Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates

Brief Review

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Summary. Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the recently recognized *Arteriviridae* family within the genus *Arterivirus*, order *Nidovirales*, which also includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). Mature viral particles are composed of an envelope 50–72 nm in diameter, with an isometric core about 20–30 nm enclosing a linear positive-stranded RNA genome of approximately 15 kb. The virions are assembled by the budding of preformed nucleocapsids into the lumen of the smooth endoplasmic reticulum and/or Golgi apparatus. The mature virions are then released by exocytosis. The viral genome contains eight open reading frames (ORFs) which are transcribed in cells as a nested set of subgenomic mRNAs. The ORF1a and ORF1b situated at the 5′end of the genome represent nearly 75% of the viral genome and code for proteins with apparent replicase and polymerase activities. The major structural proteins consist of a 25 kDa envelope glycoprotein (GP$_5$), an 18–19 kDa unglycosylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively. The N protein is the more abundant protein of the virion and is highly antigenic, which therefore makes it a suitable candidate for the detection of virus-specific antibodies and diagnosis of the disease. Four to five domains of antigenic importance have been identified for the N protein, a common conformational antigenic site for European and North American strains being localized in the central region of the protein. In cells and virions, both M and GP$_5$ occur in heterodimeric complexes linked by disulfide bonds. The

expression products of ORFs 2 and 4 are also incorporated into virus particles as additional minor membrane-associated glycoproteins designated as GP_2 and GP_4, with M_r of 29 and 31 kDa, respectively. The structural nature of the ORF3 product, a highly glycosylated protein with an apparent M_r of 42 kDa, is still being debated, in view of the apparently conflicting data on its presence in virus particles. Nonetheless, the GP_3 of North American and European strains has been shown to be antigenic, providing protection for piglets against PRRSV infection in the absence of a noticeable neutralizing humoral response. Pigs exposed to the native form of GP_5 by means of DNA immunization develop specific neutralizing and protecting antibodies. The GP_5 is involved in antigenic variability, apoptosis, and possibly antibody-dependent enhancement phenomena. The GP_4 also possesses antigenic determinants that trigger the immune system to produce neutralizing antibodies. Each of the PRRSV structural proteins carries common and type-specific antigenic determinants that permit the ability to differentiate between European and North American strains. The potential use of the PRRSV structural proteins in subunit recombinant-type vaccines is also discussed.

Introduction

The porcine reproductive and respiratory syndrome (PRRS) is an infectious disease in swine that emerged 12 years ago in the United States [48] and Canada [12, 21], but two to three years later in the European countries [1, 6, 87, 119, 121]. Today, PRRS is endemic in many, if not all swine-producing countries [2, 42]. The disease has many clinical manifestations but the two most prevalent are severe reproductive failure in sows and gilts (characterized by late-term abortions, an increased number of stillborns, mummified and weak-born pigs) [12, 15, 48 , 94, 109], and respiratory problems in pigs of all ages associated with a non-specific lymphomononuclear interstitial pneumonitis [12, 17, 43, 44, 98].

The causative virus, PRRSV, belongs to the recently recognised *Arteriviridae* family within the genus *Arterivirus*, order *Nidovirales*, along with equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV) [4, 14, 18, 29, 71, 92]. The virus is remarkably adapted to its natural host, and as other arteriviruses [91, 92], it infects almost exclusively pig monocytes and macrophages [8, 21, 117, 120, 121].

Mature PRRSV virions, as in the case of other arteriviruses [14], are enveloped and contain an icosahedral capsid [11, 22, 95] which encloses a linear positive-stranded RNA genome of approximately 15 kb [18, 71]. Despite major differences at the ultrastructural level, the link with coronaviruses, also recently grouped into the order *Nidovirales* [14, 29] was proposed on the basis of the genome of equine arteritis virus (EAV) [30], the prototype of the *Arterivirus* genus. As for EAV, the genome of the PRRSV contains eight open reading frames designated (5′ to 3′) ORF 1a, ORF 1b, and ORFs 2 to 7 [18, 71]. Aside from the genome length mRNA, expression of the viral genomes of EAV [25, 112] and PRRSV [18, 66, 70] involves the synthesis of a 3′ coterminal nested set of six to seven subgenomic mRNAs , which contain a common leader sequence. The available evidence indicates that

only the most 5′ ORF is translated from each one of these subgenomic mRNAs [102]. According to sequence data, ORFs 1a and 1b represent nearly 75% of the viral genome and code for proteins with apparent replicase and polymerase activities [30, 71, 78] whereas ORFs 2 to 6 (from 5′ to 3′ end) are predicted to encode for membrane-associated proteins, and ORF7 for a highly basic protein [61, 74, 102].

Since the isolation of PRRSV strain Lelystad (LV) in Europe and ATCC VR-2332 in the US, the presence of three major structural protein components has been identified in purified virions: the nucleocapsid protein N (14–15 kDa), the non-glycosylated membrane protein M (18–19 kDa), and the major envelope glycoprotein GP_5 (25–26 kDa) [9, 11, 83, 120], which represent the counterparts of the N, M and G_L of the EAV [26]. Evidence of the three major structural proteins has also been confirmed in the Canadian isolates IAF-exp91 and IAF-Klop [58, 61]. Peptide-specific antibodies [74], as well as monospecific antisera raised in rabbits against *Escherichia coli*-expressed proteins [62], revealed that the three major structural proteins of PRRSV are encoded by ORFs 5, 6 and 7, respectively. Three other glycoproteins encoded by ORFs 2 to 4 (GP_2, GP_3, and GP_4) have been identified as minor structural proteins [75, 114]. It has been suggested that the GP_2 may represent the counterpart of the small envelope glycoprotein (G_s) of the EAV [28]. This paper summarizes current knowledge on the molecular characteristics, biological and immunological functions of the PRRSV structural proteins. For more details on the cell biology, genome properties and replication, as well as biochemistry of arteriviruses, the readers should refer to the recent review by Snijder and Meulenberg [102], whereas pathogenesis and epidemiology of PRRSV have been more described by others [2, 42, 69, 92].

Ultrastructure and morphogenesis of the PRRS virus

The PRRSV was originally isolated on primary cultures of porcine alveolar macrophages (PAMs) [120] and so far, these cells as well as blood monocytes [117], remain the only porcine cells that can effectively be used for viral propagation *ex-vivo*. Two non-porcine permissive cell subclones, MARC-145 and CL2621 cells, both derived from the MA104 monkey kidney cell line [8, 11, 49] are also routinely used for in vitro propagation of wild and vaccine strains. Data from routine diagnosis investigations, as well as several experimental studies, have indicated that European strains of PRRSV are most successfully isolates in PAMs, whereas the great majority of North American strains can be initially isolated in the established monkey cell subpopulations [8, 67, 68]. The cytopathogenic effect (CPE) observed on both non-porcine cell lines consists of the appearance of small rounded clumps of cells raised above the remainder of the infected monolayer [11, 49]. Infected cells become progressively pycnotic and detach from the monolayer two to four days post-infection (p.i.). Infectivity titers of 10^5 to 10^7 $TCID_{50}$ (50 percent tissue culture infective dose)/ml are usually obtained from clarified tissue culture supernatants after 5 to 7 serial passages [11, 49]. On PAMs, the CPE is characterized by the rounding off, clumping, and lysis of cells. Infected
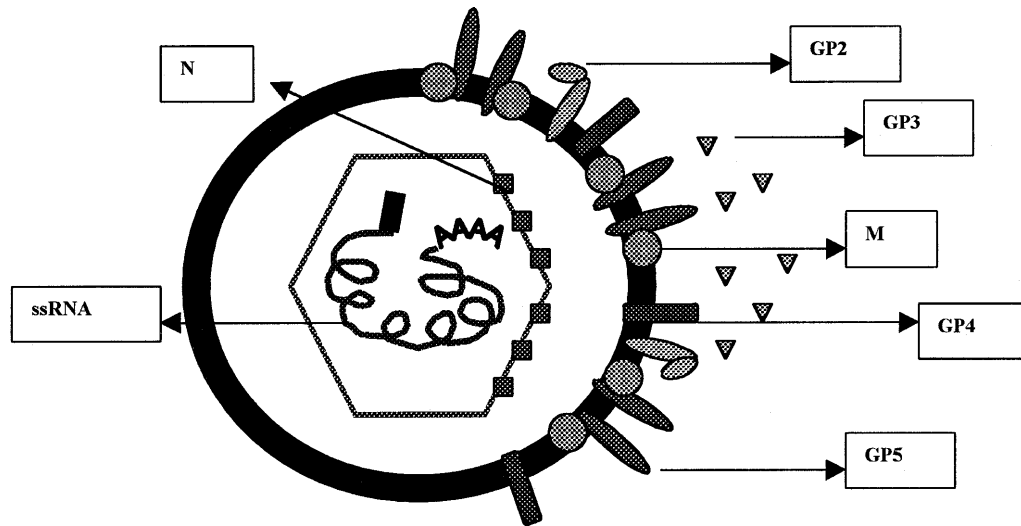
Fig. 1. Schematic representation of the porcine reproductive and respiratory syndrome virus. The virion is mostly spherical in shape, envelope, and possesses a non-segmented single-strand RNA genome that is encapsidated by the nucleocapsid protein (N), yielding an icosahedral core structure. At least four protein components are envelope-associated: the non-glycosylated matrix protein (M) and the GP_5 glycoproteins which represent major components, whereas GP_2 and GP_4 are two minor components. Both the M and GP_5 are incorporated as disulfide-linked heterodimers. The GP_3 has been also identified as another minor envelope-associated glycoprotein in case of the European LV strain, but in the case of the North American IAF-Klop strain it is rather a soluble and weakly membrane-associated protein. Adapted from Meulenberg et al. [74–76], Mardassi et al. [61–63], and Gonin et al. [40]

cells commonly display a bristling cytoplasmic membrane and granulation [21, 120]. Destruction of the monolayer is usually acheived by 72 to 96 h p.i. [11, 49, 58, 120]. In vivo, macrophages from other tissues such as heart, tonsil, spleen, turbinates and choroid plexus [43, 52, 98, 99], as well as testicular germ cells [107], have been reported also to be susceptible to PRRSV infection.

Replication of PRRSV in the susceptible cells has been described to be restricted to the cytoplasm. By indirect immunofluorescence (IIF) and immunoperoxydase, viral antigens can be detected in the perinuclear region as early as 6 h p.i. [8, 49, 126]. Nucleocapsids budding at smooth endoplasmic reticulum (ER) and/or Golgi region, and enveloped viral particles that accumulate in the lumen of the ER or Golgi vesicles, are the main features of the viral morphogenesis [21, 22, 58, 95]. The virus apparently is released by exocytosis [22, 96].

Purified extracellular virions are pleomorphic, but mostly spherical enveloped particles with a diameter of 45–70 nm [11, 58, 95]. Buoyant densities of the infectious viral particles are 1.13–1.15 g/ml in sucrose and 1.18–1.19 g/ml in CsCl [11, 58, 121]. A schematic representation of the PRRSV particle is shown in Fig.1. and the molecular characteristics of ORFs 2 to 7 products of the North American and European strains are depicted in Table 1. The non-segmented single-stranded RNA genome (15 kb) is encapsidated by the nucleocapsid protein N, yielding

Table 1. Comparative characteristics of ORFs 2 to 7 products of the North American and European strains of PRRSV

Coding area gene product	Protein	No. of aa residues		Predicted M_r (kDa)		Apparent M_r (kDa)		Glycosylation sites	
		Euro	NAm	Euro	NAm	Euro	NAm	Euro	NAm
ORF2	GP$_2$	249	265	28.4	29.4	29–30	27–29	2	2
ORF3	GP$_3$	265	254	30.6	29.0	45–50	42–45	7	7
ORF4	GP$_4$	183	178	20.0	19.6	31–35	31–35	4	4
ORF5	GP$_5$	201	200	22.4	22.4	25	24–26	2	2–5
ORF6	M[a]	173	174	18.9	19.1	18	19	2	1
ORF7	N[a]	128	123	13.8	13.6	15	14–15	1	1

Euro European strains; *NAm* North American strains
[a]In spite of potential glycosylation sites, these proteins are not glycosylated
Data were taken from Mardassi et al. [61], Meulenberg et al. [71, 74], Meng et al. [64, 65], Morozov et al. [80] and Pirzadeh et al. [90]

an icosahedral core structure of 20 to 30 nm in diameter. The lipid bilayer which surrounds the nucleocapsid contains at least four additional structural proteins: the non-glycosylated membrane protein M and the GP$_5$ glycoprotein represent the two major components of the viral envelope [62, 74], whereas GP$_2$ and GP$_4$ represent two minor components [75, 76, 114] which have not been yet characterized for North American isolates of PRRSV. The GP$_3$ is an additionnal minor structural glycoprotein which has been reported for the virion of the European prototype Lelystad strain [74, 114], but its structural nature remains controversial since the GP$_3$ of the Canadian IAF-Klop strain could not be detected in preparations of purified virus, but small amounts of the protein were detected in a soluble form in the medium [40, 63].

Major structural proteins

The nucleocapsid (N) protein

As for other arteriviruses [26], the PRRSV N protein with 123 or 128 amino acid (aa) residues (for the North American and European strains, respectively) is a small, highly basic protein with an estimated M_r of 14–15 kDa [61, 74]. It is abundantly expressed in infected cells and on the basis of SDS-PAGE and western immunoblotting profiles, it constitutes about 20–40% of the protein content of the virion [9, 58, 83]. In all PRRSV isolates analysed so far, the N-terminal half of the protein contains 26% basic residues (Arg, Lys and His) that may facilitate its interaction with the RNA genome [59, 65, 71, 74]. Preliminary studies by immunoprecipitation of the N protein from purified PRRSV, using monospecific hyperimmune serum as well as pig convalescent serum, suggested that it is predominantly present as a disulfide-linked homodimer [62, 74]. The nucleocapsid

protein is thus multimerized to form icosahedral core structures which can be observed by electron microscopy of PRRSV-infected cells [58, 95, 96]. Computer analysis and in vitro expression experiments using deletion mutants suggested that the PRRSV capsid protein possesses 6 putative beta-sheets between an unstructured N-terminal arm and an ordered C-terminus, forming a basic core structure of a jelly roll barrel [123]. Contrary to what was previously demonstrated, by constructing mutants to change cysteine residues at positions 23, 75, and 90 of a reference North American strain to proline, and introducing β-breaker mutations at positions 114, 115 and 116, it has been demonstrated that the capsid protein of PRRSV dimerizes through non-covalent interactions. These non disulfide-linked interactions involving the C-terminal domain (specially the last 11 amino acids) of the protein may form the structural basis for viral nucleocapsid formation [123, 124].

Several groups have generated anti-N monoclonal antibodies (MAbs) that recognise epitopes specific to or shared by North American and European isolates [23, 31, 79, 83, 125]. Based on the immunoreactivity of N protein deletion mutants with panels of N-specific MAbs, five domains of antigenic importance have been identified for a reference North American strain, four of them being defined by aa 30 to 52, 37 to 52, 69 to 112, and 112 to 123, respectively. Other MAbs revealed the presence of a common conformational antigenic site localised in the central region (aa 52–69) of the protein [97, 125]. The region between aa 37 and 52 is well conserved among isolates of both continents and is the most hydrophilic region of the protein. None of the N-specific MAbs have been found to be associated with virus neutralisation. It has been also demonstrated that the 11 most carboxy-terminal amino acids play a critical role in the formation of the conformational epitopes [125].
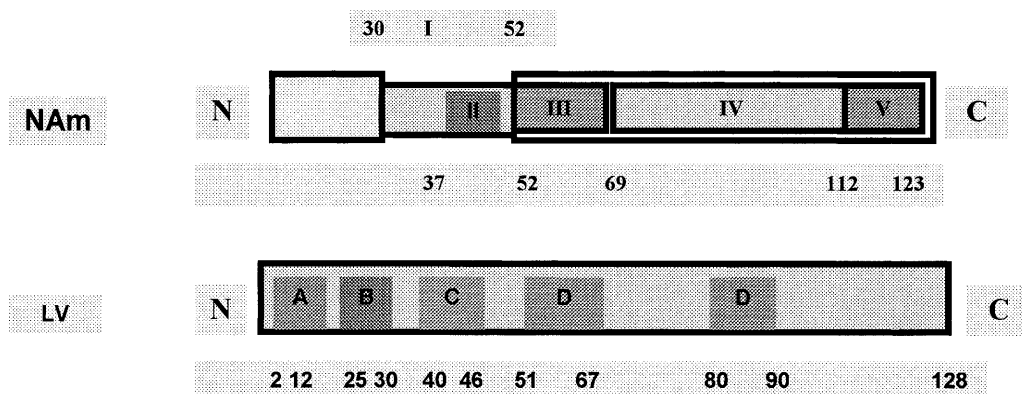

Fig. 2. Localization of antigenic sites on the nucleocapsid proteins of North American and European strains of PRRSV. The diagrams were deduced from competitive ELISA with MAbs that were raised against the native viral proteins, immunoblotting analysis with truncated *E. coli*-expressed recombinant N protein [125], pepscan analysis with solid-phase dodecapeptides and by studying the reactivities of the MAbs to chimeric PRRSV/LDV recombinant N proteins [79]

Four distinct antigenic domains were identified for the reference European LV strain [79], three of them being defined by the reactivity of anti-N MAbs with linear dodecapeptides whose core sequences consisted of aa 2–12 (site A), aa 25–30 (site B), and aa 40–46 (site C). The fourth antigenic domain (site D) is rather conformation-dependent, involving regions between aa 51–67 and aa 80–90, and was defined by MAbs which did not react with solid-phase dodecapeptides. This antigenic domain was delineated by studying the reactivities of the MAbs to chimeric PRRSV/LDV recombinant N proteins [79]. Sites A and C contain epitopes that are conserved in the European but not in the North American isolates, site B contains epitopes that are conserved in strains of both continents, and site D contains epitopes that are either conserved or not conserved in the European and North American isolates [79]. Data on epitopes mapping of the N proteins of North American and European strains of PRRSV are summarized in Fig. 2.

The major envelope proteins

The M protein

The ORF6 encoded M protein of PRRSV has an estimated M_r of 18–19 kDa, is non-glycosylated, and shows three highly hydrophobic regions in its N-terminal half [61, 71], which are assumed to represent potential membrane-spanning domains by analogy to the coronavirus M protein [100]. On the basis of its hydropathy profile (Fig. 3), a very short region of the M protein (10 to 18 amino acid
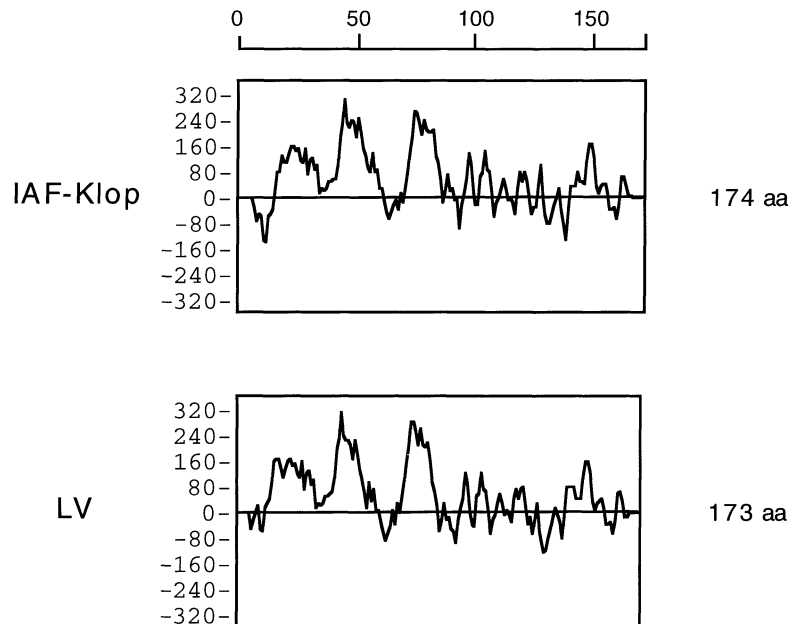
Fig. 3. Comparison of the hydropathy profiles of the M protein of a North American (IAF-Klop strain) and European (Lelystad) strains of PRRSV. The three highly hydrophobic regions at the N-terminal half are assumed to correspond to membrane spanning domains

residues) of PRRSV is probably exposed on the virion surface. As for EAV and LDV [27, 33], the M protein of PRRSV accumulates in the ER of the infected cells, where it forms disulfide-linked heterodimers with the major GP$_5$ glyco-protein [62]; cysteine residues located at the N-terminal ectodomains of both envelope proteins are probably involved in the formation of an intermolecular disulfide bridge [61, 64, 71]. In PRRSV-infected cells [62], disulfide-linked M protein homodimers have also been observed but these were not incorporated into virions; this feature has also been observed for EAV [27]. It remains to be seen if the disruption of intermolecular disulfide bonds can reduce PRRSV infectivity as it has been previously shown for LDV [33]. Because of its close association with other viral envelope-associated proteins, its membrane-associated function, and the close similarity with the molecular structure of the coronavirus M protein, an important role in virus assembly and budding has been suggested for the PRRSV M protein [62, 92].

The major envelope glycoprotein (GP$_5$)

The ORF5 of PRRSV encodes a 24.5–26 kDa envelope protein with a charac-teristic hydropathy profile and putative glycosylation sites [61, 62, 74]. In spite of significant genomic variations between the PRRSV North American isolates and LV, the hydropathy profiles of their ORF5 encoded proteins are remarkably similar [61, 64, 81]. All North American strains studied so far possess a putative signal sequence, with two additionnal putative membrane-spanning motifs situ-ated between aa residues 65–130 and 170–190 [61, 64, 81]. In the case of the prototype European LV strain of PRRSV, sequence analysis suggested that the first 32 aa residues of the ORF5 product constitute the signal peptide with a puta-tive cleavage site situated at position 32–33 [71, 74]. According to the prediction method of von Heijine [116], the putative N terminal signal sequence of North American strains of PRRSV comprises amino acid residues 1 to 25, a region which is highly variable between strains of both continents [90]. Compared to EAV [102], the putative ectodomain of the PRRSV ORF5 protein is much smaller, but as for the former this region contains N-glycosylation sites [61, 81, 90] (Fig. 4). The Quebec reference IAF-Klop strain, possess three potential glycosylation sites at aa positions 30–32, 44–46 and 51–53, respectively [61, 81, 90]. The US reference strain ATCC VR-2332 possesses an additional glycosylation site at po-sition 33–35. Only the two glycosylation sites at aa positions 44–46 and 51–53 have been conserved in the GP$_5$ of LV [71]. It is not clear at present, which of the potential glycosylation sites are occupied by oligosaccharide residues, but the GP$_5$ incorporated into the virion contains N-linked oligosaccharides of the high mannose and complex type, since it was proven to be sensitive to endoglycosi-dase F /N-glycosidase F (glyco F) digestion (giving rise to a 16.5 kDa species) and partially sensitive to endo-β-N-acetylglucosaminidase H (endo H) digestion [62, 74]. Unlike the ORF5-encoded G$_L$ protein of EAV [26], the GP$_5$ of LV as well as that of the Canadian isolate IAF-Klop does not contain *N*-acetyllactosamine, since it is totally resistant to endo-β-galactosidase [62]. As in the case of EAV [27], the GP$_5$ of PRRSV forms disulfide linked-heterodimers with the M protein,

	POSITION 26 — 39	N-glycosylation sites
IAF-Klop	A A L V [N A S] S S S S S Q L	1
MLV	· V · A [· · · N D ·] · · H ·	2
ATCC VR-2332	· V · A [· · · N D ·] · · H ·	2
ATCC VR-2385	V · · · S · N G N · G · N ·	0
IAF-BAJ	· · · · [· · · N [N ·] ·] · · ·	3
IAF-DESR	· · · · [· · ·] · T · · · H ·	1
IAF-CM	· · · · [· · ·] · · · · · · ·	1
IAF 93-653	V · · · · · N T D · · · H ·	0
IAF 93-2616	· · · · [· · · N · ·] · · H ·	2
IAF 94-3182	· V · · [· · · P [N · ·] ·] H ·	2
IAF 94-287	· · · · [· · · N [N · ·] ·] · H ·	3
ONT-TS	V · · · S · · [N · ·] · · H ·	1

Fig. 4. Detailed presentation of the hypervariable region of the major GP$_5$ envelope glycoprotein of North American strains of PRRSV located near the N terminal of the ORF5 (position 26 to 39), affecting the number of potential N-glycosylation sites (boxes); taken from Pirzadeh et al. [90] and Meng et al. [64]. MLV: attenuated U.S. vaccine ATCC VR-2332 strain (Ingelvac MLV)

since monospecific anti-GP$_5$ or anti-M sera precipitate both proteins [62]. Apparently, all the steps of GP$_5$ N-glycans processing proceed after its association with the M occurs in the ER.

As for LDV and EAV [5, 20, 38], MAbs that recognised the PRRSV ORF5 protein were reported to neutralize virus infectivity [88, 89, 118, 129]. The data obtained also suggest that at least two types of neutralizing antigenic determinants are associated with the GP$_5$ of PRRSV; some are linear and are recognized by MAbs obtained following immunization of mice with *E. coli*-expressed recombinant ORF5 protein [88], but there are also determinants that are conformation dependent [118, 129]. Since all MAbs reacted with both the glycosylated and unglycosylated forms of the viral envelope protein, it appears that glycosylation is not necessarily associated with the neutralizing epitope(s). The importance of an internal conformation is also supported by the demonstration that DNA immunization with a plasmid encoding the GP$_5$ of PRRSV under the control of human cytomegalovirus promoter can trigger the immune system of pigs for the production of anti-GP$_5$ specific neutralizing antibodies, but not parenteral inoculation with *E. coli*-expressed recombinant ORF5 protein [89].

Based on the immunoreactivities of the ORF5 protein deletion mutants and synthetic peptides corresponding to the three most hydrophilic regions of the protein with a panel of GP$_5$-specific MAbs, at least two domains of antigenic importance have been identified, one being located within the ectodomain (aa 27 to 41) and the other to the C-terminus (aa 180 to 197) (Dorval et al., pers. comm.). It appears that as for EAV [5], most important neutralizing epitopes mapped to

the putative ectodomain of the ORF5 protein since the reactivities of neutralizing convalescent pig sera were higher with the synthetic peptide corresponding to the ectodomain of the GP_5. However, the majority of convalescent pig sera that tested positively to the native viral protein and the *E. coli*-expressed recombinant ORF5 protein lost their reactivity following deletion of 50 aa residues from its C-terminus (Pirzadeh et al., pers. comm.). This suggests that the C-terminal region is the site of an important antigenic determinant and/or plays an important role in maintaining local protein conformation.

Both North American and European strains of PRRSV have been shown to induce apoptosis in susceptible monkey kidney cells and primary cultures of PAMs [104, 108]. This feature has been attributed to the ORF5 as the individual expression of the ORF5 product of a Spanish strain of PRRSV in COS-1 and BSC40 cells, using a vaccinia virus recombinant, resulted in a strong cytotoxicity associated with induction of apoptosis [104]. The GP_5-induced apoptotic activity could not be prevent by using a cell line permanently expressing the anti-apoptotic Bcl-2 protein, suggesting the existence of a target downstream the Bcl-2 family members, or the use of an alternative and unknown apoptotic pathway [104]. Transient expression experiments with plasmids carrying N- and C-terminal deletions, as well as internal fragments of the ORF5 sequence, indicated that the region in the GP_5 protein necessary to induce apoptosis is into the first 118 aa residus while the C-terminal region is not required for this activity [36]. In vivo, studies have shown that PRRSV-induced apoptosis occurs in lung and lymphoid tissues [108], but also in testis where PRRSV is able to replicate in macrophages, as well as germinal cells, causing abundant germ cell depletion and death of cells by apoptosis [107]. In all tissues, virus infection-induced apoptotic cells are more abundant than PRRSV-infected cells, thus suggesting the presence of an indirect mechanism in the induction of apoptosis for the PRRSV [107, 108]. The mechanism of PRRSV-induced apoptosis in bystander cells is not known, but may involve the local release of apoptogeneic cytokines by the infected macrophages [108].

The GP_5 of PRRSV thus appears to have an important role in virus infectivity. However, the involvement of the major envelope glycoprotein in attachment to cell receptors and/or in virus penetration into the cytoplasm of target cells still remains to be demonstrated. Recently, infection of MARC-145 cells by PRRSV could be prevented by prior incubation of the virus with heparin, and also by pretreatment of the cells with heparinase [45, 110]. Consequently, it was suggested that a heparin-like molecule on the surface of permissive cells may serve as an attachment molecule for the virus, but the role of the GP_5 in this interaction is still to be defined.

Minor structural proteins of PRRSV

The GP_2 glycoprotein

The ORF2 encoded glycoprotein of PRRSV is probably the Gs counterpart of EAV [26, 28], but since its relative molecular size (29–30 kDa) is greater than

GP$_5$ (the G$_L$ counterpart of EAV), the nomenclature used for EAV would be inappropriate. The GP$_2$ of European and North American strains of PRRSV contain 2 distinctive hydrophobic peaks and share two putative N-linked glycosylation sites [64, 74, 75, 80]. Using anti-GP$_2$ specific anti-peptide serum, it has been established that the GP$_2$ of LV is incorporated into the extracellular virion [75]. The ORF2 protein associates with intracellular membranes both in PRRSV-infected cells and upon individual expression in vivo [75], as in the case of EAV G$_s$ protein [28]. Compared to N and M proteins, the estimated quantity of incorporated GP$_2$ protein in the extracellular virion is apparently smaller favouring the concept that the 29–30 kDa protein encoded by the ORF2 of LV is a minor structural protein. The SDS-PAGE migration pattern of GP$_2$ in the presence or absence of the reducing agent β-mercaptoethanol, or the alkylating agents iodoacetamine or N-ethylmaleimide, suggests that a fraction of GP$_2$ is folded on itself via disulfide bonds, without forming homodimers, or hetero-multimers with other viral proteins [75]. Finally, the reduction of apparent M_r of GP$_2$ following glyco F treatment of extracellular virion and its resistance to endo H digestion indicate that the putative N-glycosylation site(s) of GP$_2$ are occupied by complex N-glycans [75]. Whether the ORF2 protein is indispensable for virus replication, as previously showed for EAV [113], has not yet been demonstrated.

Recently, a short region between the replicase gene and ORF2 of the EAV genome has been shown to contain the 5$'$ part of a novel gene (ORF2a) which is conserved in all arteriviruses, including PRRSV [103]. The 3$'$ part of the EAV ORF2a overlaps with the 5$'$ part of the former ORF2 (now renamed ORF2b), which encodes the Gs glycoprotein, the counterpart of GP$_2$ of PRRSV. Both ORF2a and ORF2b appear to be expressed from mRNA 2, which thereby constitutes the first proven example of a bicistronic mRNA in arteriviruses. Computer analysis revealed that the putative ORF2a protein is clearly most conserved between LDV and PRRSV [103]. In view of its predicted hydrophobic nature, its membrane association, and its structural nature as determined by analysis of purified EAV particles by radioimmunoprecipitation using a specific antiserum, this new 8 kDa non glycosylated protein has been provisionally named the envelope (E) protein for EAV. By using reverse genetics, it was found that the EAV E protein is essential for the production of infectious progeny virus [103]. It remains to be demonstrated if this essential low molecular weight structural envelope protein is also expressed in PRRSV-infected cells.

The GP$_4$ glycoprotein

The ORF4 encoded apoprotein has a predicted M_r of 19.6–20.0 kDa, containing highly hydrophobic sequences situated at its N- and C-terminal regions and four putative N-linked glycosylation sites which are conserved amongst the European and the North American strains of PRRSV. The aa sequence of the N-terminus is suggestive of a putative signal sequence [61, 64, 71, 74]. Immunoprecipitation experiments with specific anti-peptide sera revealed two different products from cell lysates and extracellular virus of CL2621 cells infected with LV. The intracellular

form has an apparent M_r of 20–28, whereas the virion-associated protein has an apparent M_r of 31–35 kDa, due to the addition of complex type N-glycans during the transport of the protein through the ER and Golgi compartments [76]. Consequently, the mature glycoprotein is largely endo H resistant, but sensitive to glyco F digestion [76]. The GP_4 protein of LV is associated with neutralizing epitopes which are not conserved amongst North American and European strains [76, 118]. Apparently, MAbs to the GP_4 are less effective in virus neutralization than MAbs to the GP_5. [118]. The neutralizing domain of the GP_4 of the European LV strain has been mapped to a hydrophilic exposed region, adjacent to its amino terminal region (aa position 40–79) which appears to be highly variable amongst the European strains [118]. Approximately 65% of PRRSV-positive sera obtained from affected pig farms in the US and Canada reacted positively by immunoblotting with the *E. coli*-expressed recombinant ORF4 proteins of reference North American strains [41, 51]. However, no correlation has been demonstrated between virus neutralization titers of convalescent pig sera and the presence of anti-GP_4 antibodies [41]. Accordingly, four MAbs which have been recently obtained to recombinant ORF4 protein of the North American VR-2385 strain expressed in insect cells lacked neutralizing activity; all of them were found to be directed against conformationally dependent epitopes [129]. Therefore, the implication of GP_4 in inducing neutralizing antibodies following PRRSV infection remains controversial.

The GP_3 glycoprotein

With approximately 54 to 60% aa identity between the North American and the European isolates, the ORF3 encoded protein is the second most heterogeous protein of PRRSV [61, 64, 71, 81]. A deletion of 12 aa in the C-terminal region of the ORF3 protein of North American strains of PRRSV is of particular interest [40, 47], making this region of the ORF3 protein highly hydrophilic for the European strains, whereas it is amphipathic for the North American strains (Fig. 5). The ORF3 protein is also the most glycosylated protein with seven N-linked glycosylation sites that have been well preserved amongst PRRSV strains from both continents. Consequently, the predicted M_r of the ORF3 protein (27–29 kDa) is remarkably smaller than its apparent M_r estimated by gel electrophoresis (42–50 kDa) [40, 61, 74].

While reactivity of MAbs specific to GP_3 of LV suggested that it is incorporated into or associated with the viral envelope [74, 114], recent studies on the ORF3 product of the Quebec IAF-Klop strain of PRRSV suggested it is rather a soluble and weakly membrane-associated protein [40, 63], and similar conclusions have been made for the LDV ORF3 protein [35]. Indeed, western immunoblotting and radioimmunoprecipitation experiments conducted with infected cell extracts showed that the ORF3 of the Quebec strain codes for a highly glycosylated protein (Glyco F sensitive) with an estimated M_r of 42 kDa, similar to that of the protein expressed by a replication-defective recombinant adenovirus (AdCMV5/ORF3) in human 293 cells [40, 63]. Although the GP_3 protein appeared to be synthesized in amounts comparable to those of the N, M, and GP_5
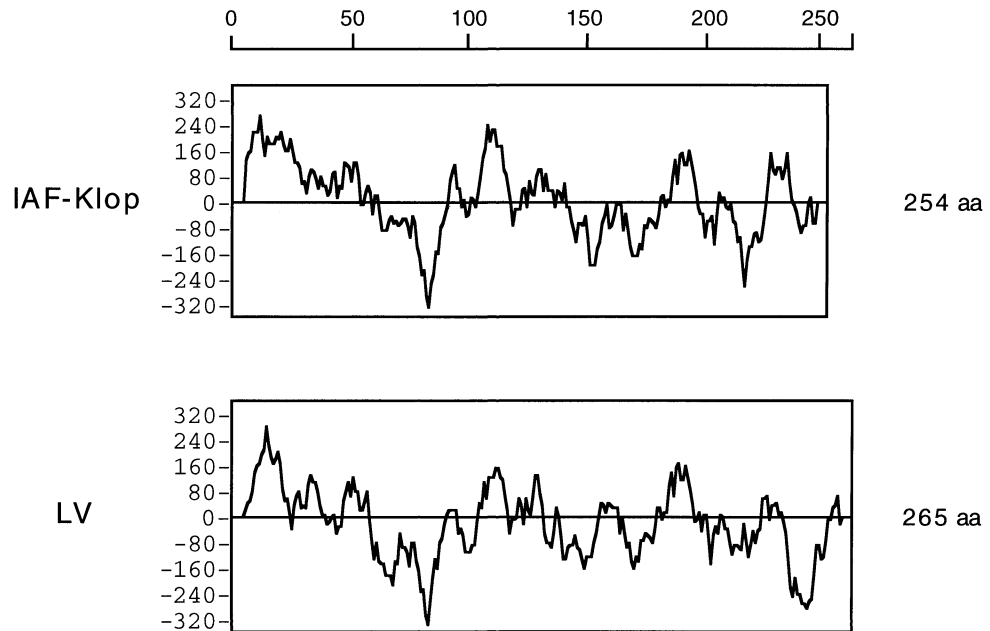
Fig. 5. Comparison of the hydropathy profiles of the GP3 North American (IAF-Klop strain) and European (Lelystad) strains of PRRSV. Sequencing analysis of the ORF3 of North American strains revealed a C-terminal deletion of 12 aa compared to LV which results in a remarkable hydrophilic region associated with the GP3 of LV, whereas the predicted product of North American strains shows an amphipathic pattern in this region [40, 47, 61, 71]

proteins in the PRRSV-infected cells, as estimated by the intensity of the protein bands obtained by SDS-PAGE, no GP3 could be detected in purified virions [40, 63]. The transport of GP3 of the Quebec strain is apparently restricted to the pre-medial Golgi compartment, presumably the ER, as no resistance to endo H have been observed at any time after the protein synthesis [40, 63].

Nonetheless, a minor fraction of GP3 was found to be able to exit the ER and transit through the secretory pathway to be secreted in the culture medium of cell cultures infected with PRRSV and AdCMV5/ORF3 as a soluble membrane-free form (sGP3) with M_r of 43 to 53 kDa [63]. The absence of a putative ER retention signal correlates with the ability of a GP3 fraction to be transport competent and finally secreted from the cell. The unglycosylated form of sGP3 comigrated with its intracellular deglycosylated counterpart (M_r of 27 kDa), suggesting that the release of a subset of GP3 out of the cell does not result from the cleavage of a putative membrane-anchor sequence [63]. Strikingly, unlike GP3, the sGP3 acquires Golgi-specific modifications of its carbohydrate side chains and folds into a disulfide-linked homodimer [63]. Brefeldin A treatment completely abolished the release of sGP3, suggesting that ER-to-Golgi compartmentalization is an obligatory step in the cellular secretion of sGP3 [63].

Tested convalescent pig sera, that were found to be seropositive to PRRSV by IIF, reacted positively with the recombinant GST-ORF3 fusion protein by im-

munoblotting [40, 41]. Secretion of a minor fraction of GP$_3$ might be an explanation for its high degree of immunogenicity in PRRSV-infected pigs. As mentioned above, the coding sequence of GP$_3$ is highly variable amongst PRRSV strains, with the majority of the aa variations occuring at the amino terminus [47, 61, 81]. Since this region carries signals that influence the destiny of most proteins, it is conceivable that the difference in the fate of GP$_3$ between North American and European strains may lie in such a portion of the protein. Further studies involving the construction of GP$_3$ chimeric viruses and the generation of mutants may certainly help in learning more about the biogenesis and the potential role of this protein. However, it can be speculated that since GP$_3$ is not packaged in the North American IAF-Klop PRRSV strain, this protein may not be necessary for initiation of viral infection.

Genomic variability of PRRSV

As for other envelope RNA viruses, a high degree of genomic variability has been reported for the arteriviruses ([102], a review), including PRRSV [59, 64, 65, 81, 82]. Percentages of aa identity of ORFs 2 to 7 of LV strain with those of PRRSV reference strains from other pig producing countries, and other *Arteriviruses*, are summarised in Table 2. Sequencing analysis demonstrated that PRRSV and LDV are more closely related to each other than they are to EAV, and that ORF3 and ORF5 proteins are the most variable proteins amongst the PRRSV isolates. The ORF2 to 7 protein sequences of the European and North American PRRSV isolates differ by 21 to 48%. Therefore, PRRSV isolates from both continents belong to two distinct genotypes that probably emerged from a common ancestor, must probably an LDV-like ancestor [61, 65, 81, 82]. Such an assumption is plausible considering the identical biochemical and morphological characteristics, the similarities in genomic organisation and replication strategies, the type of disease caused in pigs, and the infectivity of identical permissive cells. On the other hand, field isolates of each continent have a greater degree of relatedness to each other than the strains originating from the other continent [37, 46, 80, 90, 106]. Sequencing data available indicate that North American strains exhibit significant genetic diversity whereas European strains are highly conserved and genetically related to each other [37, 46, 80, 90, 106]. Field PRRSV isolates that have been associated with outbreaks of reproductive and respiratory syndromes in Asian countries have been shown to be genetically related with the North American genotype [101, 111], whereas PRRSV strains isolated in Russia belong to the European genotype [4]. By the end of summer 1996, dramatic losses (decreased reproductive performance, high mortality rates in piglets) was reported among Danish pig herds at the approximate time of reported infection with PRRS and introduction of a modified live PRRS vaccine (Ingelvac PRRS MLV) based on the American VR2332 strain [13]. The virus has spread rapidly throughout the country and approximately 40% of the Danish swine herds are now considered to be infected by either one or both of the PRRSV genotypes [55, 86].

Table 2. Percentages of amino acid identity of ORFs 2 to 7 of LV (The Netherlands strain) with those of PRRSV strains from other countries and other arteriviruses

Gene products of LV[a]	Virus strains and origin										
	PRRSV-10[b] Germany	VR-2332[c] US	VR-2385[d] US	IAF-Klop[e] Canada	DK111-92[f] Denmark	Olot/91[g] Spain	CH-1a[h] China	MD-001[i] Taiwan	Kitasato[j] Japan	LDV[k] Strain C	EAV[l] Bucyrus
ORF2	99	63	62	59	94	97	58	60	NA	32	NS
ORF3	99	60	57	54	88	94	56	55	55	28	NS
ORF4	99	70	69	68	91	94	68	67	67	30	NS
ORF5	99	55	54	52	93	93	54	54	52	47	NS
ORF6	100	79	78	81	94	97	80	79	77	53	23
ORF7	100	64	57	59	97	99	60	59	58	44	20

NS = non significant (<20%); NA = non available
Sequence data were taken from:
[a]Meulenberg et al. [71], EMBL/GenBank accession no. M96262;
[b]Conzelmann et al. [18], EMBL/GenBank accession no. L04493;
[c]Murtaugh et al. [81], EMBL/GenBank accession no. U00153;
[d]Meng et al. [64, 65], EMBL/GenBank accession no. U03040;
[e]Mardassi et al. [61], GenBank accession no. U64928, AF003345, AF003344, AF003343;
[f]Madsen et al. [55], GenBank accession no. AJ223078, before the introduction in Denmark of the american PRRSV MLV vaccine strain;
[g]PlanaDuran et al. [93], GenBank accession no. X92942;
[h]Tong et al. (unpubl.), GenBank accession no. AF132118;
[i]Chueh and Lee (unpubl.), GenBank accession no. AF121131;
[j]Takikawa (unpubl.), GenBank accession no. AB023782;
[k]Godeny et al. [39], GenBank accession no. L13298;
[l]den Boon et al. [30], GenBank accession no. X53459
This table is partly adapted from Meulenberg et al. [77]

Variability in the major non-glycosylated structural proteins

The non-glycosylated M protein is the most conserved structural protein with 78 to 81% aa identity between the North American and European PRRSV strains [37, 65, 81]. The percentage of aa identity is greater than 96% when comparing strains from the same continent [37, 65]. However, antigenic variability can be detected between strains of both continents by MAbs specific to the M protein [23, 57].

The N protein of PRRSV is also encoded by a relatively well conserved region of the viral genome, since a high degree of aa sequence identity has been observed amongst the N protein of North American (96–100%) and European (94–99%) strains [65, 106]. When compared to the homologous sequences of the European prototype strain LV, the N protein of the North American strains exhibits only 63% and 59% identity at the nucleotides (nt) and aa levels, respectively [59, 61, 65, 81]. Such relatively high divergence resulted from a number of nt substitutions, insertions or deletions, making the North American N protein five aa shorter than that of the LV strain [59]. The nt substitutions are randomly distributed, but are more frequent in the first half of the N protein gene. Two aa stretches (STAPM and SQGAS), situated respectively at the N-terminus and C-terminus of the N protein in the European strains, are missing in the N protein sequence of the North American strains [59, 65].

In view of the genomic stability of the N protein genes of PRRSV strains from the same continent and the identification of highly conserved regions shared by strains of both continents, RT-PCR methods using oligonucleotide primers of the N gene have been designed by several groups for detection of PRRSV from pig clinical specimens including lungs, lymphoid tissues, serum and semen, with higher sensitivities than isolation in cell cultures [16, 60, 86, 105].

MAbs specific to the N protein of PRRSV, recognized epitopes specific to or shared by the North American and European isolates [23, 31, 56, 79, 83]. Minor variations in the immunoreactivity patterns to N specific MAbs have been described amongst strains from the same continent [23, 31, 127]. However, the MAb SDOW17, which recognises a common epitope of the N protein of the European and North American strains, failed to react with 1 to 2% of the North American strains tested, including the tissue culture attenuated PrimePac PRRS vaccine strain (Schering-Plough Animal Health) [85]. This specific vaccine strain is recognized by MAb SR30, which reacts with all the North American strains tested to date [85]. In Canada, three distinct antigenic profiles were identified by comparing the IIF reactivities of anti-N and anti-M MAbs to field isolates, an attenuated U.S. vaccine (Ingelvac PRRS MLV) strain and two European reference strains of PRRSV [23]. As previously mentionned, a highly conserved region consisting of 54 aa residues, shared by the North American and European isolates, has been mapped to the middle third (aa position 47–93) of the N protein [79, 97, 125]. This fragment defines a discontinuous antigenic domain of the N protein [79].

The 3′-terminal noncoding region (151 nt) of the North American isolates of PRRSV is 22 nt longer than that of the European strains [59, 65]. The aligned

nt sequence of this noncoding region exhibits an overall identity of 59% with that of the European strains. RT-PCR experiments, using specific or common antisense oligonucleotide primers designed within this genomic region, permit the ability to distinguish PRRSV strains from different geographic origins [59, 60]. Furthermore, differentiation between the North American and European isolates, as well as differentiation between the field isolates and the MLV vaccine strain, could be achieved by cutting PCR-amplified products encompassing both ORF 6 and 7 genes with four restriction enzymes [37]. With respect to the restriction enzymes, *Bsa*JI and *Alu*I were deemed the most appropriate restriction enzymes for distinguishing the vaccine ATCC VR-2332 strain from the field isolates. The results obtained suggest that determining the restriction fragment length polymorphism profiles of the genomic region covering the ORFs 6 and 7 genes may be a valuable tool for differentiating between PRRSV isolates. Moreover, a certain correlation could be made between antigenic variation identified by the reactivity to anti-M and anti-N MAbs, and the RFLP profiles determined by the various restriction enzymes, suggesting that some of the nt substitutions lead to aa changes affecting the epitopes recognized by these MAbs [37]. The fact that the genomic region corresponding to ORFs 6 and 7 genes of PRRSV has been shown to be a genomic area with a higher sequence homology than ORFs 3 to 5, makes it a region of great interest to identify emerging PRRSV strains [37, 86].

Variability in the major envelope glycoprotein

Comparative studies of different European and North American PRRSV isolates show that the ORF5-encoded glycoprotein is the most heterogeneous structural polypeptide [46, 61, 64, 90, 106]. Nucleotide sequence analysis revealed 88 to 99% aa identity among strains from the same continent, and only 52–55% aa identity between North American and European strains. Thus, in view of their ORF5-encoded protein, PRRSV strains from both continents differ from each other as much as they differ from LDV (Table 2) [61, 72, 77]. Most of the aa substitutions observed amongst strains from the same continent are clustered in a hypervariable region (between aa 26 and 39) adjacent to the amino-terminal signal sequence (Fig. 4), which also involves N-linked glycosylation sites varying from none to three [3, 64, 90]. This may explain, in part, the difference in the apparent M_r of GP$_5$ of PRRSV isolates on SDS-polyacrylamide gels [57]. Such hypervariable region has been also identified for the corresponding protein of EAV and LDV [34, 38] with variations in the number of potential N-linked glycosylation sites. In the case of LDV, data suggested that the level of glycosylation of the major envelope associated glycoprotein may be implicated in the pathogenicity of the various strains [34, 53].

Recently, the reactivity patterns of strain specific hyperimmune polyclonal anti-ORF5 sera and two panels of anti-GP$_5$ MAbs indicated that GP$_5$ of North American field isolates are also subjected to antigenic variations [90, 129]. The data obtained suggest that neutralizing epitopes, independent of conformation

and glycosylation, as well as conformationally dependent epitopes, are associated with the antigenic variability of the GP_5 of PRRSV.

Restriction fragment length polymorphism (RFLP) analysis of RT-PCR amplified DNA fragments from clinical specimens encompassing ORF5 (approximately 800 bp in length) was found to be a suitable test for differentiating a PRRSV vaccine strain from other North American field strains [122]. Indeed, it has been demonstrated that the vaccine virus strain and its parent virus, ATCC VR-2332 strain, were distinguishable from field isolates by their *Mlu*I pattern, and the combined *Hinc*II/ *Sac*II gel patterns. Only the vaccine/ATCC VR-2332 viruses have an alanine (A) at residue 137 in a moderately conserved region of ORF5 [3]. The consensus sequence has a serine (S) at residue 137 for all the PRRSV field strains examined so far. This preference for S at residue 137 explains why only the vaccine/ATCC VR-2332 viruses have an apparently unique *Mlu*I site in ORF5. However, a second modified live PRRS vaccine virus (PrimePac PRRS, Schering-Plough Animal Health), is not distinguishable using these RFLP patterns.

Variability in the minor viral glycoproteins

Based on sequencing analysis data, the degrees of aa identity amongst the US PRRSV isolates varied from 91 to 99% for GP_2, 86 to 98% for GP_3, and 92 to 99% for GP_4 [46, 61, 64, 80]. With approximately 54 to 60% aa identity between the North American and European isolates of PRRSV, GP_3 is regarded as one of the least conserved proteins amongst PRRSV strains [61, 81], with most of the variations located at the N-terminus. In fact, only 29% aa identity was found within the 35 most N-terminal residues between strains from the two continents. Interestingly, despite these extensive aa changes, the potential N-linked glycosylation sites, as well as the general hydropathy profiles of the ORF3 product, are highly conserved. As previously mentioned, the GP_3 of the North American strains has a C-terminal deletion of 12 aa compared to LV [61, 64, 80].

According to a study using UK PRRSV isolates, the ORF3 product has a hydrophilic hypervariable region proximate to the C-terminal region that overlaps with ORF4, resulting in a hypervariable region situated at the N-terminal extremity of GP_4 [32, 47]. This region of GP_4 has been associated with the putative neutralizing domains of LV [76]. It was reported that a motif consisting of nine aa (positions 59–67) forms the core of this putative neutralizing domain, since isolates with aa deletion(s) or substitution(s) in these positions failed to react with a panel of MAbs specific to GP_4 of LV.

Protein specificity of the immune response and vaccines

The PRRSV is responsible for two clinical syndromes (respiratory distress and reproductive problems) for which the pathophysiology remains partly unknown. Most of the pigs are probably infected via aerosols [2, 15, 42], although the semen of infected boars is also a major source of infection of gilts and sows [16]. In both cases, PRRSV-infected animals developped humoral and cellular immune responses, but there is still some concerns as whether the immunity in convales-

cent animals is really protective. In fact, following infection of porcine alveolar macrophages and monocytes, and dissemination of the virus to the systemic lymphoid organs, the viremia can be extended and may last for about 6 weeks despite the presence of high IgG antibodies circulating [54, 84]. Virus persistence in the lymphoid tissues and semen is also an hallmark of PRRSV infection [43, 44, 52], as it is also the case for other arteriviruses [92]. Thus, cellular immunity mechanisms may also be involved in the protection against PRRSV infection. An increased in CD4$^+$ lymphocytes, T-cell dependent cytotoxicity and NK cell activity has been reported [7, 10, 115]. Furthermore, antigen-specific lymphoproliferation can be determined with in vitro assays [7, 115]. Understanding the mechanisms that lead to virus persistence is crucial for the development of an effective vaccine for the prevention of PRRSV infection.

Live attenuated (RespPRRS, Boehringer Ingelheim Inc; PrimePac PRRS, Schering-Plough Animal Health.) and killed (CyBlue, Laboritorios Sobrino, S.A.) PRRS vaccines are commercially available to protect pigs against PRRSV infection. In comparison to the killed vaccines which only provided limited protection against PRRSV infection, the attenuated live virus (MLV) vaccines shorten the viremia of virulent challenge virus in immunized pigs, and induce an immunity against the disease which lasts longer and is more effective [2, 102]. However, these MLV vaccines are still able to cause viremia and thus can spread to other pigs, as recently reported in Denmark [13, 55]. The MLV vaccines do not prevent reinfection, since the field virus does not induce a lifelong immunity either, and do not allow serological discrimination between vaccinated and naturally-infected pigs.

Several research groups have been involved in the study of the humoral immune response triggered by each of the major structural components of PRRSV, but only few information is yet available on the characterization of the cellular immune response. Alternative vaccines have been developed and tested experimentally. Genes coding for the major structural proteins of PRRSV have been cloned in *E. coli* [41, 51, 54, 62] and baculovirus [24, 50, 73] vectors to produce the viral proteins. However, immunization with isolated or combined viral proteins did not result in protective immunity [89, 93]. More recently, promising results were obtained using recombinant vaccinia viruses [10, 104, 124] and adenoviruses [40,63], but additionnal information on the importance of antigenic determinants of each of the virus structural proteins are missing in order to interprete the significance of the immunity induced by such recombinant viruses.

The kinetics of appearance of antibodies directed to the major structural proteins N, M, and GP$_5$ of PRRSV was followed in pigs naturally and experimentally exposed to PRRSV virus. Specific IgM antibody titers are first detected by IIF at the end of the first week of PRRSV infection; titers peak by day 14 to 21 p.i. (IIF titers of 256 to 1024), then rapidly decrease to undetectable levels by day 35 to 42 p.i. [54]. On the other hand, specific IgG antibody titers peak by day 21 to 28 p.i. (IIF titers of 1024 to 4096) and remain unchanged for 3 to 6 months, residual antibody titers being detected for up to one year in convalescent pigs [54, 84, 128]. Virus neutralizing (VN) antibody titers > 8 are not detected until 3 to

4 weeks p.i. [54, 128]. The results obtained by Western immunoblotting analysis with field sera, using purified virus and *E. coli*-expressed ORFs 5 to 7 gene products, showed that the early immunological response of infected pigs is directed mainly to the N protein, a positive response being detected by the end of the first week p.i. [54, 84, 128]. Antibodies directed against the envelope GP_5 protein also appear by day 7 p.i., whereas antibodies directed against the membrane M protein can only be detected by the end of the second week p.i. [54, 84]. The antibody response to the M and GP_5 varies considerably amongst pigs from the same herds and antibody titers decline much more rapidly than those directed to the N protein [84, 128].

Since the majority of antibodies produced during PRRSV infection in pigs are specific for the N protein, for which major antigenic determinants are relatively well conserved amongst strains from the same continent, the N protein has been targeted as a suitable candidate for the detection of virus-specific antibodies and diagnosis of the disease [54, 67, 84, 128]. The use of *Escherichia coli* and baculovirus systems has been reported for the genetic expression of recombinant N protein in order to apply it as antigen in indirect or competitive ELISA for the detection of antibodies against PRRSV [19, 24, 50, 73]. The sensitivities and specificities of such ELISA tests were comparable to those using purified and detergent-treated virus preparations [19, 24].

To determine the structural protein(s) of PRRSV involved in the production of virus neutralizing antibodies following clinical infection, a panel of pooled sera obtained from different pig farms in Quebec was screened by ELISA and Western immunoblotting using recombinant *E. coli*-expressed glutathione S-transferase (GST) fusion proteins [41]. A correlation between virus neutralization titers of convalescent sera and the presence of anti-GP_5 antibodies was demonstrated, but such a correlation was not found in the cases of anti-GP_3, anti-GP_4, and anti-N specific antibodies. As well, none of the anti-M MAbs yet characterized have demonstrated a neutralizing activity [23, 37, 57].

On the other hand, the role of the ORF3 product in the life cycle of arteriviruses remains unknown. Therefore, full characterization of this viral protein is required. Apart from its possible nonstructural nature, recent reports indicate that the ORF3 product is highly antigenic [40, 41], and in one challenge study, this protein was shown to provide protection for piglets against PRRSV infection in the absence of a noticeable neutralizing antibody response [93]. In the same study, it has been demonstrated that immunization of piglets with recombinant N protein does not confer protection against a challenge infection with a homologous PRRSV strain [93]. Consequently, the N protein has not been retained as a suitable candidate for a subunit recombinant vaccine.

More recently, interesting data were obtained on the putative role of the GP_5 protein in inducing a protective immune response. Indeed, DNA immunization with a plasmid encoding the GP_5 of PRRSV, under the control of a human cytomegalovirus promoter has been shown to induce anti-GP_5 specific neutralizing antibodies in pigs and BALB/c mice [89]. Peripheral blood mononuclear cells obtained from DNA vaccinated pigs underwent blastogenic transformation in the

presence of *E. coli*-expressed recombinant ORF5 encoded protein, indicating the specificity of the cellular immune response to GP_5. Following a massive intra-tracheal challenge with the virulent IAF-Klop strain of PRRSV, DNA-vaccinated pigs were protected from generalised viraemia and the development of typical macroscopic lung lesions [89]. These clinical manifestations were present in unvaccinated, virus-challenged controls, and in pigs that were immunized with *E.coli*-expressed GST-ORF5 recombinant fusion protein. Similar studies have not been yet conducted for the ORF4- and ORF2-encoded proteins. Particularly, the role of GP_4 in protection should not be neglected in view of data which have been obtained with neutralizing anti-GP4 MAbs suggesting that this protein is most probably exposed at the surface of the virion and, thus, may be involved in virus-cell interactions [76].

The role of cell-mediated immunity (CMI) in protection against viral diseases has been widely documented. The identification of antigens recognized by T cells has proven to be useful in the design of subunit vaccines used for the induction of an effective immune response to viruses. In the past few years, it has been demonstrated that infected pigs develop specific lymphocyte proliferation and delayed-type hypersensitivity responses to PRRSV [7, 89, 115]. Recently, expression of individual polypeptides of the PRRSV isolate ATCC VR-2332 as a fusion protein using the vaccinia expression system, and the electrophoretic separation of the PRRSV structural polypeptides, allowed for identification of the M protein as the major PRRSV polypeptide recognized by porcine T cells [10].

Conclusion

The role of the various PRRSV envelope glycoproteins in viral attachment to permissive cells, viral pathogenesis, apoptosis, antibody dependent enhancement phenomena, cellular immune response, and protection, has yet to be determined. Understanding the properties of the structural proteins and their role in virus assembly remains as one of the major challenges facing the study of the molecular biology of this new porcine virus. The recently developed infectious cDNA clones of PRRSV [78], construction of chimeric viruses between EAV/LDV and PRRSV [103], and the generation of deletion mutants [36, 123, 125], are extremely valuable tools for use in studies focusing on the various functions of the viral structural proteins. Production of MAbs to the minor structural glycoproteins and to the conformationally dependent epitopes of the GP_5 major envelope glycoprotein are still needed to understand the biological functions of the various structural components of the virus. Epitope mapping is necessary to identify the antigenic domains of the structural proteins involved in the development of a protective immune response. Epitope mapping is also necessary to identify the antigenic variability associated with virus persistence. Genetically engineered vaccines can be designed to discriminate between vaccinated and naturally-infected pigs [89, 93]. However, to elicit a protective response, researchers will have to target antigens which are not only involved in the production of virus neutralizing antibodies, but also those antigens that are involved in the induction of an effective CMI response

[10]. The role of mucosal immunity should also not be neglected, as it may be largely involved in the establishment of protective immunity against opportunistic infections that are favoured following early pulmonary alveolar macrophages depletion by PRRSV. Eliciting effective mucosal immunity to PRRSV could also interfere with the establishment of a persistent infection.

Acknowledgements

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