown by Ohuchi *et al.* (1997) for the influenza virus HA protein, the *N*-glycans adjacent to the receptor binding site can control the receptor affinity of the virus. Another level at which the GP_{2a}-linked sugars might act is the immune system. Several studies have shown that the oligosaccharides can shield critical epitopes from immune recognition (see for example Back *et al.*, 1994; Huso *et al.*, 1988; Johnson *et al.*, 2003). Such an effect might explain why no monoclonal antibodies have been obtained against the PRRSV GP_{2a} protein. Furthermore, in a recent study on the efficacy of DNA vaccination against PRRSV infection using a set of constructs expressing the structural protein genes, no antibodies were detected against the GP_{2a} protein (I. F. A. van der Linden, H. A. R. van Wijk, E. H. J. Wissink, P. A. van Rijn, J. J. M. Meulenberg and P. J. G. M. Steverink, unpublished results). It would therefore be interesting to evaluate whether the antigenicity of single- or non-glycosylated GP_{2a} in the natural host is qualitatively and quantitatively different from that of the wild-type form. Results of such studies might also be relevant for an understanding of the protective features of the Primepac vaccine strain.

The GP_5 protein of LV has two *N*-glycosylation sites, at positions 46 and 53, both of which we showed to be actually occupied in the wild-type protein. As for GP_2, these two sites are highly conserved in North American- and European-type PRRSV strains. They are also conserved in non-neuropathogenic strains of LDV (Chen *et al.*, 2000), while in neuropathogenic strains the site corresponding to position 46 is always lacking. Despite this high conservation of the sugar attached to N53, preventing glycosylation at this site had no severe effects on mutant particle formation or on the infectivity of such particles. As argued above for GP_{2a}, this glycan probably has its significance in the natural host.

Preventing glycosylation of the GP_5 protein at position N46 had dramatic effects at two different levels. Both for the single mutant and for the mutant lacking both glycans, virion production and virion infectivity were strongly reduced. The former effect may have a number of reasons. First of all, the absence of the oligosaccharide can lead to improper folding of the GP_5 protein. *N*-Glycans located near the N terminus of a protein, more specifically those within the 50 most N-terminal amino acids, often play a

pivotal role in the folding process by interacting with folding chaperones in the endoplasmic reticulum (ER) (reviewed by Helenius & Aebi, 2001). Furthermore, *N*-glycans located in the vicinity of a cysteine residue have been suggested to protect these cysteines from improper and premature formation of disulphide bonds (Daniels *et al.*, 2003; Hebert *et al.*, 1997). Actually, the *N*-glycan at position 46 is very close to Cys^{50}, the residue presumed to be involved in GP_5–M protein linkage (Verheije, 2002). In this respect it is remarkable that the sole PRRSV strains known to lack the *N*-glycan at position 46 instead have a nearby glycosylation site at position 37 (Mateu *et al.*, 2003; Stadejek *et al.*, 2002), suggesting that these glycans can adopt each other's function. As GP_5–M disulphide-bond formation is essential for arterivirus assembly (Snijder *et al.*, 2003; Verheije, 2002), an effect of the GP_5-N46Q mutation on this process might explain the reduced mutant virion production levels.

Improper folding of the GP_5 protein caused by the mutation may also affect the intracellular transport of the protein. Only properly folded proteins pass the quality control system of the ER and are allowed to exit the compartment. The GP_5 protein has to move to and accumulate at the site of virion budding, which is located in the ER, the intermediate compartment or the Golgi complex (Dea *et al.*, 2000; Magnusson *et al.*, 1970; Pol & Wagenaar, 1992). Assuming that GP_5–M heterodimerization occurs in the ER, impaired transport of the dimeric complexes will affect the efficiency of budding. The transport of budded virions through the Golgi apparatus to the plasma membrane might also suffer from improper folding of the GP_5 protein. Any of these transport defects will result in reduced release of virions from the infected cell.

The GP_5-N46Q mutation also significantly lowered the specific infectivity of the mutant viruses. This would have been a logical observation if the GP_5 protein, or the GP_5–M complex, was involved in targeting of the virus to its receptor on the host cell. However, studies in which the ectodomain of the GP_5 protein of EAV (Dobbe *et al.*, 2001) or of the M protein of PRRSV (Verheije *et al.*, 2002) was replaced by corresponding domains from other arteriviruses showed that these changes do not lead to altered viral tropism. A role of GP_5 in interaction with the primary receptor is therefore unlikely. Still, there are several steps in the post-binding process of virus entry that might be hampered by the N46Q mutation and thus cause the reduced viral infectivity. One early step might involve the interaction with heparin-like molecules at the target cell surface described by Delputte *et al.* (2002). Other steps might, for instance, involve the process of endocytosis of bound virions and, once within the endosome, the merging of the virion and endosomal membranes that leads to the delivery of the nucleocapsid into the cell cytoplasm. Clearly, we will need to learn more about the molecular details of these processes in order to understand the mechanisms behind our observations.

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