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# The major envelope protein, $GP_5$, 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 $GP_5$ 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 $GP_5$ 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 $GP_5$ 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 $Pro^{24}$ such that the epitope remains intact. Our results demonstrate the presence of a neutralization epitope in the N-terminal ectodomain of the $GP_5$ protein of PRRSV and imply a role for the ectodomain of $GP_5$ 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 $GP_5$ and ORFs 2–4 the minor envelope glycoproteins $GP_2$, $GP_3$ and $GP_4$, 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 $GP_5$ 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 $GP_4$ protein have also been found to be

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neutralizing (Meulenberg *et al.*, 1997), although mAbs against the $GP_5$ protein appeared to be much more effective (Weiland *et al.*, 1999).

The $GP_5$ protein is a glycoprotein of approximately 200 amino acids with an apparent molecular mass of 25 kDa. The $GP_5$ 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 $GP_5$ proteins of the American and European isolates are very similar. For North American strains, it has been demonstrated that the $GP_5$ 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 $GP_5$ 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 $GP_5$ 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 $GP_5$ is the homologue of the $G_L$ 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 $GP_5$ 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 $GP_5$ 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 $GP_5$ protein has been reported to be involved in LDV persistence and pathogenicity (Chen *et al.*, 2000). On the other hand, the importance of $GP_5$ 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 $GP_5$ 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. Pepscan 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 $GP_5$ protein of the PPV strain, belong to a set of 15 mAbs that all react against the same antigenic region on the $GP_5$ 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 $GP_5$ 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 *Pac*I 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 *Xba*I and *Not*I were added upstream and downstream of ORF5, respectively. The PCR fragments were digested with *Xba*I and *Not*I 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 *Xba*I and *Xho*I 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 *Xcm*I–*Blp*I 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

| Designation primer* | Nucleotide position† | Sequence primer‡ | Purpose |
|---|---|---|---|
| LV46 (+) | 13160 | GCCGTCGGTACCCCTCAGTACAT | Fusion PCR full-length construct |
| LV107 (−) | 14508 | CCGAAGCCCGGGTACTAGAGTG | Fusion PCR full-length construct |
| LV275 (+) | 13493 | GCTCTAGAGCCGCCACCATGAGATGTTCTCACAAATTGGG | Incorporation of *Xba*I + consensus Kozak sequence |
| LV282 (−) | 14079 | ATAGTTTAGCGGCCGCCTAGGCCTCCCATTGCTCAGC | Incorporation *Not*I |
| LV285 (+) | 13549 | GGCTTTTTTTGCTGccTACCGGCTTGTCC | $Cys^{24}$→$Pro^{24}$ |
| LV286 (−) | 13549 | GGACAAGCCGGTAggCAGCAAAAAAAGCC | $Cys^{24}$→$Pro^{24}$ |
| LV287 (+) | 13772 | CGGTCTCGGCGtTGTATCCACTGCAGG | $Ala^{97}$→$Val^{97}$ |
| LV288 (−) | 13772 | CCTGCAGTGGATACAaCGCCGAGACCG | $Ala^{97}$→$Val^{97}$ |
| LV289 (+) | 13789 | CCACTGCAGGAcTTGTTGGCGGG | $Phe^{103}$→$Leu^{103}$ |
| LV290 (−) | 13789 | CCCGCCAACAAgTCCTGCAGTGG | $Phe^{103}$→$Leu^{103}$ |
| LV291 (+) | 13490 | CCGCTCGAGCGGCTGCAGAACCAATGC | Incorporation of *Xho*I |
| LV292 (+) | 13486 | GCAGAACCAATGCATTGGCAATATGAGATG | Fusion PCR pCIneo constructs |
| LV293 (−) | 14091 | CTAGTCTAGACTAGCTAGGCCTCCC | Incorporation of *Xba*I |

*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 *Bst*XI and *Nhe*I and ligated into the similarly digested plasmid pABV651, a cDNA clone encompassing the structural genes of PRRSV LV. From the resulting clones, the *Aat*II–*Hpa*I fragment was excised and introduced into pABV437, resulting in full-length construct 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 $CO_2$ 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 transcribed *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 $TCID_{50}$ $ml^{-1}$) 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 $TCID_{50}$ 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 $GP_5$ 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 $Tran^{35}S$-labelled $GP_5$ 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 $Tran^{35}S$ 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 desoxycholate $l^{-1}$ (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.

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

## RESULTS

### A proline residue at position 24 of the $GP_5$ protein is essential for binding of neutralizing mAbs

A set of neutralizing mAbs directed against the $GP_5$ 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 $GP_5$ 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 $GP_5$ 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 $GP_5$ protein expressed from pABV789, containing the ORF5 gene of the PPV strain, was recognized by the neutralizing mAbs, whereas the $GP_5$ protein expressed from pABV786, containing the ORF5 gene of the LV strain, was not detected (Fig. 2). The $GP_5$ protein expressed from pABV803, containing the mutation encoding a proline at position 24 of $GP_5$, was recognized by the mAbs, whereas the other three mutant $GP_5$ proteins, expressed from pABV804, -805 and -806, were not detected. 3AH9 recognized all mutant $GP_5$ 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 $GP_5$ 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 $GP_5$ proteins by radioimmunoprecipitation

In order to examine expression and processing of the mutant $GP_5$ proteins, immunoprecipitations were performed for radiolabelled $GP_5$ proteins. BHK-21 cells were transfected with the ORF5 plasmids and labelled with [$^{35}$S]methionine and [$^{35}$S]cysteine. $GP_5$ proteins were immunoprecipitated

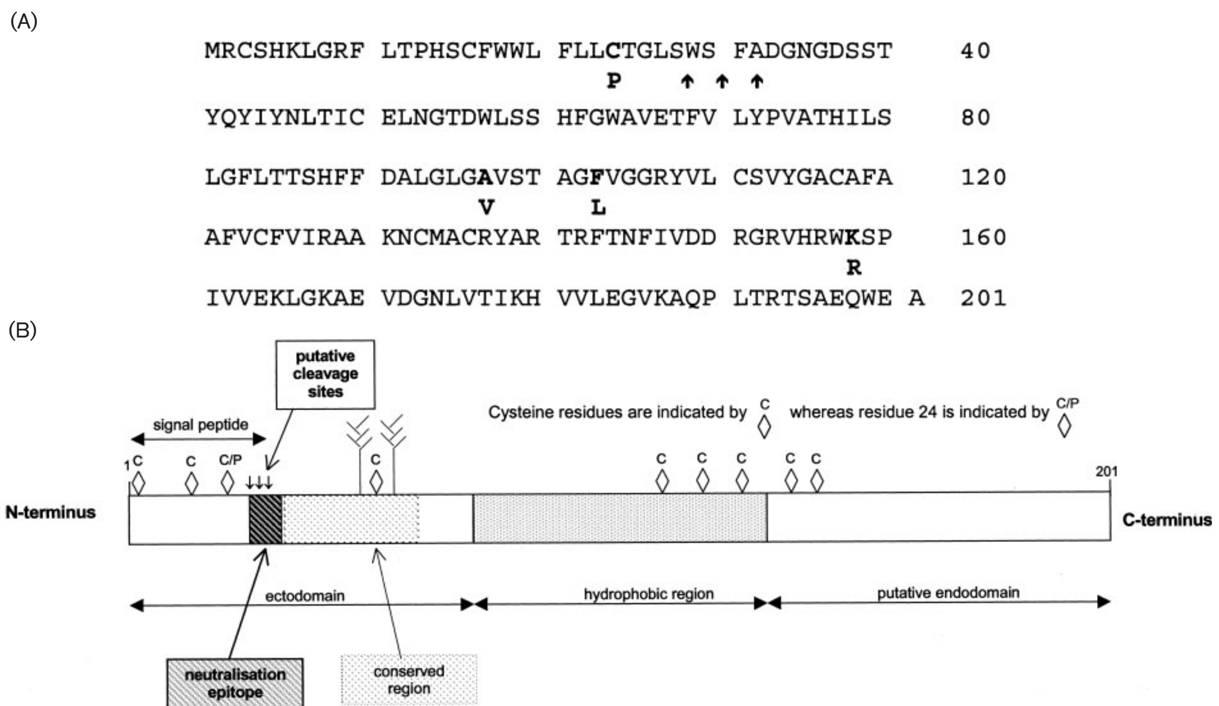


**Fig. 1.** (A) Amino acid sequences of $GP_5$ of PRRSV. The four amino acid residues in the $GP_5$ sequence of PPV that differ from the sequence of the LV strain are indicated below the LV sequence. Putative signal peptide cleavage sites are depicted by arrows (↑). (B) Diagram of the $GP_5$ protein.

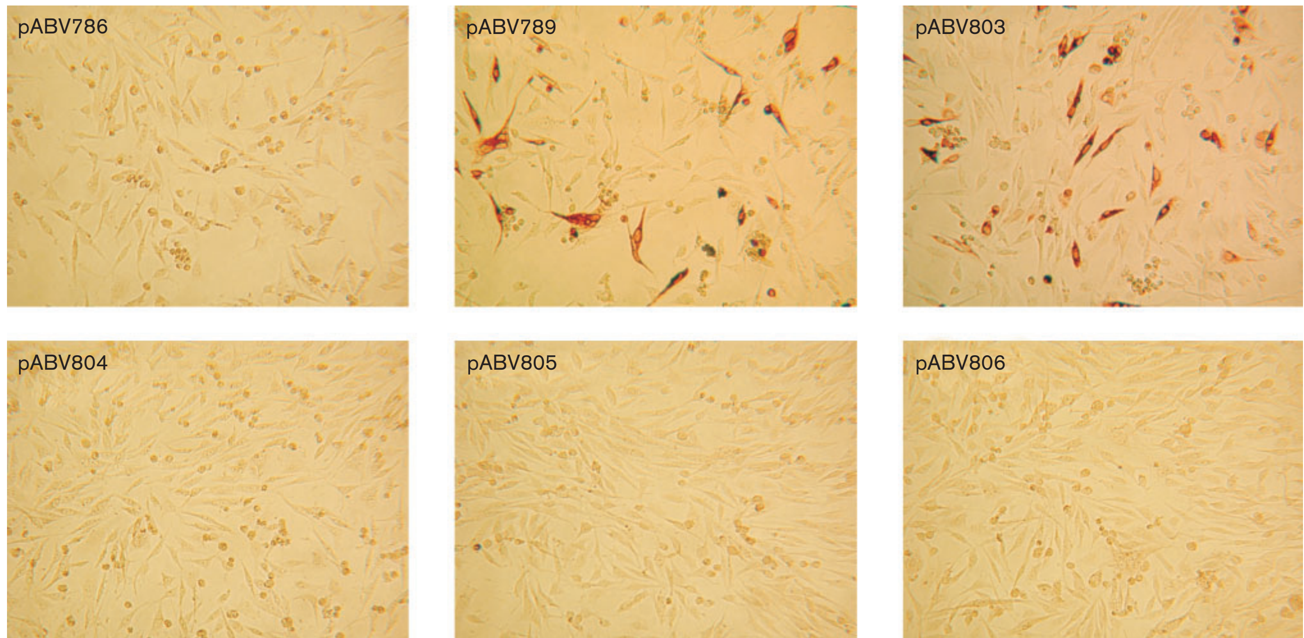


**Fig. 2.** Expression of mutant $GP_5$ proteins in BHK-21 cells transfected with plasmids pABV786 (LV), pABV789 (PPV), pABV803 ($Pro^{24}$), pABV804 ($Val^{97}$), pABV805 ($Leu^{103}$) and pABV806 ($Arg^{158}$). An immunoperoxidase monolayer assay was performed with PPV-specific mAb P10/a46 directed against $GP_5$; mAbs P10/b38 and P4/a2-19 gave a similar staining pattern (data not shown).

with either mAb P10/a46 or with p703, an anti-peptide serum directed against aa 145–161 of the LV $GP_5$ protein. Subsequently, the precipitates were analysed by 14 % SDS-PAGE. P10/a46 precipitated the $GP_5$ protein expressed from plasmids pABV789 and pABV803 (Fig. 3), whereas p703 precipitated the $GP_5$ proteins expressed from all ORF5 plasmids. This was in agreement with the immunostaining results. The apparent molecular mass of all precipitated $GP_5$

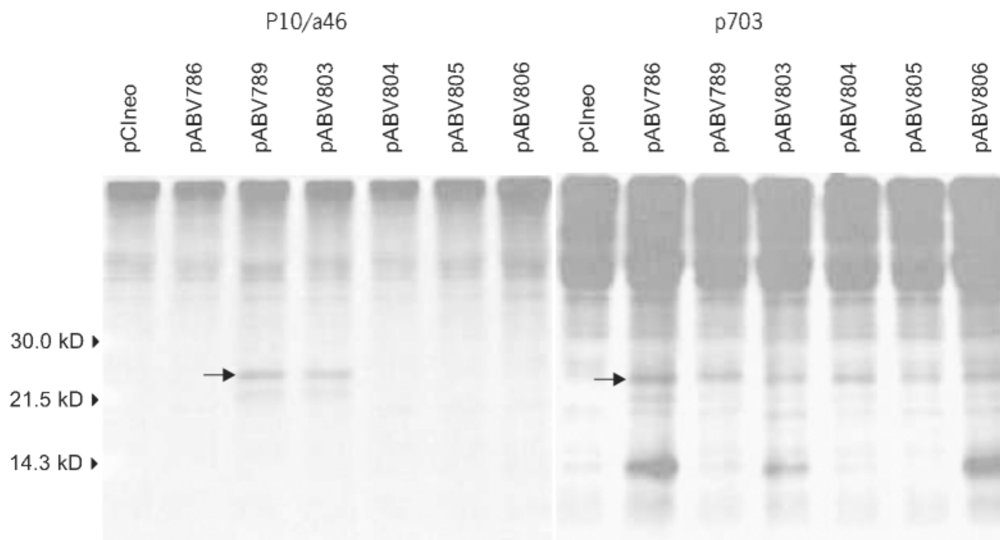


**Fig. 3.** Radioimmunoprecipitation analysis of expressed mutant $GP_5$ proteins with anti-$GP_5$ mAb P10/a46 and peptide serum p703. Proteins were immunoprecipitated from lysates of BHK-21 cells transfected with pABV786 (LV), pABV789 (PPV), pABV803 ($Pro^{24}$), pABV804 ($Val^{97}$), pABV805 ($Leu^{103}$) and pABV806 ($Arg^{158}$). BHK-21 cells transfected with the pCIneo vector were used as negative control. The immunoprecipitated $GP_5$ proteins were analysed by 14 % SDS-PAGE. The $GP_5$ 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 $GP_5$ proteins expressed from pABV789 and -803 were not markedly different from that of the other $GP_5$ 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 $GP_5$ proteins

In order to specify further the boundaries of the neutralization epitope of the $GP_5$ protein, a set of overlapping 12-mer peptides covering the complete $GP_5$ amino acid sequence of the PPV strain was tested for reactivity with anti-$GP_5$ 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 $GP_5$ 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 $TCID_{50}$ 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 $GP_5$ 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

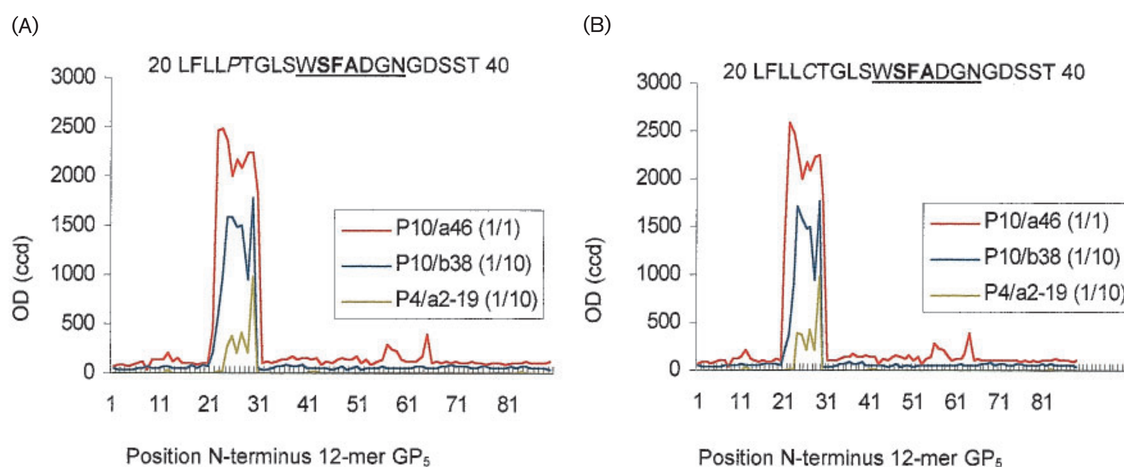


**Fig. 4.** Pepscan analyses of PRRSV $GP_5$ proteins. Overlapping 12-mer peptides derived from the $GP_5$ sequences of PRRSV LV and of the PPV strain were tested for reactivity with anti-$GP_5$ mAbs P10/a46, P10/b38 and P4/a2-19 (Slootstra *et al.*, 1997). (A) Overlapping 12-mer peptides derived from the $GP_5$ sequence of the PPV strain. (B) Overlapping 12-mer peptides derived from the $GP_5$ sequence of PRRSV LV. Optical densities (OD) were determined with a ccd-camera. The values are logarithmic values in a range of 0 to 4000. The Pepscan epitope is underlined, while the core sequence is printed in bold letters.

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(Δpro)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, $G_L$, 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 $GP_5$ and M protein are essential for LDV infectivity, suggesting that the heterodimeric $GP_5$–M complexes might be involved in receptor binding (Faaberg *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 $GP_5$ in PRRSV infection in more detail.

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## REFERENCES

**Balasuriya, U. B., Maclachlan, N. J., De Vries, A. A., Rossitto, P. V. & Rottier, P. J. (1995).** Identification of a neutralization site in the major envelope glycoprotein (GL) of equine arteritis virus. *Virology* **207**, 518–527.

**Cavanagh, D. (1997).** *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. *Arch Virol* **142**, 629–633.

**Chen, Z., Li, K. & Plagemann, P. G. (2000).** Neuropathogenicity and sensitivity to antibody neutralization of lactate dehydrogenase-elevating virus are determined by polylactosaminoglycan chains on the primary envelope glycoprotein. *Virology* **266**, 88–98.

**Chirnside, E. D., de Vries, A. A., Mumford, J. A. & Rottier, P. J. (1995).** Equine arteritis virus-neutralizing antibody in the horse is induced by a determinant on the large envelope glycoprotein GL. *J Gen Virol* **76**, 1989–1998.

**Conzelmann, K. K., Visser, N., Van Woensel, P. & Thiel, H. J. (1993).** Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. *Virology* **193**, 329–339.

**Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B. & Rogan, D. (2000).** Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Arch Virol* **145**, 659–688.

**Dekker, A., Leendertse, C. H., van Poelwijk, F., Rebel, J. M. & Moormann, R. J. (2000).** Chimeric swine vesicular disease viruses produced by fusion PCR: a new method for epitope mapping. *J Virol Methods* **86**, 131–141.

**Delputte, P. L., Vanderheijden, N., Nauwynck, H. J. & Pensaert, M. B. (2002).** Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol* **76**, 4312–4320.

**Dobbe, J. C., van der Meer, Y., Spaan, W. J. & Snijder, E. J. (2001).** Construction of chimeric arteriviruses reveals that the ectodomain of the major glycoprotein is not the main determinant of equine arteritis virus tropism in cell culture. *Virology* **288**, 283–294.

**Faaberg, K. S., Even, C., Palmer, G. A. & Plagemann, P. G. (1995).** Disulfide bonds between two envelope proteins of lactate dehydrogenase-elevating virus are essential for viral infectivity. *J Virol* **69**, 613–617.

**Gewurz, B. E., Ploegh, H. L. & Tortorella, D. (2002).** US2, a human cytomegalovirus-encoded type I membrane protein, contains a non-cleavable amino-terminal signal peptide. *J Biol Chem* **277**, 11306–11313.

**Glaser, A. L., de Vries, A. A. F. & Dubovi, E. J. (1995).** Comparison of equine arteritis virus isolates using neutralizing monoclonal antibodies and identification of sequence changes in GL associated with neutralization resistance. *J Gen Virol* **76**, 2223–2233.

**Gonin, P., Pirzadeh, B., Gagnon, C. A. & Dea, S. (1999).** Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. *J Vet Diagn Invest* **11**, 20–26.

**Indik, S., Valicek, L., Klein, D. & Klanova, J. (2000).** Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **81**, 2497–2502.

**Kapur, V., Elam, M. R., Pawlovich, T. M. & Murtaugh, M. P. (1996).** Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. *J Gen Virol* **77**, 1271–1276.

**Kozak, M. (1987).** An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* **15**, 8125–8148.

**Kwang, J., Zuckermann, F., Ross, G., Yang, S., Osorio, F., Liu, W. & Low, S. (1999).** Antibody and cellular immune responses of swine following immunisation with plasmid DNA encoding the PRRS virus ORFs 4, 5, 6 and 7. *Res Vet Sci* **67**, 199–201.

**Li, K., Chen, Z. & Plagemann, P. (1998).** The neutralization epitope of lactate dehydrogenase-elevating virus is located on the short ectodomain of the primary envelope glycoprotein. *Virology* **242**, 239–245.

**Mardassi, H., Mounir, S. & Dea, S. (1995).** Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. *Arch Virol* **140**, 1405–1418.

**Mardassi, H., Massie, B. & Dea, S. (1996).** Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. *Virology* **221**, 98–112.

**Meulenberg, J. J. M. & Petersen-den Besten, A. (1996).** Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP(2) encoded by ORF2 is incorporated in virus particles. *Virology* **225**, 44–51.

**Meulenberg, J. J., Hulst, M. M., de Meijer, E. J., Moonen, P. L., den Besten, A., de Kluyver, E. P., Wensvoort, G. & Moormann, R. J. (1993).** Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology* **192**, 62–72.

**Meulenberg, J. J., Petersen den Besten, A., De Kluyver, E. P., Moormann, R. J., Schaaper, W. M. & Wensvoort, G. (1995).** Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology* **206**, 155–163.

**Meulenberg, J. J., van Nieuwstadt, A. P., van Essen Zandbergen, A. & Langeveld, J. P. (1997).** Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. *J Virol* **71**, 6061–6067.

**Meulenberg, J. J., Bos-de Ruijter, J. N., Wensvoort, G. & Moormann, R. J. (1998).** An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. *Adv Exp Med Biol* **440**, 199–206.

**Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997).** Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* **10**, 1–6.

**Nothwehr, S. F. & Gordon, J. I. (1989).** Eukaryotic signal peptide structure/function relationships. Identification of conformational features which influence the site and efficiency of co-translational proteolytic processing by site-directed mutagenesis of human pre(delta pro)apolipoprotein A-II. *J Biol Chem* **264**, 3979–3987.

**Ostrowski, M., Galeota, J. A., Jar, A. M., Platt, K. B., Osorio, F. A. & Lopez, O. J. (2002).** Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol* **76**, 4241–4250.

**Pirzadeh, B. & Dea, S. (1997).** Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. *J Gen Virol* **78**, 1867–1873.

**Pirzadeh, B. & Dea, S. (1998).** Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **79**, 989–999.

**Rodriguez, M. J., Sarraseca, J., Fominaya, J., Cortes, E., Sanz, A. & Casal, J. I. (2001).** Identification of an immunodominant epitope in the C terminus of glycoprotein 5 of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **82**, 995–999.

**Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Slootstra, J. W., Puijk, W. C., Ligtvoet, G. J., Kuperus, D., Schaaper, W. M. & Meloen, R. H. (1997).** Screening of a small set of random peptides: a new strategy to identify synthetic peptides that mimic epitopes. *J Mol Recognit* **10**, 217–224.

**Snijder, E. J. & Meulenberg, J. J. (1998).** The molecular biology of arteriviruses. *J Gen Virol* **79**, 961–979.

**Snijder, E. J., van Tol, H., Pedersen, K. W., Raamsman, M. J. & de Vries, A. A. F. (1999).** Identification of a novel structural protein of arteriviruses. *J Virol* **73**, 6335–6345.

**Stadejek, T., Stankevicius, A., Storgaard, T., Oleksiewicz, M. B., Belak, S., Drew, T. W. & Pejsak, Z. (2002).** Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. *J Gen Virol* **83**, 1861–1873.

**Verheije, M. H., Welting, T. J. M., Jansen, H. T., Rottier, P. J. M. & Meulenberg, J. J. M. (2002).** Chimeric arteriviruses generated by swapping of the M protein ectodomain rule out a role of this domain in viral targeting. *Virology* **303**, 364–373.

**von Heijne, G. (1986).** A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**, 4683–4690.

**Weiland, E., Wieczorek-Krohmer, M., Kohl, D., Conzelmann, K. K. & Weiland, F. (1999).** Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. *Vet Microbiol* **66**, 171–186.

**Wensvoort, G., Terpstra, C., Boonstra, J., Bloemraad, M. & Van Zaane, D. (1986).** Production of monoclonal antibodies against swine fever virus and their use in laboratory diagnosis. *Vet Microbiol* **12**, 101–108.

**Wu, W. H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R. R., Christopher-Hennings, J. & Nelson, E. A. (2001).** A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. *Virology* **287**, 183–191.

**Yang, L., Frey, M. L., Yoon, K. J., Zimmerman, J. J. & Platt, K. B. (2000).** Categorization of North American porcine reproductive and respiratory syndrome viruses: epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization. *Arch Virol* **145**, 1599–1619.

**Yoon, K.-J., Zimmerman, J. J., Swenson, S. L. & 7 other authors (1995).** Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. *J Vet Diagn Invest* **7**, 305–312.

**Zhang, Y., Sharma, R. D. & Paul, P. S. (1998).** Monoclonal antibodies against conformationally dependent epitopes on porcine reproductive and respiratory syndrome virus. *Vet Microbiol* **63**, 125–136.