The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain

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A set of neutralizing monoclonal antibodies (mAbs) directed against the GP5 protein of European type porcine reproductive and respiratory syndrome virus (PRRSV) has been produced previously (Weiland et al., 1999). This set reacted with a plaque-purified virus (PPV) subpopulation of Dutch isolate Intervet-10 (I-10), but not with the European prototype PRRSV LV. In order to map the neutralization epitope in the GP5 protein of the PPV strain, the ORF5 nucleotide sequence of PPV was determined. When the amino acid sequence derived from this nucleotide sequence was compared with that of PRRSV LV, four amino acid differences were found. Using site-directed mutagenesis, we showed that a proline residue at position 24 of the GP5 sequence of the PPV strain enabled recognition by the neutralizing mAbs. Pepscan analysis demonstrated that the epitope recognized by the neutralizing mAbs stretched from residues 29 to 35. Surprisingly, the reactivity of the mAbs in the Pepscan system was independent of the presence of a proline in position 24. Moreover, residue 24 is located within the predicted signal peptide, implying that either the signal peptide is not cleaved or is cleaved due to the presence of Pro24 such that the epitope remains intact. Our results demonstrate the presence of a neutralization epitope in the N-terminal ectodomain of the GP5 protein of PRRSV and imply a role for the ectodomain of GP5 in the infection of PRRSV.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus that belongs to the Arteriviridae family, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian haemorrhagic fever virus (SHFV) (Meulenberg et al., 1993). The Arteriviridae family belongs to the order Nidovirales, together with the Coronaviridae (Cavanagh, 1997). PRRSV causes respiratory problems and anorexia in pigs and abortion in sows. The virus has a restricted tropism for cells of the monocyte/macrophage lineage, presumably by a cell-specific receptor that has not yet been identified. The virus has a plus-strand polyadenylated RNA genome of about 15 kb, which contains at least nine partially overlapping open reading frames (ORFs). ORF1a and ORF1ab constitute about 80% of the genome and encode the virus replication machinery including the RNA-dependent RNA polymerase. ORFs 2–7 are located in the 3’ terminal region of the genome and encode structural proteins (Meulenberg et al., 1995; Meulenberg & Petersen-den Besten, 1996). ORF7 encodes the nucleocapsid protein N, ORF6 the non-glycosylated membrane protein M, ORF5 the major envelope glycoprotein GP5 and ORFs 2–4 the minor envelope glycoproteins GP2, GP3 and GP4, respectively (Meulenberg et al., 1995). Within ORF2, a second ORF is present that expresses a non-glycosylated protein (Wu et al., 2001), as has also been reported for EAV (Snijder et al., 1999).

PRRSV neutralization is correlated with antibodies directed against the GP5 protein, both in vivo (Gonin et al., 1999; Kwang et al., 1999; Pirzadeh & Dea, 1998; Yoon et al., 1995) and in vitro (Pirzadeh & Dea, 1997; Weiland et al., 1999; Yang et al., 2000; Zhang et al., 1998). Monoclonal antibodies (mAbs) against the GP4 protein have also been found to be
neutralizing (Meulenberg et al., 1997), although mAbs against the GP5 protein appeared to be much more effective (Weiland et al., 1999).

The GP5 protein is a glycoprotein of approximately 200 amino acids with an apparent molecular mass of 25 kDa. The GP5 protein is the most variable protein among PRRSV isolates, with only 51–55% amino acid sequence identity between European and North American isolates (Indik et al., 2000; Kapur et al., 1996; Mardassi et al., 1995), with the largest differences observed in the N terminus. Despite these differences, the hydropathy profiles of the GP5 proteins of the American and European isolates are very similar. For North American strains, it has been demonstrated that the GP5 protein is present as part of a disulphide-linked heterodimer with the M protein in the virion (Mardassi et al., 1996). The N terminus of the GP5 protein contains a predicted signal peptide of about 32 amino acids according to the prediction of von Heijne (1986), which is followed by a hydrophilic stretch of about 40 amino acids. This domain contains two or three potential N-linked glycosylation sites at residues 37, 46 and 53 (Indik et al., 2000; Meulenberg et al., 1995; Stadejek et al., 2002) and a highly conserved core sequence (aa 38–55) potentially involved in heterodimer formation with the M protein (Verheije et al., 2002). This N-terminal domain is presumed to be exposed on the outside of the virion and is therefore designated the ectodomain. The ectodomain is followed by a long hydrophobic region of about 60 amino acids that is presumed to span the membrane either one or three times. Whether the N-terminal ectodomain constitutes the only exposed part of the protein or whether a second ectodomain – positioned more C-terminally – exists is presently unclear (Stadejek et al., 2002). The last 70 C-terminal amino acids are thought to form the endodomain (Meulenberg et al., 1995). The GP5 protein, possibly as a heterodimeric complex with the M protein, is presumed to play a role in attachment to host-cell receptors (Dea et al., 2000; Delputte et al., 2002; Snijder & Meulenberg, 1998) and in virus assembly (Verheije et al., 2003).

PRRSV GP3 is the homologue of the G1 protein of EAV and the VP-3P protein of LDV. For EAV and LDV, neutralizing epitopes have been mapped to the ectodomain of their respective GP3 homologues (Balasuriya et al., 1995; Chirnside et al., 1995; Glaser et al., 1995; Li et al., 1998). Furthermore, disruption of the disulphide bonds between the GP3 and the M protein of LDV resulted in loss of viral infectivity, suggesting that the heterodimers are involved in receptor binding (Faaberg et al., 1995). In addition, the ectodomain of the LDV GP3 protein has been reported to be involved in LDV persistence and pathogenicity (Chen et al., 2000). On the other hand, the importance of GP5 in receptor binding, at least for EAV, has been questioned (Dobbe et al., 2001).

The aim of this study was to determine the location of a neutralizing epitope in the GP5 protein of European type PRRSV. Neutralizing mAbs described by Weiland et al. (1999) were found to be specific for a plaque-purified virus (PPV) subpopulation of the Dutch PRRSV isolate I-10. Hence the nucleotide sequence of the ORF5 gene of PPV was determined and compared with that of PRRSV LV, which does not react with these mAbs. Site-directed mutagenesis was used to identify the amino acid residues essential for recognition of the native protein by the neutralizing mAbs. Pepsan analysis further defined the boundaries of the epitope bound by these mAbs.

**METHODS**

**Cells and viruses.** Baby hamster kidney (BHK-21) cells were grown in BHK-21 medium (Gibco BRL) with 5% foetal bovine serum (FBS), 10% tryptose phosphate broth (Gibco BRL), 20 mM HEPES pH 7.4 (Gibco BRL), 200 mM glutamine, 100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹. Porcine alveolar lung macrophages (PAMs) were maintained in MCA-RPMI 1640 medium (Gibco BRL) containing 10% FBS, 50 U penicillin ml⁻¹ and 50 U streptomycin ml⁻¹. The PPV and EPV (gained by end-point dilution) subpopulations of the Dutch isolate I-10 and the escape mutant viruses have been described previously (Weiland et al., 1999).

**Monoclonal antibodies and antisera.** Three mAbs, P10/a46, P10/b38 and P4/a2-19, directed against the GP5 protein of the PPV strain, belong to a set of 15 mAbs that all react against the same antigenic region on the GP5 protein (Weiland et al., 1999). Monoclonal antibody 3AH9 was raised against aa 170–201 of European type PRRSV (Rodriguez et al., 2001). Peptide serum p703 was raised against an LV-specific peptide consisting of aa 145–161 of the GP3 protein (Meulenberg et al., 1995).

**Construction of mutant ORF5 genes in the pCIneo mammalian expression vector.** Plasmid p5a6 containing the ORF5 gene of the PRRSV PPV strain (Conzelmann et al., 1993) was generously provided by K. Conzelmann and has been described previously (Weiland et al., 1999). The ORF5 sequences of pABV437, the PAd mutant of the genome-length cDNA clone of LV (Meulenberg et al., 1998), and of plasmid p5a6 were amplified using oligonucleotides LV275 and LV282, located upstream and downstream of ORF5, respectively (Table 1). The nucleotide sequence directly upstream of the start codon of ORF5 was modified to a consensus Kozak sequence (Kozak, 1987). In addition, the restriction sites Xbal and NotI were added upstream and downstream of ORF5, respectively. The PCR fragments were digested with Xbal and NotI and ligated into the corresponding sites of the pCIneo mammalian expression vector (Promega). This resulted in plasmids pABV786 and pABV789 containing the ORF5 genes of LV and p5a6, respectively.

Subsequently, three different codons resulting in single amino acid substitutions were introduced into the LV ORF5 sequence by site-directed mutagenesis using a fusion PCR (Dekker et al., 2000). The sequences of the primers that were used are shown in Table 1. Individual parts were amplified with the forward or the reverse primer containing the desired mutation. The two mutated PCR products were hybridized and amplified with two primers outside the mutation. The mutated fragments were then digested with Xbal and XhoI and were reintroduced into the pCIneo vector, resulting in plasmids pABV803, pABV804 and pABV805, respectively.

The fourth difference between both ORF5 genes, codon 158 (Lys→Arg), was introduced in pABV786 by exchange of a XcmI–BglII fragment between plasmids pABV786 and pABV789. This resulted in construct pABV806.
Table 1. Primers used for site directed mutagenesis and to clone the ORF5 fragments in the pCIneo mammalian expression vector

<table>
<thead>
<tr>
<th>Designation primer*</th>
<th>Nucleotide position†</th>
<th>Sequence primer‡</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV46 (+)</td>
<td>13160</td>
<td>GCCGTCGGTACCCCTGCACTGACAT</td>
<td>Fusion PCR full-length construct</td>
</tr>
<tr>
<td>LV107 (−)</td>
<td>14508</td>
<td>CCGAAGCGCGGGTACTAGTG</td>
<td>Fusion PCR full-length construct</td>
</tr>
<tr>
<td>LV275 (++)</td>
<td>13493</td>
<td>GCTCTAGAGCGCGCCACATTGGCAATATGAGATGTCCTGACCTGCTGCCAATGCACCATGAGATG</td>
<td>Incorporation of XhoI + consensus Kozak sequence</td>
</tr>
<tr>
<td>LV282 (−)</td>
<td>14079</td>
<td>ATAGTTTATCGGGCGCGCCTAGGGCTCCATTGGCTACGGC</td>
<td>Incorporation NotI</td>
</tr>
<tr>
<td>LV285 (+)</td>
<td>13549</td>
<td>GCCGTTTTTTGTCGCAGTTACCGCGCCCTGTCGCC</td>
<td>Cys24→Pro24</td>
</tr>
<tr>
<td>LV286 (−)</td>
<td>13549</td>
<td>GGAGAAGCGGTTAGCATCGACAAAAAACGCC</td>
<td>Cys24→Pro24</td>
</tr>
<tr>
<td>LV287 (+)</td>
<td>13772</td>
<td>CGGTTCTAGGGCTGATCCCACTGGCGAGG</td>
<td>Ala97→Val97</td>
</tr>
<tr>
<td>LV288 (−)</td>
<td>13772</td>
<td>CCGTCAGGGATACAGCGGCGAAGCCG</td>
<td>Ala97→Val97</td>
</tr>
<tr>
<td>LV289 (+)</td>
<td>13789</td>
<td>CCCTAGGCGAGAACTTGTTGCGGG</td>
<td>Phe103→Leu103</td>
</tr>
<tr>
<td>LV290 (−)</td>
<td>13789</td>
<td>TCGGGAACACTAGCTAGCTAGCTAGCCTGCC</td>
<td>Phe103→Leu103</td>
</tr>
<tr>
<td>LV291 (+)</td>
<td>13490</td>
<td>CCGTCAGGGGCTGACGAAATCCTGCC</td>
<td>Incorporation of XhoI</td>
</tr>
<tr>
<td>LV292 (+)</td>
<td>13486</td>
<td>GCAGAACCATAGTGGCAATATGAGTG</td>
<td>Fusion PCR pCIneo constructs</td>
</tr>
<tr>
<td>LV293 (−)</td>
<td>14091</td>
<td>CTAGCTAGCTAGCTAGCTAGCTAGCCTGCC</td>
<td>Incorporation of XhoI</td>
</tr>
</tbody>
</table>

*Positive-sense primers and negative-sense primers are marked by (+) and (−), respectively.
†The position of each primer with respect to the nucleotide sequence of PRRSV LV (GenBank accession no. M96262).
‡The restriction sites are indicated by single underlining and the consensus Kozak sequence is indicated by double underlining. The underlined nucleotides in lower case are mutated compared with the original genome sequence of PRRSV LV.

Construction of a full-length neutralization-sensitive PRRSV cDNA clone. In order to introduce a proline at aa 24 into pABV437, a fusion PCR was performed with primers LV46 and LV286 and with primers LV285 and LV107, respectively. The mutated fragments were then digested with BstXI and NheI and ligated into the similarly digested plasmid pABV651, a cDNA clone encompassing the structural genes of PRRSV LV. From the resulting clones, the AatI–HpaI fragment was excised and reintroduced into pABV437, resulting in full-length constructs pABV911. Plasmid constructs were amplified and purified using the Qiagen plasmid mini kit. Recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989).

Sequence analysis. Fragments generated by PCR were analysed by nucleotide sequencing. Sequences were determined with the PRISM Ready Dye Deoxy Terminator cycle sequencing kit and the ABI PRISM 310 Genetic Analyser (Perkin Elmer).

DNA transfection. BHK-21 cells seeded in 24-well plates were transfected with plasmids using Lipofectamine (Gibco BRL). Transfection mix was removed after 4 h and replaced with complete BHK-21 medium, and cells were incubated for another 20 h at 37 °C in a CO₂ incubator.

Production of infectious virus from full-length genomic cDNA clones. To obtain infectious virus, the full-length genomic cDNA clones pABV437 and pABV911 were transfected in vitro. The RNAs were then transfected into BHK-21 cells using Lipofectin (Gibco BRL). The culture supernatant of BHK-21 cells was harvested 24 h after transfection and the supernatant was subsequently used to inoculate PAMs. After 1 h, the inoculum was removed and fresh culture medium was added. At 24 h post-infection (p.i.), the supernatant was harvested and virus titres (expressed as TCID₅₀ ml⁻¹) were determined on PAMs.

Virus neutralization assay. The sensitivity of viruses vABV437 and vABV911 to neutralization was determined in an immunoperoxidase monolayer assay (IPMA) 24 h p.i. PAMs were plated into 96-well microtitre plates and incubated overnight at 37 °C. To determine the neutralization index, twofold serial dilutions of culture supernatant of mAB P10/a46 were mixed with 100 TCID₅₀ of the viruses, incubated at 37 °C for 1 h and subsequently transferred to the PAMs. At 24 h p.i., cells were washed with PBS, dried and stored at −20 °C until an IPMA was performed. Monoclonal antibody 122.17 was used to detect the expression of the PRRSV N protein.

Immunoperoxidase monolayer assay. Cells were fixed with cold 96 % methanol for 25 min and the GP₃ protein expressed by the various ORF5 constructs was detected with mAB P10/a46, P10/b38 or P4/a2-19 in an IPMA as described by Wensvoort et al. (1986).

Radioimmunoprecipitation. Peptide serum 703 (p703) and mAB P10/a46 were used to precipitate Tran²⁵S-labelled GP₃ proteins from lysates of BHK-21 cells transfected with the different ORF5 constructs. BHK-21 cells transfected with the pCIneo vector were used as negative control. Cellular proteins were labelled with Tran²⁵S label in MEM-E without methionine and cysteine (Gibco BRL), supplemented with 1 % l-glutamine, 100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹ for 4 h after starving with the same medium without label for 30 min. Cells were lysed in PBS containing 1 g SDS l⁻¹, 10 ml Triton X-100 l⁻¹ and 5 g sodium deoxycholate l⁻¹ (PBS-TDS) for 10 min on ice. Cell lysates were mixed with either an equal volume of hybridoma supernatant or 15 µl of p703 and incubated overnight at 4 °C. Subsequently, protein A–Sepharose (Amersham Pharmacia Biotech) was added and lysates were incubated at 4 °C for another 2 h. Immunoprecipitates were then washed with PBS-TDS, resuspended in loading buffer and samples analysed by 14 % SDS-PAGE. Gels were dried and immunoprecipitated proteins were visualized by autoradiography.

Pepsan analysis. Pepscan analysis was performed using overlapping 12-mer peptides as described in Slootstra et al. (1997). Optical densities (OD) were determined with a cc-d detector. The values are logarithmic values in a range of 0 to 4000.
RESULTS

A proline residue at position 24 of the GP5 protein is essential for binding of neutralizing mAbs

A set of neutralizing mAbs directed against the GP5 protein of PRRSV has been produced previously (Weiland et al., 1999). This set reacted with a PPV subpopulation of Dutch isolate I-10, but not with the EPV subpopulation of I-10, gained by end-point dilution (Weiland et al., 1999), or with the prototype European PRRSV LV. Plasmid p5a6, containing the ORF5 fragment of the PPV strain, has been generated previously (Weiland et al., 1999). The nucleotide sequence of the ORF5 gene in plasmid p5a6 was determined and compared with that of PRRSV LV, since this sequence was already available. Nine nucleotide differences were identified, which resulted in four amino acid differences in the GP5 protein, i.e. aa 24 (Cys→Pro), 97 (Ala→Val), 103 (Phe→Leu) and 158 (Lys→Arg) (Fig. 1A).

Subsequently, coding sequences for these four amino acids were individually introduced into the ORF5 gene of PRRSV LV in the pCIneo mammalian expression vector (pABV786), resulting in pABV803, pABV804, pABV805 and pABV806, respectively. In addition, the PPV ORF5 gene was introduced into the pCIneo vector (pABV789).

The generated ORF5 constructs were transfected into BHK-21 cells. At 24 h post-transfection, the expressed GP5 proteins were analysed by immunostaining with the three mAbs P4/a2-19, P10/a46 and P10/b38. Monoclonal antibody 3AH9, directed against aa 170–201 of European type PRRSV (Rodriguez et al., 2001), was used as a positive control. As expected, the GP5 protein expressed from pABV789, containing the ORF5 gene of the PPV strain, was recognized by the neutralizing mAbs, whereas the GP5 protein expressed from pABV786, containing the ORF5 gene of the LV strain, was not detected (Fig. 2). The GP5 protein expressed from pABV803, containing the mutation encoding a proline at position 24 of GP5, was recognized by the mAbs, whereas the other three mutant GP5 proteins, expressed from pABV804, -805 and -806, were not detected. 3AH9 recognized all mutant GP5 proteins, confirming that absence of immunostaining with the neutralizing mAbs was not due to low expression levels and/or aberrant processing of the other mutant GP5 proteins (data not shown). Thus, aa 24 is essential for recognition by the three neutralizing mAbs, due to either the absence of the cysteine or the presence of the proline.

Analysis of mutant GP5 proteins by radioimmunoprecipitation

In order to examine expression and processing of the mutant GP5 proteins, immunoprecipitations were performed for radiolabelled GP5 proteins. BHK-21 cells were transfected with the ORF5 plasmids and labelled with [35S]methionine and [35S]cysteine. GP5 proteins were immunoprecipitated

(A)

MRCSHKLGFLTPHSCFWLPLLCTGLSWSPADDNGDSST 40
YQIYINLTCBLENQDNLSSHFGLWAVETFVLYVPVATHILS 80
LGFLTTSKFFDALGLQAVSTAGFVGGRYVLCSVYGACAFA 120
AFVCFPIRAAKNCMACKYARTRPTNIVDPRGRVRHWRKSPR 160
IVYKLGKAEVDGLNVTIHKVVLEGVKAQP LTRTSAESQEWE A 201

putative cleavage site

Cysteine residues are indicated by whereas residue 24 is indicated by

signal peptide

N-terminus

ectodomain neutralization epitope

conserved region

hydrophobic region

putative endodomain

(B) Diagram of the GP5 protein.

Fig. 1. (A) Amino acid sequences of GP5 of PRRSV. The four amino acid residues in the GP5 sequence of PPV that differ from the sequence of the LV strain are indicated below the LV sequence. Putative signal peptide cleavage sites are depicted by arrows (↑). (B) Diagram of the GP5 protein.
with either mAb P10/a46 or with p703, an anti-peptide serum directed against aa 145–161 of the LV GP5 protein. Subsequently, the precipitates were analysed by 14 % SDS-PAGE. P10/a46 precipitated the GP5 protein expressed from plasmids pABV789 and pABV803 (Fig. 3), whereas p703 precipitated the GP5 proteins expressed from all ORF5 plasmids. This was in agreement with the immunostaining results. The apparent molecular mass of all precipitated GP5

Fig. 2. Expression of mutant GP5 proteins in BHK-21 cells transfected with plasmids pABV786 (LV), pABV789 (PPV), pABV803 (Pro26), pABV804 (Val87), pABV805 (Leu103) and pABV806 (Arg158). An immunoperoxidase monolayer assay was performed with PPV-specific mAb P10/a46 directed against GP5; mAbs P10/b38 and P4/a2-19 gave a similar staining pattern (data not shown).

Fig. 3. Radioimmunoprecipitation analysis of expressed mutant GP5 proteins with anti-GP5 mAb P10/a46 and peptide serum p703. Proteins were immunoprecipitated from lysates of BHK-21 cells transfected with pABV786 (LV), pABV789 (PPV), pABV803 (Pro26), pABV804 (Val87), pABV805 (Leu103) and pABV806 (Arg158). BHK-21 cells transfected with the pCIneo vector were used as negative control. The immunoprecipitated GP5 proteins were analysed by 14 % SDS-PAGE. The GP5 protein is indicated by an arrow. Protein size markers (kDa) are shown on the left.
proteins seemed to be comparable, indicating that expression and processing of the GP5 proteins expressed from pABV789 and -803 were not markedly different from that of the other GP5 proteins. In addition, p703 precipitated proteins with an apparent molecular mass of approximately 14 kDa from the lysates of BHK-21 cells transfected with pABV786, -803 and -806. These proteins were most likely non-specifically coprecipitated.

**Pepscan analysis of the PRRSV GP5 proteins**

In order to specify further the boundaries of the neutralization epitope of the GP5 protein, a set of overlapping 12-mer peptides covering the complete GP5 amino acid sequence of the PPV strain was tested for reactivity with anti-GP5 mAbs P10/a46, P10/b38 and P4/a2-19 (Slootstra et al., 1997). All three mAbs reacted specifically with peptides that had the sequence WSFADGN (residues 29–35; Fig. 4A, B, underlined), while the core sequence consisted of residues 30–32 (SFA; Fig. 4A, B, bold). In addition, a small set of twelve 12-mer peptides containing a cysteine at position 24 was tested for reactivity. Surprisingly, these peptides gave comparable OD values in the Pepscan analysis, i.e. the reactivity of the mAbs in the Pepscan system was independent of the presence of a proline at position 24.

**A proline residue at position 24 of GP5 results in sensitivity to neutralization in vitro**

To investigate whether the presence of a proline at position 24 indeed results in sensitivity to neutralization in vitro, a mutant full-length PRRSV LV cDNA clone was generated in which the codon for the cysteine at position 24 was changed into a codon for proline. The mutant construct pABV911 as well as the parental LV clone pABV437 were used to transcribe viral RNAs in vitro, which were subsequently transfected into BHK-21 cells. Porcine alveolar macrophages (PAMs) were then infected with the virus present in the culture supernatants of the transfected cells, the supernatants of the macrophages were harvested 24 h p.i. and the virus titres of the resulting viruses vABV437 and vABV911 were determined by end-point titration. Subsequently, a neutralization assay was performed in which 100 TCID₅₀ of the two viruses were mixed with serial dilutions of hybridoma culture supernatant of neutralizing mAb P10/a46. The results showed that P10/a46 was able to neutralize vABV911 completely up to a dilution of 1:1280, whereas the infection of parental LV clone was not inhibited.

In conclusion, a proline residue at position 24 enables recognition of a neutralization epitope that is located in the N-terminal ectodomain.

**DISCUSSION**

In the present study, a neutralization epitope was identified in the GP5 protein of one of the European strains of PRRSV. Using site-directed mutagenesis, we identified the residue at position 24 as being essential for recognition by the neutralizing mAbs. The epitope recognized in Pepscan analysis stretched from residues 29 to 35. Furthermore, the three neutralizing mAbs, P10/a46, P10/b38 and P4/a2-19, each recognized the same epitope, as was previously suggested by the results of Weiland et al. (1999).

Remarkably, the reactivity of the three neutralizing mAbs in the Pepscan system was independent of the presence of a proline at position 24.
of a proline at position 24, since the set of 12-mers containing a Cys$^{24}$ and the 12-mers containing a Pro$^{24}$ gave comparable OD values in the Pepscan analysis. Moreover, residue 24 is located within the signal peptide predicted by the SignalP computer algorithm (Nielsen et al., 1997). Several authors have reported that signal peptide cleavage is predicted to occur between aa 32 and 33 (Meulenberg et al., 1995; Rodriguez et al., 2001), i.e. in the core of the epitope. In view of our results, however, it is more likely either that the signal peptide is not cleaved off and the amino acid residue at position 24 is itself part of the epitope in the native protein, or that signal peptide cleavage occurs more N-terminally.

Examples of non-cleaved predicted signal peptides exist (Gewurz et al., 2002). Assuming that the signal peptide of PRRSV GP$_5$ is not cleaved, both residue 24 and the part of the epitope recognized by the Pepscan analysis would be present in the GP$_5$ protein. A proline at position 24, a helix-breaking residue, might be responsible for exposing the epitope, whereas a cysteine at position 24 might hide the epitope, possibly by forming a disulphide bond with another cysteine residue in the GP$_5$ protein.

However, sequence data from one of the escape mutant viruses showed that the GP5 protein of this virus possessed a leucine at position 24, emphasizing the importance of the proline (E. Weiland, unpublished data). Therefore, a more likely explanation might be that the signal peptide might be cleaved but that Pro$^{24}$ affects the site of this signal peptide cleavage. Nothwehr & Gordon (1989) introduced proline residues at various positions in the signal peptide of human pre(Apro)apolipoprotein A-II and observed that the site of cleavage was affected by the location of a proline, i.e. there was a tendency to maintain a distance of four to five residues between the proline and the site of cleavage. Furthermore, they reported that two or more potential cleavage sites might compete for recognition by the signal peptidase, although one site is preferred. Using the SignalP computer algorithm for both the Cys$^{24}$ and the Pro$^{24}$ GP$_5$ proteins, probable cleavage sites are located between residues 28 and 29, 30 and 31, and 32 and 33 (Fig. 1B). In the Cys$^{24}$ protein, the preferred cleavage site is located between residues 32 and 33. In the Pro$^{24}$ protein, however, the proline promotes the signal peptidase to cleave between residues 28 and 29. This would result in a mature glycoprotein that was two to three amino acids larger and thus the presence of the epitope in the Pro$^{24}$ protein, in contrast to the Cys$^{24}$ protein. Obviously, N-terminal sequencing of both mature glycoproteins should give the definitive answer.

Our data indicated that the identified neutralization epitope is located at the N terminus of the GP$_5$ ectodomain, comprising residues 29–35 (Fig. 1B). The presence of both linear and conformation-dependent neutralization epitopes in the GP$_5$ protein of PRRSV has been described by others, although their locations in the protein have not been determined (reviewed by Dea et al., 2000). Recently, both a non-neutralization and a neutralization epitope located at the N terminus of the GP$_5$ of North American PRRSV strains were identified by Ostrowski et al. (2002), comprising residues 27–31 and residues 37–45, respectively. The neutralization epitope is located in an area that is conserved among PRRSV isolates (Fig. 1B) and between PRRSV and LDV. The VP-3P protein of LDV also contains a neutralization epitope in its ectodomain that is mapped between residues 37 and 60 (Li et al., 1998). Interestingly, this epitope comprises the residues that form the main recognition site in the epitope described by Ostrowski et al. (2002). The ectodomain of the EAV ORF5 protein, G$_1$, is much longer and shows far less homology with the LDV and PRRSV GP5 ectodomains. Nevertheless, it also contains three overlapping neutralization epitopes, just upstream of the first transmembrane segment (Balasuriya et al., 1995; Chirnside et al., 1995; Glaser et al., 1995). The presence of neutralizing epitopes in the GP$_5$ ectodomains of PRRSV, LDV and EAV leads to the suggestion that the GP$_5$ ectodomains play an important role in arterivirus infection. Another argument that pleads for such a role of the GP$_5$ ectodomain is the fact that the disulphide bonds between the LDV GP$_3$ and M protein are essential for LDV infectivity, suggesting that the heterodimeric GP$_5$–M complexes might be involved in receptor binding (Faabeerg et al., 1995). In addition, N-glycans associated with the ectodomain of the LDV GP$_5$ protein determine LDV neuropathogenicity and sensitivity to antibody neutralization (Chen et al., 2000).

However, the strongest argument against such a role for GP$_5$ is that the exchange of the putative ectodomain of the EAV equivalent of GP$_5$ with that of PRRSV GP$_5$ does not alter its tropism (Dobbe et al., 2001). Moreover, recent research in which the ectodomain of the M protein of PRRSV was replaced by that of other arteriviruses revealed that the tropism of the resulting chimeric viruses had remained unchanged (Verheije et al., 2002). These authors concluded that the heterodimeric GP$_5$–M complexes are essential for arterivirus assembly, but that they do not determine host-cell specificity.

To explain these contradictory results, the role of GP$_5$ in PRRSV infection of PAMs should be further investigated. Recently, Delputte et al. (2002) reported that PRRSV binds to glycosaminoglycans at the cell surface of PAMs and that this binding is probably mediated by the heterodimeric GP$_5$–M complexes. These complexes might thus initiate PRRSV infection of PAMs by attachment to a low-affinity receptor, followed by interaction of other PRRSV glycoprotein(s) with a high-affinity receptor that determines tropism. Our data suggest that neutralizing antibodies that recognize the ectodomain of GP$_5$ may interfere with this initial step of the PRRSV infection. It is unlikely that the neutralization epitope identified in this study plays an important role in PRRSV infection, because the sequence is variable (Stadejek et al., 2002) and it is easily lost from the population (Weiland et al., 1999). The neighbouring highly conserved region, however, containing the neutra-
lization epitope identified by Ostrowski et al. (2002) and implicated in heterodimerization with the M protein (Verheije et al., 2002), might play a role in PRRSV infection. Further studies will be necessary to clarify the role of GP₅ in PRRSV infection in more detail.

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