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The complex co-translational processing of glycoprotein GP5 of type 1 porcine reproductive and respiratory syndrome virus

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ABSTRACT

GP5 and M, the major membrane proteins of porcine reproductive and respiratory syndrome virus (PRRSV), are the driving force for virus budding and a target for antibodies. We studied co-translational processing of GP5 from an European PRRSV-1 strain. Using mass spectrometry, we show that in virus particles of a Lelystad variant, the signal peptide of GP5 was absent due to cleavage between glycine-34 and asparagine-35. This cleavage site removes an epitope for a neutralizing monoclonal antibody, but leaves intact another epitope recognized by neutralizing pig sera. Upon ectopic expression of this GP5 in cells, signal peptide cleavage was however inefficient. Complete cleavage occurred when cysteine-24 was changed to proline or an unused glycosylation site involving asparagine-35 was mutated. Insertion of proline at position 24 also caused carbohydrate attachment to asparagine-35. Glycosylation sites introduced downstream of residue 35 were used, but did not inhibit signal peptide processing. Co-expression of the M protein rescued this processing defect in GP5, suggesting a novel function of M towards GP5. We speculate that a complex interplay of the co-translational modifications of GP5 affect the N-terminal structure of the mature proteins and hence its antigenicity.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV, a plus-strand RNA virus categorized in the order Nidovirales, family *Arteriviridae*) is currently the most relevant viral pathogen in pigs worldwide, with significant economic impact. PRRSV infection causes abortion and stillbirth in pregnant sows as well as respiratory disease and poor growth performance in piglets (An et al., 2011; Chand et al., 2012). PRRSV was previously divided into two distinct genotypes termed “European” and “North American”, but because of the low nucleotide identity (approx. 50%) they are now classified as two species, PRRSV-1 and PRRSV-2, respectively (Kuhn et al., 2016). The early isolates Lelystad virus (LV, type 1, (Meulenberg et al., 1993; Wensvoort et al., 1991)) and VR-2332 (type 2, (Collins et al., 1992)) serve as respective prototype strains. Since their discovery, both genotypes have spread worldwide. PRRSV has diversified rapidly by mutation and recombination, including the occurrence of highly pathogenic variants in China ((Tian et al., 2007), related to type 2) and Eastern Europe ((Karniychuk et al., 2010), related to type 1).

The glycoprotein 5 (GP5) is the major envelope protein of PRRSV. It comprises an N-terminal signal peptide followed by a short ectodomain of roughly 30 residues, a hydrophobic transmembrane region and an endodomain. GP5 forms a disulphide-linked complex involving cysteine 50 (Veit et al., 2014) with the non-glycosylated M protein. This GP5/M complex is relevant for virus assembly/budding as well as binding of virions to cellular attachment factors (Van Breedam et al., 2010; Wissink et al., 2005; Zhang and Yoo, 2015). For equine arteritis virus (EAV), the prototype arterivirus, it was shown that heterodimerization of M with GP5 is required for their transport from the endoplasmic reticulum (ER) to the Golgi apparatus, the viral budding site (de Vries et al., 1995). M might also affect the reactivity of antibodies against GP5: An escape mutant of PRRSV was described that had become resistant to a broadly neutralizing antibody owing to a deletion of one amino acid in the ectodomain of M, with likely implications on the interaction with GP5 (Trible et al., 2015). This is in line with former studies showing that GP5/M heterodimerization is critical for the expression of the neutralization epitopes of arteriviruses (for review see (Balasuriya and MacLachlan, 2004; Dea et al., 2000; Lopez and Osorio,

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2004)).

GP5 is targeted to the rough endoplasmic reticulum (ER) by the signal peptide and then translocated into the ER lumen through the "translocon", a hetero-oligomeric complex serving as channel in the ER membrane. During translocation, a number of co-translational modifications are performed on the nascent protein, most importantly N-glycosylation on asparagine residues in the sequence context N-X-S/T ("sequon"), cleavage of the signal peptide, and oligomerisation. The enzyme complexes for N-glycosylation (oligosaccharyl transferase, OST) and signal peptide cleavage (signal peptidase, SPase) are associated with the translocon (but not necessarily in the same complex) to perform their activity once a nascent polypeptide chain becomes accessible (Dudek et al., 2015; Shrimal et al., 2015).

Signal peptides, typically ~30 residues in length, consists of an N-region containing positively charged residues, a hydrophobic H-region and a small C-region with the cleavage site (Auclair et al., 2012). The N-terminus of a nascent protein is inserted as a hairpin into the translocon. The N-region of the signal peptide is oriented to the cytosolic site, the H-region forms an α-helix that is bound to a hydrophobic groove located outside the translocon, and the C-region is located entirely inside the channel. Thus, at some point during translocation the signal peptide must adopt a more extended conformation such that the cleavage site becomes accessible to the signal peptidase (Li et al., 2016; Voorhees and Hegde, 2016).

Signal peptides are often, but not always cleaved by signal peptidase, the catalytic subunit of which is a serine-like protease. Whether and where a signal peptide is cleaved depends primarily on the presence of small and neutral amino acids (Ala, Gly, Ser, Thr, Cys) at the −1 and −3 position with respect to the cleavage site (Von Heijne, 1983). Some more distant features that can influence signal peptide cleavage have been identified: In prokaryotic signal peptides the boundary between the H-region and the C-region is marked by a helix-breaking residue (mostly Pro at the −6 position), which is required for efficient cleavage (Auclair et al., 2012; Shen et al., 1991). Conversely, a proline residue at the +1 position (but not any other amino acid) prevents cleavage of the signal peptide; such a construct was also shown to be an effective inhibitor of signal peptidase (Cui et al., 2015). Another feature that may influence signal peptide cleavage is the concurrent attachment of a carbohydrate chain through N-glycosylation at sites near the signal peptide cleavage site. A bulky glycan can obstruct signal peptide cleavage, as we showed for the glycoprotein of another Arterivirus, GP3 of equine arteritis virus (EAV), where glycosylation at a unique overlapping sequon adjacent to the signal peptide completely blocked signal peptide cleavage. Deletion of both sites causes complete cleavage and virus particles containing GP3 without a signal peptide did not show a replication-defect in cell culture (Matczuk et al., 2013; Matczuk and Veit, 2014).

Thus, the glycoproteins of arteriviruses appear to follow unusual, in part unpredictable co-translational processing schemes. This processing warrants close investigation, not least because antibody epitopes were hypothesised to lie within the C-region of the signal peptide, particularly in case of GP5 of PRRSV (Ostrowski et al., 2002; Plagemann, 2004a,b; Plagemann et al., 2002; Popescu et al., 2017). Thus, signal peptide cleavage may affect the presence of such epitopes in the mature protein. For GP5 of various PRRSV-2 strains, we previously determined that signal peptide cleavage occurs efficiently in transfected cells. Mass spectrometry of GP5 present in virus particles of the VR 2332 strain revealed that cleavage occurs at either of two sites, leading to retention or removal, respectively, of an antibody epitope situated between these two sites (Thaa et al., 2013). Deep sequencing of RNA isolated from pigs that showed virus rebound after experimental infection revealed various nonsynonymous nucleotide exchanges, especially in ORF5. The region involved correspond to the N-terminal region of GP5, the exchanged amino acids are located around the signal peptide cleavage site, but also within the signal peptide. The most exchanges were identified at day 28 after infection, shortly before virus reappeared in the blood and thus may be the result of virus escape from neutralizing antibodies (Chen et al., 2016; Evans et al., 2017).

For type 1 PRRSV signal peptide processing of GP5 could also have implications regarding the presence of a neutralizing antibody epitope. Monoclonal GP5-directed antibodies from mouse raised against the strain Intervet 10 (I10) neutralised infectivity of a plaque-purified subpopulation of this strain (PPV), but failed to neutralise the closely related Lelystad virus (Weiland et al., 1999). Sequencing revealed four amino acid differences between PPV-GP5 and LV-GP5, but only the cysteine-to-proline exchange at position 24 (located within the signal peptide) affected antibody reactivity. Surprisingly though, position 24 is not part of the antibody epitope, which was identified by Pepscan analysis to encompass residues 29–35 (WSFADGN) of GP5, thus situated around the potential signal peptide cleavage sites. It was hypothesised–but never assessed experimentally—that cysteine at position 24 of GP5 may cause signal peptide cleavage at a site downstream of the antibody epitope, resulting in mature GP5 lacking the epitope (Wissink et al., 2003). Sera from PRRSV-infected pigs did not recognize the WSFADGN epitope of the monoclonal antibody questioning its physiological relevance. The pig sera rather bind to a not precisely defined epitope (encompassing residues 38–54) located in the middle of the ectodomain of GP5 (Plagemann, 2004a,b).

The purpose of this study was to explore the molecular requirements for co-translational processing of GP5 of PRRSV-1 and to identify the exact signal peptide cleavage site to provide a better understanding of the primary structure of the N-terminal region of the mature GP5 protein. Thousands of different GP5 nucleotide sequences present in the database show the enormous variability of PRRSV and its evolution (Murtaugh et al., 2010) and nucleotide exchanges occurring in GP5 during PRRSV infection in pigs have been reported (Chen et al., 2016; Evans et al., 2017; Faaberg et al., 2006; Vu et al., 2011; Wei et al., 2012a). However, studies which investigate GP5 on the protein level are rather rare, but relevant to identify the amino acids (and their modifications) actually to be found in the mature protein and thus exposed to the immune system.

2. Materials and methods

2.1. Cells, viruses, plasmids and protein processing analysis

Cell culture of CHO-K1 (Chinese hamster ovary cells) and MARC-145 (simian kidney epithelial cells derived from MA-104) cells were maintained as described (Thaa et al., 2013). The isolate of Lelystad virus (LV) was described previously (Costers, 2008; Costers et al., 2010). The GP5 gene of LV, reverse-transcribed from LV-RNA and cloned into pExpr-IBA3 (IBA Life Sciences) (Costers, 2008; Costers et al., 2010), was used as template for PCR to subclone the GP5 gene into the expression vector pCMV-TnT (Promega) using the *Xho*I and *Not*I restriction sites. The sequence encoding a C-terminal HA tag was included in the reverse primer; the start codon of the overlapping ORF5a protein (Firth et al., 2011; Johnson et al., 2011) was disrupted by an A-to-G mutation in the forward primer, a silent mutation regarding the GP5-encoding sequence. Site-directed mutagenesis was performed by overlap-extension PCR (Green and Sambrook, 2012). The M–YFP vector was generated by subcloning the LV-M gene (GenBank M96262), obtained by gene synthesis (Eurofins), into pEYFP-N1 using *Xho*I and *Kpn*I. Accuracy of the constructs was verified by sequencing.

Signal peptide cleavage was essentially assessed as described before (Thaa et al., 2013). Briefly, transfected cells in 6-well plates were trypsinsed at 24 h after transfection, pelleted and resuspended in 80 µL glycoprotein denaturing buffer (0.5% SDS, 40 mM DTT) and boiled for 10 min at 100 °C. Typically, 15 µL of this lysate was digested with PNGase F or Endo H (New England BioLabs, 2.5–5 units/µL, 4 h at 37 °C) according to the manufacturer's instructions, with control samples left untreated. Samples were then subjected to SDS-PAGE and Western blot following standard procedures (Thaa et al., 2013), using

rabbit-anti-HA tag (ab9110, Abcam, Cambridge, UK, 1:5000) and a horseradish peroxidase-coupled anti-rabbit secondary antibodies (Sigma).

In vitro transcription/translation was performed with the TNT Quick Coupled Transcription/Translation System (Promega) as described (Thaa et al., 2013). For immunoprecipitation, cells were lysed with RIPA buffer (20 mM Tris·HCl, 150 mM NaCl, 10 mM EDTA, 10 mM iodoacetamide, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, pH 7.4), followed by incubation with rabbit-anti-GFP antibody (Molecular Probes, A11122) at 4 °C for 16 h with agitation, addition of protein A–sepharose (Sigma), further incubation for 2 h at 4 °C, pelleting and washing with RIPA and elution with SDS-PAGE loading buffer prior to SDS-PAGE and Western blot.

2.2. Mass spectrometry

Viruses were pelleted from supernatants of infected MARC-145 cells as described (Thaa et al., 2013). Proteins were reduced with 10 mM DTT (in 50 mM ammonium bicarbonate buffer for 45 min at 56 °C), alkylated with iodoacetamide (55 mM) and deglycosylated with PNGase F for 4 h at 37 °C prior to SDS-PAGE and Coomassie staining. GP5 was identified by Western blot with the monoclonal anti-GP5 antibody 3AH9 (Rodriguez et al., 2001) using an aliquot run on an adjacent lane. GP5 was excised from the gel followed by either in-gel chymotrypsin (in 50 mM ammonium bicarbonate buffer for 20 h at 25 °C) or elastase (in 50 mM ammonium bicarbonate at 37 °C overnight) digest, peptide extraction with trifluoroacetic acid (TFA) in acetonitrile, drying and reconstitution in 0.1% TFA/5% acetonitrile. NanoLC-ESI–MS/MS was performed on an Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) equipped with an Ultimate 3000 NCS-3500RS liquid chromatography system (Thermo Scientific). MS data were acquired in a data-dependent strategy selecting MS/MS fragmentation events based on the precursor abundance in the MS scan. MS and MS/MS spectra were used to search against a custom-made database containing all proteins of the SwissProt database, including the full-length LV-GP5 sequence (Costers, 2008). In addition, the database contained all possible N-terminally truncated sequences of GP5 resulting from signal peptide cleavage site prediction. Asn/Asp amino acid exchanges were used as variable modifications. For identification of GP5 peptides, the processed MS/MS spectra were compared with the theoretical fragment ions of GP5 peptides using the MASCOT server version 2.2.2 (Matrix Science), allowing a maximum of six mixed chymotrypsin cleavages, with a mass tolerance of precursor and sequence ions set to 10 ppm and 0.35 Da, respectively.

2.3. Bioinformatics resources

Signal peptide cleavage site predictions were performed with SignalP 4.1 (Petersen et al., 2011), (http://www.cbs.dtu.dk/services/SignalP/). Sequence alignments were done using EMBOSS (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Graphical representations of multiple sequence alignment were generated with WebLogo (Crooks et al., 2004), (http://weblogo.berkeley.edu/logo.cgi).

3. Results

3.1. Bioinformatic predictions of the N-terminal ectodomain of GP5

Fig. 1A shows the sequence of the N-terminal region of GP5 employed in this study, cloned from a laboratory variant of the PRRSV-1 prototype strain Lelystad (Costers, 2008; Costers et al., 2010). Of note, GP5 in our variant of Lelystad virus carries an amino acid substitution at position 37 (aspartic acid to serine, (Costers, 2008)) as compared to the sequence of the original Lelystad isolate (Meulenberg et al., 1993). Importantly, this creates a glycosylation sequon (N-X-S/T) at position 35 adjacent to one of the predicted signal peptide cleavage sites; 35NGD37 in the original LV isolate is mutated to 35NGS37. The two other glycosylation sites at positions 46 and 53 are present in both variants of the Lelystad virus and conserved in all PRRSV-1 strains. See supplementary Table 1 for the complete GP5 sequences. The region from amino acid tyrosine 41 to cysteine 50 in the conserved ectodomain forms a β-sheet structure according to secondary structure predictions with Jpred; the preceding region (34–40) is unfolded and more variable. Residue 50 is the only cysteine in the ectodomain and forms the disulphide bond to the M protein (Veit et al., 2014). The N-terminal region comprises the features of a canonical signal peptide (positively charged N-region, hydrophobic H-region predicted to form an α-helix, C-region, see Fig. 1A). Adjacent to the signal peptide is a hypervariable region (see also Fig. 3A) containing mainly the amino acids N, S and T. It was shown that GP5 can rapidly acquire or loose potential glycosylation sites in this region during virus evolution in pigs (Chang et al., 2002; Kwon et al., 2008).

Several positions between residues 27 and 35 fulfil the requirements for a signal peptide cleavage site (small/uncharged residues at positions −3 and −1). We employed the SignalP algorithm (Petersen et al., 2011) to predict cleavage efficiency (Fig. 1B). According to this analysis, the most probable cleavage site is located between A32 and D33, which is within the monoclonal antibody epitope 29WSFADGN35. Other potential sites (albeit predicted with lower probability) are located with a regular spacing of two amino acids upstream and downstream of A32 (26G27L, 28S29W, 30S31F, 34G35N, 36G37S). Cleavage at the most N- or C-terminal sites would either leave the antibody epitope entirely unaffected or would completely remove it, respectively.

3.2. Signal peptide cleavage of GP5 in transfected cells

To analyse signal peptide cleavage we did not utilize the classical approach using *in vitro* translation in the presence of microsomes (Blobel and Dobberstein, 1975) since (due to inefficient translocation and glycosylation) it usually produces multiple GP5 bands that complicate interpretation of the data (Thaa et al., 2013). Instead, we expressed GP5 in cells and compared the SDS-PAGE mobility of the deglycosylated protein with the product synthesized *in vitro*, which remains unglycosylated, but retains the signal peptide. We generated an expression plasmid comprising GP5, cloned from the laboratory variant of Lelystad having the glycosylation sequon at position 35, fused at the C-terminus with an HA tag for subsequent antibody detection. We also mutated cysteine 24 in the signal peptide to proline (GP5 C24P). This exchange corresponds to the sequence found in the Intervet 10 virus strain which was neutralised by a monoclonal antibody that failed to neutralise Lelystad virus (Weiland et al., 1999; Wissink et al., 2003). We hypothesised that the identity of residue 24 might influence signal peptide cleavage to affect the presence of the epitope in mature GP5.

To biochemically assess signal peptide cleavage, MARC-145 cells, which are permissive for PRRSV, were mock-transfected or transfected with either GP5 wt or GP5 C24P, cell lysates were prepared 24 h post-transfection, digested with the enzyme peptide:N-glycosidase F (PNGase F) to remove all N-linked glycans and subsequently analysed by SDS-PAGE and Western blot (Fig. 2A). For comparison, GP5 wt was also generated from the same plasmid by *in vitro* transcription/translation in the absence of membranes. The product of this reaction is neither processed by signal peptide cleavage nor N-glycosylated and thus represents a “size marker” for unprocessed GP5 wt, running at an apparent molecular weight of ~18 kDa (lane 1). Western blot of PNGase treated samples revealed two bands for GP5 wt (lane 6), the upper of these bands corresponding in size to the unprocessed GP5 “size marker” (lane 1) and thus representing deglycosylated GP5 with signal peptide, while the lower band ran faster and thus corresponds to the deglycosylated protein lacking the signal peptide. Since the two bands were of roughly the same intensity, the signal peptide of GP5 wt was cleaved with low efficiency. In contrast, the deglycosylated GP5 C24P

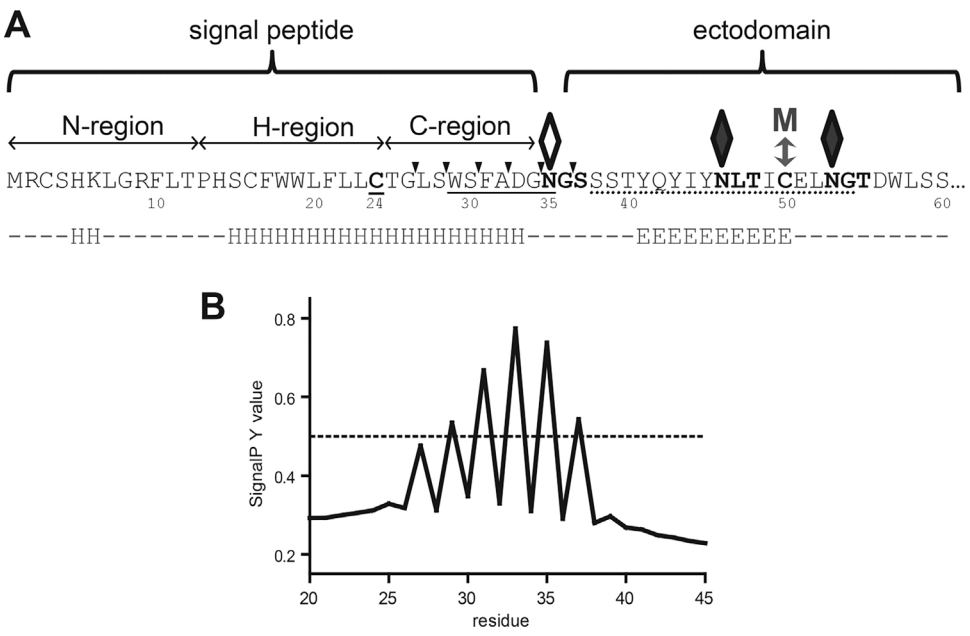

Fig. 1. Predicted signal peptide cleavage of PRRSV-GP5.

(A) Amino acid sequence of the N-terminal part (signal peptide plus ectodomain) of the GP5 employed in this study. Relevant features are highlighted: Potential N-glycosylation sequons in bold (black lozenges indicates conserved sites, the white lozenge marks the non-conserved glycosylation site), cysteine 50 (forms the disulphide bond to M) in bold, residue 24 bold and underlined, possible signal peptide cleavage sites derived from the prediction in (B) indicated by triangles. The epitope WSFADGN recognized by a monoclonal antibody (Wissink et al., 2003) and the epitope recognized by pig sera encompassing residues 38–54 (Plagemann, 2004a; Plagemann, 2004b) are underlined with a continuous and dotted line, respectively. Secondary structure prediction (Jpred) in the bottom line (H: α-helix, E: β-sheet).

(B) SignalP 4.1 signal peptide cleavage prediction for GP5. The "Y value" as a measure of signal peptide cleavage probability at the indicated residue is displayed; threshold (0.5) indicated by the dotted line.

mutant displayed almost exclusively the lower band, representing protein without signal peptide (lane 7). We conclude that the removal of a cysteine or the insertion of proline at position 24 positively affected signal peptide processing.

In the glycosylated samples (without PNGase F digestion), GP5 wt displayed two major bands (lane 3), whereas the C24P variant (lane 4) only showed one band, migrating similarly than the *upper* GP5 wt band. Considering that GP5 C24P lacks the signal peptide and that both the signal peptide and one carbohydrate chain typically contribute around 2.5 kDa to the apparent molecular weight of a glycoprotein, it can be concluded that GP5 C24P carried one additional glycan compared to GP5 wt–i.e., two glycans in the wt version, but three glycans in the C24P mutant of GP5.

N-glycosylation at position 35 might inhibit signal peptide cleavage by steric hindrance as described for GP3 of EAV (Matczuk et al., 2013). Therefore, we changed the sequon N35-G36-S37 to NGD (GP5 NGD). This GP5 now corresponds to the sequence of GP5 of the original Lelystad strain (GenBank M96262.2). After performing the processing analysis, the deglycosylated proteins showed that the signal peptide was (almost) completely cleaved in GP5 NGD, irrespective of whether residue 24 was cysteine or proline (Fig. 2B). The glycosylated versions of the two proteins did not markedly differ in size, which indicates that the two conserved glycosylation sites were used in each case. In sum, a glycosylation site at position 35 plus a cysteine at position 24 within the signal peptide prevented complete signal peptide processing in GP5.

3.3. Glycosylation sites and signal peptide cleavage

We next asked whether the N-glycosylation sequon at position 35 is used and also whether N-glycosylation sequons introduced at downstream positions in the vicinity (36, 37 or 38) would have a similar effect on signal peptide cleavage. Such glycosylation sites can easily be gained by a single amino acid mutation in the hypervariable region. In fact, the majority of "European" PRRSV-1 GP5 sequences comprise an N37-S38-S39 sequon, revealed by sequence alignment of 988 PRRSV-1 GP5 sequences deposited at ncbi.nlm.nih.gov (Fig. 3A).

We expressed a panel of GP5 mutants where either no asparagine was present in this region (GP5 N35S) or where N was in a sequon context at either position 35, 36, 37 or 38. Residue 24 was cysteine as in GP5 wt. These mutants did not markedly differ from each other in bioinformatic prediction analysis using SignalP (not shown). Biochemical processing analysis was performed for these mutants (Fig. 3B). After PNGase F digestion, a double-band pattern was detected for the N35 variant, as observed before (Fig. 2A). All the other variants of GP5 displayed only one major band corresponding in the size to the lower band of the N35 variant, indicating that the signal peptide was completely cleaved. Small differences in electrophoretic mobility suggest that the position of signal peptide cleavage might be shifted. Based on the migration pattern without PNGase F treatment (Fig. 3B, top panel), we conclude that the additional glycosylation sites in the N36, N37 and N38 variants of GP5 were used. The N35 sequon however was not used: The upper band of the glycosylated N35 variant displayed a higher molecular weight as the N35S mutant owing to the signal peptide.

Thus, partial prevention of signal peptide processing in GP5 required the unused sequon NGS at position 35. Hence, the obstruction of signal peptide cleavage was not due to a glycan at N35, but due to some other feature. To analyse whether the glycine at position 36, a residue known to disrupt α-helical structures, affects signal peptide processing we changed it to alanine (GP5 G36A). Furthermore, we also investigated the contribution of position 24 within the signal peptide. To determine whether the presence of cysteine 24 is relevant for obstruction of signal peptide cleavage, for example by forming an intramolecular disulphide bond, we exchanged it to a serine (GP5 C24S). Either of these mutations was introduced into GP5 with a glycosylation sequon at position 35 and analysed with the processing assay (Fig. 3C). The result shows that neither of these mutations had an effect on signal peptide cleavage and glycosylation, both GP5 C24S and GP5 G36A displayed a partially uncleaved signal peptide and two carbohydrates like the corresponding wildtype. We conclude that the glycine at position 36 had no effect on signal peptide cleavage and that it is the insertion of a proline (and not the removal of the cysteine) at position 24 that allowed complete signal peptide cleavage.

3.4. Identification of signal peptide cleavage site of GP5 by mass spectrometry

Next, we analysed signal peptide cleavage site of GP5 present in virus particles. The Lelystad variant having the glycosylation sequon at position 35 was pelleted from the supernatant of infected MARC-145 cells. When the virus particles were analysed by Western blot with a GP5 antibody (Fig. 4A), a band at an apparent molecular mass of ~22 kDa was detected. Upon PNGase F treatment, only one band with a molecular mass of ~14 kDa was observed, suggesting that virions

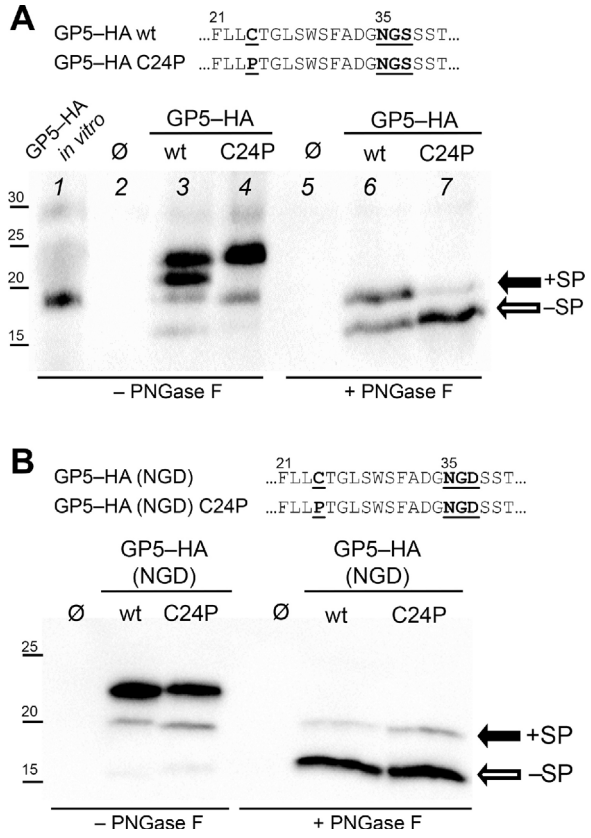

Fig. 2. Processing analysis of GP5 in transfected cells.
(A) MARC-145 cells were mock-transfected (Ø) or transfected with the indicated HA-tagged GP5 constructs (residues 21–40 are displayed). GP5-HA wt has an identical amino acid sequence as GP5 from a variant of PRRSV Lelystad with a glycosylation sequon at position N35. Cells were lysed at 24 h post-transfection, subjected to PNGase F digestion to remove N-linked glycans (lanes 5–7) or left untreated (lanes 2–4) and analysed by SDS-PAGE/Western blot (anti-HA tag). The product of *in vitro* transcription/translation of GP5-HA wt (= unprocessed protein, not glycosylated, containing the signal peptide) is shown in lane 1. Arrows indicate the position of deglycosylated proteins with (black) and without (white) signal peptide (SP), respectively. Note that there are also minor GP5 bands visible in the samples without PNGase F digestion. They probably represent un-glycosylated GP5 with (upper band) and without (lower band) signal peptide.
(B) Processing analysis as in (A) with the indicated versions of GP5-HA-constructs. The GP5-HA (NGD) has an identical amino acid sequence as the GP5 from the original PRRSV isolate Lelystad (Meulenberg et al., 1993), differing from the GP5 wt in Fig. 2A by aspartic acid instead of serine at position 37. All images are representative of three independent experiments. Molecular weight markers displayed on the left-hand side.

contain only GP5 with cleaved signal peptide.

To identify the cleavage site deglycosylated GP5 was excised from the gel and in-gel digested with chymotrypsin or elastase. The resulting peptides were subjected to mass spectrometry (LC-ESI–MS/MS). In two independent virus preparations, GP5 was identified with 65–80% sequence coverage (percentage referring to GP5 with signal peptide). We failed to detect any peptides comprising residues located N-terminally to residue 35. A peptide starting with N35 was detected in both elastase and chymotrypsin-digested samples (…adg|NGSSSTYQYIY|nlt…, displayed in Fig. 4B). Thus, N35 is likely to be the N-terminus of mature GP5 in virus particles since it does not match a canonical chymotrypsin cleavage site (Keil, 1992). Conversely, the cleavage site…**ADG**|N… corresponds to the amino acid pattern recognized by signal peptidase. Thus, the signal peptide of GP5 in the virus was efficiently cleaved at G34|N35, one of the highly likely sites predicted by SignalP (see Fig. 1B), albeit not the site with the highest cleavage probability (A32|S33). This result also implies that the GP5 molecules incorporated into virus particles lacked the neutralizing epitope (residues 29–35), consistent with the result that this virus was not neutralised by monoclonal antibodies directed against this epitope (Wissink et al., 2003). However, the epitope recognized by pig sera (encompassing residues 38–54) is present in the mature GP5 protein from virus particles.

An identical cleavage site was identified in GP5 of an Intervet 10 strain (Fig. 4C), which contains the sequence N35-G36-D37 and therefore no glycosylation sequon at N35. We identified two peptides starting with N35 (sequence N^{35}GDSSTYQYI and N^{35}GDSSTYQYIYNLT), but no peptides with residues N-terminal to N35 in GP5 of our virus sample upon elastase digest. Sequencing of the ORF5 mRNA from this Intervet 10 strain revealed that the signal peptide of GP5 contains a cysteine at position 24 (data not shown) and not the proline that is required for neutralization of the plaque-purified variant of Intervet 10 by the monoclonal antibody (Wissink et al., 2003).

3.5. Signal peptide cleavage of GP5 in cells co-expressing M

One obvious difference between virus infection and our transfection approach is that the dimerisation partner for GP5, the M protein, was not present in the latter. To address whether the processing defects of GP5 may be rescued by co-expression of M, we co-transfected cells with the plasmid encoding GP5–HA wildtype together with M, fused at the C-terminal cytoplasmic tail to the yellow-fluorescent protein (YFP). Control samples were transfected with M–YFP alone, GP5–HA on its own or GP5–HA plus native (unfused) YFP. Western blot (anti-HA tag, Fig. 5A) showed the prominent double-band pattern of GP5–HA when expressed on its own or together with YFP, indicating incomplete signal peptide cleavage. Upon co-expression of M–YFP however, the pattern of GP5–HA instead comprised a lower sharp band, at the same position as the band of glycosylated GP5–HA without signal peptide, plus a blurry signal at higher apparent molecular weight.

Blurry signals containing several bands are due to heterogeneous glycosylation of GP5 with complex carbohydrates by Golgi-located glycosyltransferases (Li et al., 2015). Indeed, the upper signal in GP5–HA co-expressed with M–YFP was retained upon treatment with endoglycosidase H (Endo H, Fig. 5B). This enzyme removes high-mannose N-linked glycans, but not the modified glycans present on glycoproteins after passage through the medial-Golgi. Thus, a fraction of GP5 molecules was transported to the medial-Golgi (or further) when co-expressed with M, consistent with a previous demonstration for GP5 of EAV using immunofluorescence microscopy (de Vries et al., 1995).

To substantiate an interaction between GP5 and M, we performed immunoprecipitation under denaturing, but non-reducing conditions using an anti-GFP antibody to pull down M–YFP and analysed samples by Western blot with anti-HA antibodies to visualise co-precipitated GP5–HA (Fig. 5C). GP5–HA signals were exclusively detected for the condition where M–YFP and GP5–HA were co-expressed.

Finally, we analysed whether co-expression with M facilitates signal peptide processing of GP5. The band pattern of PNGase F-digested samples (Fig. 5D, upper part) clearly shows that co-expression of GP5–HA with M–YFP led to complete cleavage of the signal peptide. However, when cysteine 50 in GP5–HA was exchanged by serine (C50S) to prevent disulphide-bond formation with M (Veit et al., 2014), signal peptide cleavage of GP5 remained inefficient also upon co-expression with M. Blotting of the same samples with an anti-GFP antibody showed that the expression levels of M-YFP are very similar regardless of whether GP5-HA wt or GP5-HA C50S were co-expressed (Fig. 5D, lower part).

4. Discussion

The signal peptide of GP5 present in virus particles of a variant of the Lelystad strain, the prototype strain of “European” PRRSV-1, was completely cleaved between glycine 34 and asparagine 35 as determined by mass spectrometry (Fig. 4). This also removed the epitope WSFADGN (residues 29–35, determined by Pepscan analysis) for a

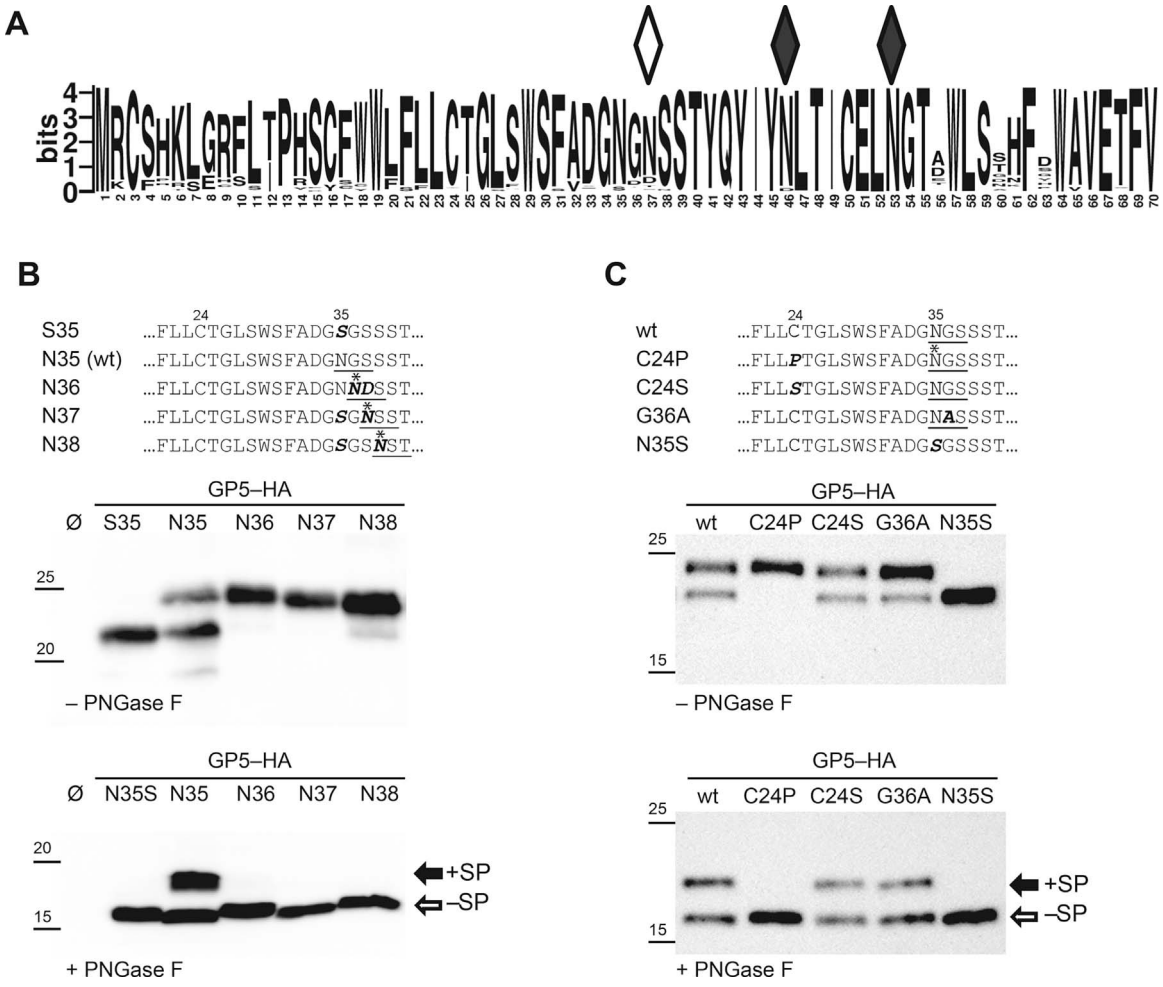

Fig. 3. In-depth analysis of sequence features and glycosylation sites influencing signal peptide cleavage in GP5.

(A) Multiple sequence alignments of 988 GP5 sequences of PRRSV type 1 (extracted from ncbi.nlm.nih.gov, first 70 residues), displayed as sequence logo. The size of the amino acids corresponds to their frequency with the "bits" being a measure of sequence conservation (the higher, the more conserved; (Crooks et al., 2004)). The black lozenge indicates the conserved N-glycosylation sites, the white lozenge the non-conserved glycosylation site in proximity to the signal peptide (cf. Fig. 1A). Note that GP5 proteins from most PRRSV-1 strains have a glycosylation sequon at position 37 and a cysteine at position 24.

(B) Processing analysis of mutants of GP5-HA with additional glycosylation sites at the indicated positions. N35 (wt) is the GP5 from the variant of the Lelystad virus having a glycosylation site at position 35 already analysed in Fig. 2A. Amino acid exchanges relative to this GP5 are in bold and italics. Potential glycosylation sites are underlined. Used glycosylation sites are marked with an asterisk.

(C) Processing analysis of GP5-HA with mutations at residue 24 or in the glycosylation sequon N35-G36-S37 as indicated. Wt is the GP5 from the variant of the Lelystad virus having a glycosylation site at position 35 already analysed in Fig. 2A. Amino acid exchanges relative to this GP5 are in bold and italics. Potential glycosylation sites are underlined. Used glycosylation sites are marked with an asterisk. GP5 was expressed in CHO-K1 cells. See legend to Fig. 2 for details.

monoclonal antibody which did not neutralise the Lelystad strain of PRRSV, confirming the assumption that the epitope is absent in mature GP5 present in virus particles (Weiland et al., 1999; Wissink et al., 2003). However, the epitope recognized by pig sera (encompassing residues 38–54) is present in the mature GP5 protein of PRRSV-1 (Plagemann, 2004a,b).

The signal peptide of GP5 of "European" PRRSV-1 is thus larger (34 amino acids) compared to GP5 of "North American" PRRSV-2, where two cleavage sites (after residues 26 and 31, respectively) were determined using the same methodology (Thaa et al., 2013). However, there is likely more variability in the location of the cleavage site(s) in different PRRSV strains. GP5 contains a cluster of putative cleavage sites (small amino acids with a regular spacing of two amino acids) in the C-region of the signal peptide (Fig. 1) which might alternatively be used if a mutation occurs at the initially preferred cleavage site. In addition, attachment of carbohydrates to asparagine 36, 37 and 38 (note that asn 37 is a glycosylation sequon in most PRRSV-1 strains, Fig. 3A) also seemed to influence the position of signal peptide cleavage since the corresponding GP5 mutants have slightly different SDS-PAGE mobility after deglycosylation (Fig. 3B).

Upon expression of wildtype GP5 of the same virus strain by transfection, in the absence of the M protein, we however observed that a large fraction of the protein still contained the signal peptide (Fig. 2). A systematic biochemical analysis revealed a variety of different features that inhibited efficient signal peptide cleavage: Incomplete cleavage of the signal peptide was observed in the context of the sequences NGS or NAS at the N-terminus of the mature protein (Fig. 2A and 3C), while amino acids not encoding a glycosylation sequon (NGD (Fig. 2B), SGS, NND and SGN (Fig. 3) allowed complete cleavage. However, the glycosylation sequon NGS was not used and thus sterical hindrance of the cleavage site by a bulky carbohydrate was not the reason for incomplete processing. Furthermore, insertion of additional glycosylation sites at position 36, 37 or 38 led to modification of the respective sites with a glycan and seemed to influence the position of signal peptide cleavage but did not inhibit signal peptide processing itself (Fig. 3B).

The inhibitory effect of the glycosylation sequon was rescued by insertion of a proline at position 24 within the signal peptide (−11 relative to the cleavage site, Fig. 2A). This is reminiscent of bacterial

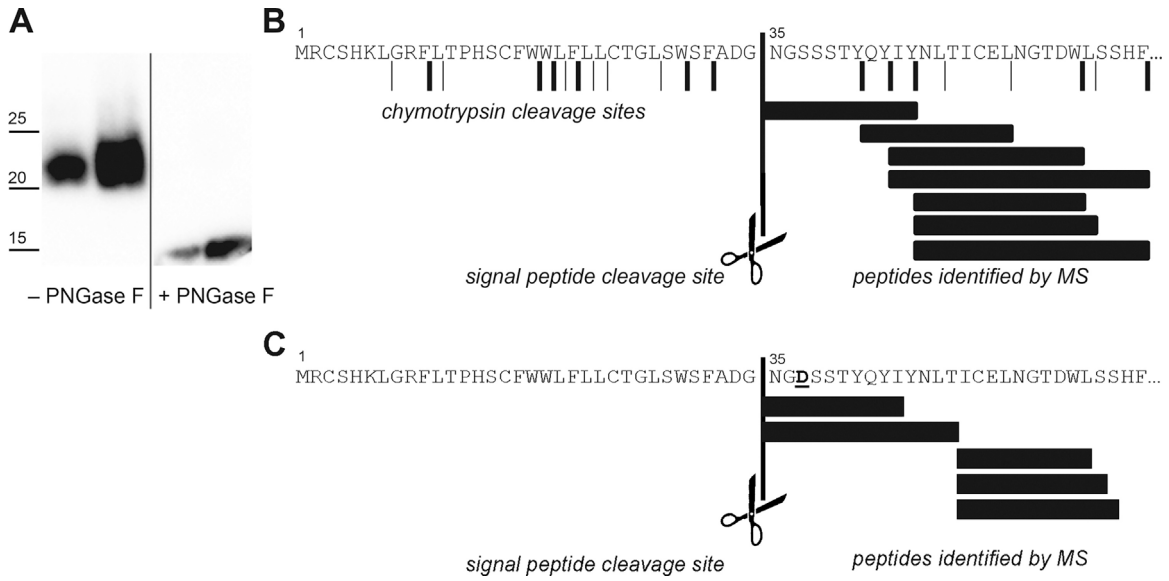

Fig. 4. Signal peptide cleavage site identification in GP5 by mass spectrometry.
(A) Western blot analysis with anti-GP5 antibodies of PRRSV particles (variant of the Lelystad virus having a glycosylation site at position 35) grown in MARC-145 cells, treated with PNGase F (right panel) or left untreated. Two independent virus preparations are shown.
(B) Result of nanoLC-ESI–MS/MS analysis of deglycosylated LV-GP5. The N-terminal protein sequence with chymotrypsin cleavage sites is shown. Thick bars indicate cleavage after aromatic and thin bars cleavage after leucine residues. Identified peptides are displayed as black horizontal bars. The N-terminal end of the first peptide (35–45) does not correspond to a chymotryptic product and hence derives from signal peptide cleavage at G34|N35. The epitope WSFADGN recognized by a monoclonal antibody (Wissink et al., 2003) and the epitope encompassing residues 38–54 recognized by pig sera (Plagemann, 2004a; Plagemann, 2004b) are underlined with a continuous and dotted line, respectively.
(C) Result of nanoLC-ESI–MS/MS analysis of deglycosylated Intervet 10 GP5. Peptides identified after elastase digestion, an enzyme without pronounced substrate specificity, are displayed as black horizontal bars. The sequence of the ectodomain of this GP5 is identical to LV-GP5 except an exchange of serine 37 to aspartic acid (marked in bold), which disrupts the potential glycosylation site at residues 35. Alignment of all identified peptides with the sequence of the plaque-purified variant (PPV) of I10 revealed that from the four amino acid differences between GP5 of PPV and LV, the two residues in the transmembrane region (positions 97 and 103) of PPV were present in our sample. However, one of the identified peptides did not match the sequence of PPV (Wissink et al., 2003); position 158 in the cytoplasmic tail was not arginine, but lysine as in LV-GP5. Note also that the signal peptide contains a cysteine at position 24 (and not the critical proline (Wissink et al., 2003)) as revealed by sequencing of the mRNA of ORF5.

signal peptides where a proline at position −6 is required for efficient cleavage. We assume, as suggested for prokaryotic signal peptides (Shen et al., 1991) that the proline enables the α-helical H-region of the signal peptide to adopt a more extended conformation. Since the crystal structure of the translocon containing a nascent polypeptide chain revealed that the signal peptide cleavage site is located entirely inside the channel (Li et al., 2016) the ability to adopt a more extended conformation might be a prerequisite to expose it to the lumenal signal peptidase.

For GP5 proteins of several PRRSV-2 strains we did not observe such inefficient signal peptide cleavage in transfected cells, although they contain a (fairly conserved) cysteine at position 24 and a (only partly used) glycosylation site downstream of the signal peptide cleavage site (Thaa et al., 2013). However, GP5 of PRRSV-2 strains contain a conserved proline at position 22 that might facilitate efficient signal peptide cleavage if the assumed mechanism to enable cleavage is correct. Nevertheless, considering the large amino acid variability between PRRSV strains and the unknown mechanism for inefficient signal peptide cleavage we do not want to exclude that GP5 of certain PRRSV-2 strains are also incompletely processed inside cells.

The insertion of proline at position 24 also caused complete glycosylation at residue N35 (Fig. 2), all other GP5 variants having a glycosylation sequon at N35 were never glycosylated at this site. Thus, features within the signal peptide (and thus at a distance of nine amino acids from a putative glycosylation site) determine by an unknown mechanism whether the sequon N35 is used. Attachment of glycans is supposed to affect the antigenicity of Gp5 by glycan shielding (Faaberg et al., 2006; Vu et al., 2011; Wei et al., 2012b). Glycan shielding of epitopes might occur not only by mutations within the hypervariable region that create additional glycosylation sites (Chang et al., 2002; Kwon et al., 2008), but also by mutations within the signal peptide that cause attachment of carbohydrates to an already existent sequon. This might be relevant since PRRS viruses that escaped antibody neutralization in pigs acquired several amino acid exchanges in the middle of the signal peptide; a putative carbohydrate attachment site was already present (Chen et al., 2016).

The most relevant data for signal peptide cleavage is our observation that co-expression of GP5 with its dimerisation partner, the M protein, rescued signal peptide cleavage defects of GP5 (Fig. 5). Complete cleavage of the signal peptide required the formation of a disulphide bond between cysteine 50 in GP5 with cysteine 9 in M, which is the only cysteine in the small ectodomain of M (9 residues). To our knowledge, this is the first demonstration that a protein of a hetero-oligomeric complex facilitates signal peptide cleavage of its binding partner. Mechanistically, binding to M might pull the signal peptide cleavage site of GP5 out of the translocon such that it becomes accessible to the signal peptidase.

From the perspective of the virus, the interaction of GP5 with M may be a “rescue mechanism” to allow complete processing of suboptimal variants of GP5. Most (~95%) PRRSV-1 strains have the “suboptimal” cysteine at position 24 in GP5 (Fig. 3A). An exchange with a more suitable amino acid, for example a proline, might not be possible because of sequence requirements for the essential ORF5a protein (Firth et al., 2011; Johnson et al., 2011; Robinson et al., 2013), which is translated from an alternative reading frame that overlaps with the GP5-encoding gene. In addition, the hypervariable region can rapidly acquire potential glycosylation sites during virus evolution (Chang et al., 2002; Kwon et al., 2008). GP5/M dimer formation could help certain GP5 variants to cleave their signal peptide and thus ensures that only properly processed GP5/M complexes (where the signal peptide of GP5 is cleaved) are incorporated into virus particles. Our observation adds another facet to the pivotal role of GP5/M heterodimerization for the biology of PRRSV and *Arteriviridae* in general. It does not only facilitate transport of these proteins from the ER to the

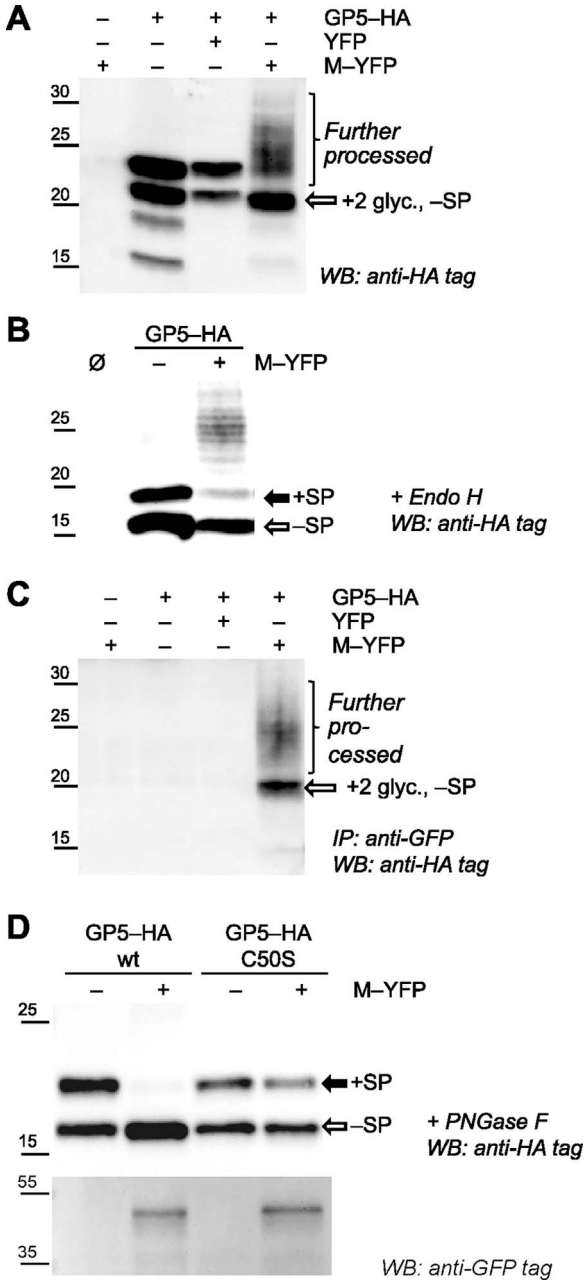

Fig. 5. Rescue function of the M protein regarding processing defects in GP5.
(A) The indicated combinations of GP5-HA (from the variant of PRRSV Lelystad with a glycosylation sequon at position N35) and M–YFP (or YFP alone) were expressed in CHO-K1 cells, followed by SDS-PAGE and Western blot (anti-HA tag). The white arrow points to GP5 containing two carbohydrates and a cleaved signal peptide (+2 gly, −SP). The minor protein bands in the samples without PNGase F digestion probably represent unglycosylated GP5 with (upper band) and without (lower band) signal peptide (cf Fig. 2A).
(B) Endo H digestion: cell lysates of mock-transfected CHO-K1 cells (Ø), cells transfected only with GP5–HA or co-transfected with GP5–HA and M–YFP were digested with Endo H prior to SDS-PAGE and Western blot (anti-HA tag).
(C) The indicated combinations of GP5-HA and M–YFP (or YFP alone) were expressed in CHO-K1 cells. M–YFP was immunoprecipitated with anti-GFP antibodies from cell lysates and co-precipitated GP5–HA was detected by Western blot (anti-HA tag).
(D) CHO-K1 cells were transfected with GP5–HA (either wt or the GP5 mutant C50S which cannot form a disulfide-bond with M) in the absence or presence of M–YFP, subsequently lysed and subjected to digestion with PNGase F, SDS-PAGE and Western blot with anti-HA tag antibodies (upper part) or anti-GFP tag antibodies (lower part) to identify GP5-HA and M-YFP, respectively.

viral budding site (de Vries et al., 1995), but is also required for virus budding and viral infectivity (Faaberg et al., 1995; Wieringa et al., 2004; Wissink et al., 2005) and for the formation of (conformation dependent) neutralizing antibody epitopes (Balasuriya and MacLachlan, 2004; Dea et al., 2000; Lopez and Osorio, 2004).

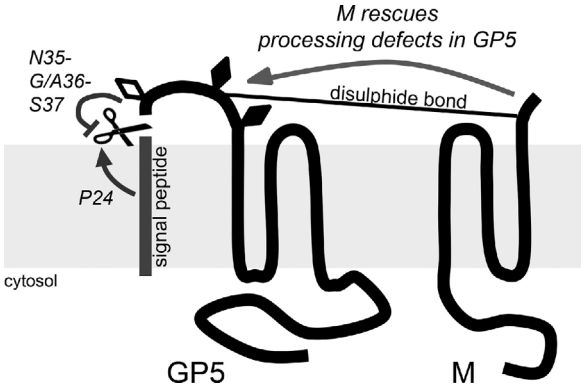

Fig. 6. Schematic representation of GP5 processing.
An unused glycosylation site located at the N-terminus of mature GP5 ($N^{35}G/A^{36}S^{37}$) plus a cysteine at position 24 cause inefficient cleavage of the signal peptide. Either mutation of the glycosylation site or exchange of cysteine 24 by proline restores complete signal peptide cleavage. Insertion of proline also causes attachment of carbohydrate to N35 (white lozenge). Oligomerization and disulphide formation of GP5 with M also rescues the processing defect.

In conclusion, we report that GP5 present in PRRSV-1 virions is cleaved between glycine-34 and asparagine-35. In transfected cells signal peptide cleavage is inefficient if GP5 contains an unused glycosylation site at Asn35 located at the N-terminus of the mature protein plus a cysteine residue at position 24 within the signal peptide. Exchange of either Cys24 with proline or mutation of the glycosylation sequon allowed complete signal peptide cleavage. The processing defect was rescued by oligomerisation of GP5 with its binding partner, the M protein (see Fig. 6 for a schematic display). Thus, a complex interplay of the co-translational modifications N-glycosylation, signal peptide cleavage and oligomerisation with the M-protein determines the amino acids present at the N-terminus of mature Gp5 and also the usage of N-glycosylation sites. This unpredictable interplay might affect removal of antibody epitopes or their shielding by glycans and hence might influence the antigenicity of GP5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2017.08.004.

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