The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis: A Review

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating viral disease affecting swine industry worldwide. Its viral etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV), possesses a RNA viral genome which contains nine open reading frames (ORFs). The ORF1a and ORF1b replicase-associated genes encode the polyproteins pp1a and pp1ab, respectively. The pp1a is processed in nine non-structural proteins (nsp): nsp1α, nsp1β, and nsp2 to nsp8. Proteolytic cleavage of pp1ab generates products nsp9 to nsp12. The pp1a cleavage products possess proteolytic activities and are responsible for the processing and cleavage of pp1a and pp1ab into nsp products; whereas nsp9 to nsp12 are involved in virus transcription and replication. The 3' end of the viral genome encodes four minor and three major structural proteins. The GP_{2a}, GP_3 and GP_4 (encoded by ORF2a, 3, and 4), are glycosylated membrane associated minor structural proteins. The fourth minor structural protein, the E protein (encoded by ORF2b), is an unglycosylated and membrane associated protein. The viral envelope contains two major structural proteins: a glycosylated major envelope protein GP_5 (encoded by ORF5), and an unglycosylated membrane M protein (encoded by ORF6). The third major structural protein is the nucleocapsid N protein (encoded by ORF7). All PRRSV non-structural and structural proteins are essential for virus replication, and PRRSV infectivity is relatively intolerant to subtle changes within the structural proteins. Overall, the available data indicate that PRRSV virulence is multigenic and resides in both the non-structural and structural viral proteins. In the following manuscript, the molecular characteristics, biological and immunological functions of the PRRSV structural and non-structural proteins and their involvement in the virus pathogenesis are summarized.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is present worldwide and is the most economically important infectious disease of swine production (Neumann et al., 2005). PRRS disease was first described in the United States in 1987 (Keffaber, 1989; Loula, 1991) and a few years later in the Netherlands (Wensvoort et al., 1991). The disease has many clinical manifestations but the two most prevalent are severe reproductive failure in sows and gilts (characterised by late-term abortions, an increased number of stillborns, mummified and weak-born pigs) (Albina, 1997; Bilodeau et al., 1991; Christianson, 1992; Keffaber, 1989; Loula, 1991; Pol et al., 1991) and respiratory problems in pigs of all ages associated with a non-specific lymphomononuclear interstitial pneumonitis (Albina, 1997; Bilodeau et al., 1991; Collins et al., 1992; Halbur et al., 1996b; Keffaber, 1989; Loula, 1991; Rossow, 1994).

The etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV) was identified in 1991 by investigators in the Netherlands and shortly after in the United States (Benfield et al., 1992; Collins et al., 1992; Wensvoort et al., 1991). The PRRSV is an enveloped, single-stranded positive sense RNA virus, approximately 50–65 nm in diameter that is classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus of mice (LDV), and simian hemorrhagic fever virus (SHFV) (Benfield et al., 1992; Cavanagh, 1997)**.** PRRSV genome is approximately 15 kb in length. The RNA viral genome is capped at the 5' end and polyadenylated at the 3' termini and encodes at least nine open reading frames (ORFs) (Dea et al., 2000a), each of which is expressed via the generation of a 3'-coterminal nested set of subgenomic (sg) mRNAs (Allende et al., 1999; Gorbalenya et al., 2006). ORFs1a and 1b, which take almost 2/3 of the genome,

encode non-structural proteins (nsp), such as RNA polymerase, that are required for virus replication (Allende et al., 1999). At least 12 nsps are generated as a result of serial cleavages of two polyproteins expressed from ORF1a and ORF1ab, which RNA is transcripted by a ribosomal frame shift between ORF1a and 1b (Snijder, 1998; Snijder, Meulenberg, 1998). Three N-glycosylated minor envelope proteins (GP_{2a}, GP_3, and GP_4) are translated from ORF2a, 3, and 4 and form heterotrimers by disulfide linkage (Wissink et al., 2005). ORF2b, which is completely embedded in ORF2a, encodes another non-glycosylated minor protein named E (Wu et al., 2001). ORF5 encodes the major envelope glycoprotein (GP_5) that forms a heterodimer with the membrane non-glycosylated protein (M) encoded by ORF6 (Mardassi et al., 1996). The viral capsid is composed of only one nucleocapsid protein (N), which is encoded by ORF7 and is highly immunogenic in infected animals (Dea et al., 2000a).

The virus is genetically, antigenically, and pathogenically heterogenic (Dea et al., 2000a; Meng, 2000). Currently, PRRSV isolates are divided into two distinct genotypes, represented by Lelystad virus (LV) in Europe (Wensvoort et al., 1991) and ATCC VR-2332 in North America (Benfield et al., 1992; Dea et al., 1992). Even though European (EU) and North American (NA) isolates possess several biological and immunological similarities, including a nearly identical genome organization, both strains are genetically and antigenically distinct (Magar et al., 1995; Mardassi et al., 1994a; Nelsen et al., 1999). Sequence analyses have shown approximately a 60% nucleotide identity between the Type I (European - EU) and Type II (North American - NA) genotypes (Allende et al., 1999; Forsberg, 2005; Hanada et al., 2005; Nelsen et al., 1999; Plagemann, 2003). Several studies have shown that a high degree of genetic variability exists within the NA-type of PRRSV (Andreyev et al., 1997; Gagnon, Dea, 1998; Kapur et al., 1996;

Meng et al., 1995a; Morozov et al., 1995; Pirzadeh et al., 1998). While early studies suggested that a lower degree of variability might exist in EU PRRSV type (Drew et al., 1997; Suarez et al., 1996b), later studies in some countries, such as Denmark and Italy, have reported a high divergence within EU-type isolates (Forsberg et al., 2001; Forsberg et al., 2002; Madsen et al., 1998; Nielsen et al., 2000; Oleksiewicz et al., 2000). Recently, the emergence of potential highly pathogenic variant of PRRSV as the possible cause for large-scale outbreaks with high mortality in China was reported in 2006 (Li et al., 2007; Tian et al., 2007). It is now obvious that PRRSV isolates showed considerable genetic variation and could be classified in several phylogenetic clusters within each genotypes (Fang et al., 2007; Kiss et al., 2006; Mateu et al., 2006; Yoshii et al., 2005).

In the following manuscript, the molecular characteristics, biological and immunological functions of the PRRSV structural and non-structural proteins and their involvement in the virus pathogenesis are described.

Virus morphogenesis

Earlier studies indicate that the N protein forms a spheric icosahedral capsid core of 20-30 nm in diameter, which is surrounded by a lipid envelope containing the viral envelope proteins, yielding a relatively smooth spherical virion of about 60 nm in diameter (Benfield et al., 1992; Dea et al., 2000a; Doan, Dokland, 2003a, b). Recently, Spilman et al. (Spilman et al., 2009) have described the structure of PRRSV virions based on cryo-electron microscopy (EM) analysis and tomographic reconstruction of virions grown in MARC-145 cells. They have reported that the virus has a pleomorphic morphology, a spherical to oval shape with a size

ranging from about 50 to 65 nm, a hollow, layered core of around 40 nm diameter and a smooth outer surface studded with a few envelope protein complexes. The structural analysis indicates that the PRRSV core consists of an helical nucleocapsid (see schematic representation in Figure 1) wrapped up into a hollow ball (Spilman et al., 2009), unlike previously mentioned studies (Benfield et al., 1992; Dea et al., 2000a; Doan, Dokland, 2003a, b). The latest results are not surprising since other members of the *Nidovirales,* such as coronavirus, have been known to possess a helicoidal capsid. Being an enveloped virus, PRRSV survivability outside the host is affected by temperature, pH and exposure to detergents. It is known that PRRSV can survive for extended intervals at temperatures ranging from -70 to -20^{O}C (Benfield et al., 1992); nonetheless, viability decreases with increasing temperature. Specifically, recovery of PRRSV has been reported for up to 20 min at 56^{O}C, 24h at 37^{O}C, and 6 days at 21 ^{0}C (Benfield et al., 1992). The PRRSV remains stable at pH ranging from 6.5 to 7.5 but, infectivity is reduced at pH<6.0 or >7.65 (Bloemraad et al., 1994). Detergents are effective at reducing infectivity of the virus and lipid solvents such as chloroform and ether are particularly efficient at disrupting the viral envelope and eliminate infectious virions (Benfield et al., 1992). Even if PRRSV is relatively fragile in the environment, appropriate weather (wind, T^{O}C, humidity, etc.) may favour the transmission of the virus through aerosols up to 4.7 km distance (Dee et al., 2009).

Cells pathogenesis

PRRSV is generally believed to have a very restricted cell tropism both *in vivo* and *in vitro*. *In vivo*, the virus mainly infects well-differentiated cells of the monocyte-macrophage lineage, in particular porcine alveolar macrophages (PAM), the primary target cells of virus and

interstitial macrophages in other tissues such as heart, thymus, spleen and Peyer's patches, hepatic sinusoids, renal medullary interstitium, and adrenal gland (Beyer et al., 2000; Duan et al., 1997; Halbur et al., 1995; Halbur et al., 1996b). In addition to macrophages, PRRSV RNA and nucleocapsid protein were found by *in situ* hybridization (ISH) and immunohistochemistry, respectively, in testicular germ cells, endothelial cells in the heart, interdigitating cells in the thymus, dendritic cells in the spleen and Peyer's patches (Halbur et al., 1996b; Sur et al., 1997). In experimentally infected gnotobiotic pigs, PRRSV antigens were found in bronchiolar epithelial cells, arteriolar endothelial cells, monocytes as well as interstitial, alveolar, and intravascular macrophages using an immuno-gold-silver immunohistochemical staining (Rossow et al., 1996). PRRSV RNAs and antigens were also found in bronchiolar epithelial cells (Pol et al., 1991), epithelium-like cells of alveolar ducts (Magar et al., 1993), and pneumocytes (Cheon et al., 1997; Pol et al., 1991) in the naturally infected pigs whereas it was not found in these types of cells in the experimentally infected pig (Teifke et al., 2001). Tissues such as lung, lymphoid tissues, Peyer's patches, and kidney were also the preferable organ targets of PRRSV infection (Haynes et al., 1997; Sur et al., 1996).

PRRSV was originally isolated on primary cultures of PAMs (Wensvoort et al., 1991) and so far, these cells as well as blood monocytes (Voicu et al., 1994), remain the only porcine cells that can effectively be used for viral propagation. Two non-porcine permissive cell subclones of MA104 monkey kidney cell line, the MARC-145 and CL2621 cells (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993) are also routinely used for *in vitro* propagation of wild and vaccine PRRSV strains. Flow cytometric and fluorescence antibody (FA) analyses of PRRSV antigen expression revealed distinct primary and secondary infection phases in MARC-145 cells (Cafruny et al., 2006). PRRSV antigen is randomly expressed in a small amount of

cells during the primary phase of infection (up to about 20–22 h p.i.), but the logarithmic infection phase (days 2–3 p.i.) is characterized by secondary spread to clusters of cells. The formation of secondary clusters of PRRSV-infected cells preceded the development of CPE in MARC-145 cells. The primary and secondary PRRSV infection phases are inhibited by colchicine and cytochalasin D, which demonstrate a critical role of the cytoskeleton in viral permissiveness as well as cell-to-cell transmission (Cafruny et al., 2006). Cellular expression of actin also appeared to correlate with PRRSV resistance, suggesting a second role of the actin cytoskeleton as a potential barrier to cell-to-cell transmission (Cafruny et al., 2006).

How the virus enters into the cells was firstly reported in 1996 (Kreutz, Ackermann, 1996). It was postulated that since the direct fusion of the PRRSV envelope with the cellular membrane was not observed at any time, PRRSV entry most occurs by receptor-mediated endocytosis. In 1998, this hypothesis was confirmed (Duan et al., 1998) and a PRRSV receptor was identified on PAM by generation of PAM-specific monoclonal antibodies. Several viral receptors for PRRSV have been described, including heparin sulphate for binding, sialoadhesin for binding and internalization, and vimentin (Delputte, Nauwynck, 2004; Delputte et al., 2007a; Delputte et al., 2007b; Delputte et al., 2002; Kim et al., 2006). However, additional factors are needed because the expression of both receptors in non-permissive cells results in virus internalization but not in virus uncoating and productive infection. Recently, a molecule that is expressed exclusively on cells of a monocytic lineage the CD163, has been identified as a possible cellular receptor for PRRSV (Calvert et al., 2007; Patton et al., 2009; Van Gorp et al., 2008). Furthermore, susceptibility of macrophages to PRRSV infection was previously shown to be associated with high expression of CD163 (Lopez-Fuertes et al., 2000). The role of CD163 and its interaction with sialoadhesin during PRRSV infection of macrophages were investigated

192 (Van Gorp et al., 2008). Both sialoadhesin and CD163 are involved in infection of macrophages
193 with different PRRSV strains (Van Gorp et al., 2008). Thus, sialoadhesin is confirmed as a
194 PRRSV internalization receptor and CD163 is shown to be involved in PRRSV infection,
195 probably during uncoating (Van Gorp et al., 2008). Finally, non-permissive cells expressing both
196 sialoadhesin and CD163 are clearly more susceptible to PRRSV infection and produce more
197 virus (10 to 100 time more) compared with cells expressing only CD163 (Van Gorp et al., 2008).
198 The interaction between the viral glycoproteins and CD163 receptor where characterized using
199 co-immunoprecipitation (co-IP) assay using monospecific antibodies (Das et al., 2010). It was
200 found that the GP_{2a} and GP_4 proteins interact with the CD163 molecule (Das et al., 2010). The
201 carboxy-terminal 223 residues of CD163 molecule are not required for interactions with either
202 the GP_{2a} or the GP_4 protein, although these residues are required for conferring susceptibility to
203 PRRSV infection in BHK-21 cells. Overall, it was postulated that the GP_4 protein is critical for
204 mediating interglycoprotein interactions and along with GP_{2a}, serves as the viral attachment
205 protein that is responsible for mediating interactions with CD163 for virus entry into susceptible
206 host cell (Das et al., 2010). The CD151 molecule was identified by RNA-ligand screening of a
207 MARC-145 cell expression library to be a PRRSV 3' UTR RNA-binding protein
208 (Shanmukhappa et al., 2007). The CD151 is a member of the tetraspanin superfamily, which has
209 several cellular functions that include cell signaling, cell activation and platelet aggregation
210 (Fitter et al., 1999; Hasegawa et al., 1998; Sincock et al., 1999). Transfection of CD151 rendered
211 BHK-21, a non-susceptible cell line, susceptible to PRRSV infection. The transfection of siRNA
212 against CD151 inhibited PRRSV infection into MARC-145 cells. Additionally, polyclonal anti-
213 CD151 antibody (Ab) completely blocked PRRSV infection into MARC-145 cells

(Shanmukhappa et al., 2007). These results suggest that CD151 plays a critical role in PRRSV infection *in vitro*.

PRRSV induces apoptosis both *in vitro* and *in vivo* (Choi, Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller, Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996a; Sur et al., 1997; Sur et al., 1998). PRRSV infection of both MARC-145 cells and PAM results in apoptosis which is characterized by morphological changes, DNA fragmentation and specific caspase activation. PRRSV seem to be able to induce apoptosis directly (in infected cells) or indirectly (in bystander non infected cells) in infected animals. For example, GP_5 protein of PRRSV has been shown to be an apoptosis inducer in GP_5 expressing cells (Gagnon et al., 2003; Suarez et al., 1996a). However, others have shown that PRRSV induces apoptosis mostly in uninfected bystander cells both *in vitro* and *in vivo* and that the majority of apoptotic cells in lung lavages, lungs, and lymphoid tissues are not positive for PRRSV antigens and RNA (Choi, Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller, Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996a; Sur et al., 1997; Sur et al., 1998). It was reported that alveolar macrophages from PRRSV-infected pigs show a significantly increased apoptotic rate (22–34%) compared to porcine circovirus 2 infected alveolar macrophages (3%) (Chang et al., 2005). Given the fact that only 5–10% of alveolar macrophages were PRRSV-infected, these authors suggested that TNF-α or GP_5 released from PRRSV-infected cells induce apoptosis in non-infected cells. Another study from the same group demonstrated that increased FasL expression in PRRSV-infected macrophages caused apoptosis in co-cultured swine splenic lymphocytes (Chang et al., 2007). A previous report showed that the majority of apoptotic cells are characterized by both early and late apoptosis markers (Lee, Kleiboeker, 2007). Ultimately, these authors demonstrated that PRRSV induce apoptosis through a mitochondrial-mediated pathway. Two oppositely directed

237 sets of reactions are switched on in PRRSV-infected macrophages *in vitro*: at first, the balance is
238 driven towards anti-apoptosis, but finally, PRRSV-infected macrophages die by apoptosis
239 (Costers et al., 2008). Both anti- and pro-apoptotic effects were not only observed in PRRSV-
240 infected macrophages, but also in PRRSV-infected MARC-145 cells (Costers et al., 2008).

The nucleocapsid protein (N)

The unglycosylated N protein possesses 123 to 128 amino acids (aa) depending on the genotype of the strains (NA versus EU, respectively) with a molecular weight of 15 kDa, and is encoded by ORF7 viral gene (Figure 2). The N protein of PRRSV is a multifunctional protein (Table 1). It is a basic protein with an isoelectric point of 10.4 and was shown to be a serine phosphoprotein (Wootton et al., 2002). Given that EAV and coronavirus nucleocapsid proteins are also phosphoproteins (Laude et al., 1995; Zeegers et al., 1976), phosphorylation of the nucleocapsid protein appears to be a common feature of nidoviruses. Phosphorylation of the N protein occurs, at position 120 (Figure 3) in PRRSV-infected cells and also in ORF7 gene-transfected cells, where the N protein is synthesized in the absence of other viral components (Wootton et al., 2002). Evolutionary conservation of this post translational modification suggests that phosphorylation of the N protein may be of significant biological importance for the virus and, therefore, it warranties further investigation. Multifunctional proteins are often regulated by phosphorylation, and this phenomenon is particularly common to positively charged, nucleic acid binding proteins that make up the nucleocapsids of the coronavirus (Laude et al., 1995; Yoo et al., 1992), hepatitis B virus (Roossinck, Siddiqui, 1987), influenza virus (Arrese, Portela, 1996; Yang, Zhang, 1999), rabies virus (McNabb, Courtney, 1992; Yang, Zhang, 1999), herpes simplex virus (McNabb, Courtney, 1992), and parvovirus (Maroto et al., 2000). Since it is well documented that phosphorylation is involved in regulating the activities of proteins at multiple levels, including nucleic acid binding, oligomerization, and nuclear transport, phosphorylation may likewise affect these properties of the PRRSV N protein.

263 The N protein is highly immunogenic in pigs (Loemba et al., 1996; Meulenberg et al.,
264 1995a, b) and in mice (Drew et al., 1995; Nelson et al., 1993; Rodriguez et al., 1997; van
265 Nieuwstadt et al., 1996). Several groups have generated anti-N monoclonal antibodies (mAbs)
266 that recognise epitopes specific to or shared by NA and EU isolates (Dea et al., 1996; Drew et
267 al., 1995; Meulenberg et al., 1998; Nelson et al., 1993; Wootton et al., 1998). Based on the
268 immunoreactivity of N protein deletion mutants with panels of N-specific mAbs, five domains of
269 antigenic importance have been identified for a reference NA strain, four of them being localized
270 at aa 30 to 52, 37 to 52, 69 to 112, and 112 to 123, respectively (Figure 3). Other mAbs revealed
271 the presence of a common conformational antigenic site localised in the central region (aa 52–
272 69) of the protein (Rodriguez et al., 1997; Wootton et al., 1998). Mutational analysis revealed
273 that the carboxyl-terminus of N plays a critical role in the formation of the conformational
274 epitopes, and a domain comprised of the 11 C-terminal-most aa (residues 112 to 123) (Figure 3)
275 was shown to be essential for binding of N-specific conformation-dependent mAbs (Meulenberg
276 et al., 1998; Wootton et al., 2001; Wootton et al., 1998). The region between aa 37 and 52 is well
277 conserved among isolates of both genotypes and is the most hydrophilic region of the protein
278 (Figure 3). None of the N-specific mAbs have been found to be associated with virus
279 neutralisation. Four distinct antigenic domains were identified for the reference EU LV strain
280 (Meulenberg et al., 1998). Three sites, designated A–C, contain linear epitopes, and these were
281 mapped between aa 2–12, 25–30, and 40–46, respectively. However, the fourth region,
282 designated domain D, contains conformation-dependent or discontinuous epitopes that are
283 (partially) composed of aa 51–67 and 80–90 (Meulenberg et al., 1998). The early immunological
284 response generated in PRRSV-infected pigs is directed mainly against the N protein, and this
285 response, which can be detected as early as one week postinfection (Loemba et al., 1996),

declines at a much lower rate than the one directed against the major structural proteins M and GP_5 (Yoon et al., 1995). Since the majority of antibodies produced during PRRSV infection in pigs are directed against the N protein, for which major antigenic determinants are highly conserved, the N protein has been targeted as a suitable candidate for the detection of virus-specific antibodies and diagnosis of the disease.

One of the fundamental role of the viral capsid protein is to provide a protective enclosure for the viral genome during the extracellular phase of the virus life cycle. The N protein is the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions (Wootton, Yoo, 2003). NA PRRSV N protein contains three highly conserved cysteine residues at aa positions 23, 75 and 90. Systematic mutation of the three cysteine residues at positions 23, 75, and 90, either alone or in combination, revealed that the cysteine at position 23 (Figure 3) is involved in the formation of intermolecular disulfide bonds between N proteins (Wootton, Yoo, 2003). Subsequently, the functional significance of the N cysteine residues for PRRSV infectivity was assessed using an infectious cDNA clone (Lee et al., 2005). Each cysteine at positions 23, 75, and 90 was replaced with serine and the individual mutation was incorporated into the cDNA clone such that three independent cysteine mutants were constructed. When transfected, the wild type and C75S clones induced cytopathic effects and produced infectious virus with indistinguishable plaque morphology. In contrast, the C23S mutation completely abolished infectivity of the clone, indicating that C23-mediated N protein homodimerization plays a critical role in PRRSV infectivity (Lee et al., 2005). The C90S mutation also appeared to be lethal for virus infectivity. This is an unexpected observation since cysteine 90 is irrelevant to the N protein homodimerization and because cysteine 90 is absent among the EU PRRSV isolates. The EU isolates of PRRSV contain only two cysteines at

positions 27 (involved in the formation of intermolecular disulfide bonds between N proteins) and 76. The N protein dimer (Figure 1) was shown to be stable both in the presence and absence of intermolecular disulfide linkages, indicating that non-covalent interactions also play a role in dimerization. Using a series of N protein deletion mutants fused to glutathione S-transferase (GST) in a pull-down assay, aa 30 to 37 were shown to be essential for non-covalent N-N interactions (Figure 3). Furthermore, since RNase A treatment markedly decreased N protein-binding affinity, it appears that at least *in vitro*, RNA may be involved in bridging N-N interactions. Upon translation in the cytoplasm, the N protein interacts with itself via non-covalent binding. As N is transported to the lumen of the endoplasmic reticulum (ER) and the Golgi complex, where its environment is oxidative and PRRSV maturation occurs, the N–N interaction becomes disulfide linked (Snijder, Meulenberg, 1998; Wootton, Yoo, 2003). Together, these findings demonstrate that the N protein possesses self-associative properties, and these likely provide the basis for PRRSV nucleocapsid assembly.

PRRSV, like several other RNA viruses, replicates in the cytoplasm (Benfield et al., 1992; Mardassi et al., 1994b). However, a fraction of the N protein has been found to localize into the nucleus and nucleolus during infection of PAM and MARC-145 cells (Rowland et al., 1999). Two conserved aa stretches, which are similar to sequences that resemble two classical types of nuclear localization signal (NLS), have been identified (Figure 3) in the N protein at aa positions 10 to 13 and 41 to 47 (NLS-1 or cryptic NLS and NLS-2 or functional NLS, respectively) (Rowland et al., 1999; Rowland et al., 2003). The inactivation of NLS-1 by site-directed mutagenesis or the deletion of the first 14 aa did not affect N protein localization to the nucleolus. The substitution of key lysine residues with uncharged amino acids in NLS-2 blocked nuclear/nucleolar localization. Site-directed mutagenesis identified the KKNKK sequence as

forming the core localization domain within NLS-2. The NLS-2 was shown to be functional and sufficient for the translocation and accumulation of N in the nucleolus (Rowland et al., 1999; Rowland et al., 2003; Rowland, Yoo, 2003). Proteins that localize to the nucleolus typically possess a nucleolar localization signal (NoLS) motif. A region covering aa 41–72 (Figure 3) was found to contained a NoLS sequence (Rowland et al., 2003; Yoo et al., 2003). The nucleolar localization of the N protein has also been reported for EAV (Tijms et al., 2002) and for several members of the *Coronaviridae* (Chen et al., 2002; Wurm et al., 2001). The arterivirus and coronavirus N proteins, when expressed alone or fused to the red-shifted EGFP, localize to the nucleolus, demonstrating that translocation across the nuclear pore complex (NPC) and accumulation in the nucleolus are independent of other viral proteins (Hiscox et al., 2001; Rowland, Yoo, 2003; Tijms et al., 2002; Wurm et al., 2001).

Since the Golgi apparatus is thought to be the maturation site of arteriviruses, it has been postulated that the PRRSV N protein plays dual roles during virus infection; a virion structural role in the cytoplasm and a non-structural role in the nucleus and/or nucleolus. The N protein NLS-null PRRSV-infected pigs had a significantly shorter mean duration of viremia than wild-type-infected pigs and increased production of neutralizing antibodies in infected pigs (Lee et al., 2006a; Lee et al., 2006b). However, MARC-145 cells infected with the NLS-null mutant virus produced cytopathology indistinguishable from that of wild-type virus, but with lower viral titers and smaller size plaques formations (Lee et al., 2006a; Lee et al., 2006b). Furthermore the N protein nuclear localization is associated with the virulence of PRRSV since modification in the NLS caused attenuation of the virus (Lee et al., 2006a; Lee et al., 2006b; Pei et al., 2008) suggesting that the N protein localization in the nucleus may play a role in viral pathogenesis. The precise mechanism of N protein trafficking within a cell and to the nucleolus is unknown,

355 although N protein has been shown to interact with the small nucleolar RNA (snoRNA)-
356 associated protein fibrillarin and thus may potentially localise to the nucleolus via this interaction
357 (Yoo et al., 2003). Using an *in vitro* pull-down assay, the N protein was able to bind importin-α
358 and importin-β nuclear transport proteins (Rowland, Yoo, 2003). The formation of an N
359 protein/importin complex using *in vitro* translated N demonstrates that N protein binding is
360 independent of other viral proteins. The GST-bead pull-down assay was used, to determine if the
361 principal export shuttle protein, the chromosome region maintenance 1 (CRM1), could bind N
362 protein (Rowland, Yoo, 2003). Radiolabeled N protein, prepared from virus infected cells or by
363 *in vitro* translation, was incubated with the GST-human CRM1 fusion protein immobilized on
364 glutathione-Sepharose beads. The results showed that CRM1 indeed bound to PRRSV N protein
365 (Rowland, Yoo, 2003). On the opposite (Tijms et al., 2002), it was reported that EAV N protein
366 localized to all nucleoli when cells were treated with leptomycin B (LMB), an inhibitor of
367 CRM1-dependent export. In conclusion, the N protein nuclear transport is importin-α/β-based
368 and CRM1-dependent. It is interesting that a virion structural protein from a cytoplasm-
369 replicating RNA virus interacts with a host cell transcription factor. It was shown previously that
370 the PRRSV N protein can interact with fibrillarin and co-localizes with fibrillarin in the
371 nucleolus (Yoo et al., 2003). For NA N protein, eight amino acids (IAQQNQSR) at positions 30
372 to 37 were identified as the binding domain with fibrillarin. Interestingly, this string of aa was
373 previously mapped as the region which participated in the formation of the N-N interaction
374 (Wootton, Yoo, 2003). This region corresponds to a relatively hydrophilic region of N and forms
375 part of the conformational epitope recognized by mAb SDOW17 (Wootton et al., 2001). The
376 interaction of viral capsid with the cellular transcription factor implicates a possible regulation of
377 host cell gene expression by the N protein during PRRSV infection (Song et al., 2009).

Localization of the virus capsid proteins and replication proteins in the nucleus and/or nucleolus appears to be an emerging feature of positive strand RNA viruses (Song et al., 2009). The reason why positive strand RNA virus capsid proteins localize in the nucleolus is unknown, and several hypotheses have been proposed including; i) as part of a cellular defense mechanism to sequester viral proteins away from sites of virus replication and assembly; ii) to recruit nucleolar proteins to facilitate virus replication; iii) to usurp cellular processes by disrupting the nucleolar proteome; or iv) only because the viral protein contains motifs which mimic nucleolar localization signals (NoLSs) (Hiscox, 2002, 2003, 2007; Rowland, Yoo, 2003; Uchil et al., 2006; Weidman et al., 2003).

In resume, the N protein is the sole component of the PRRSV capsid. In addition, it is a highly immunogenic protein which makes it a suitable candidate for the detection of virus-specific antibodies and subsequently for the diagnosis of the disease. The PRRSV N protein plays dual roles during virus infection; 1) a virion structural function and 2) a non-structural role in the nucleus/nucleolus, indicating that the N protein localization in these compartments may play a fundamental role in viral pathogenesis such as the regulation of cell genes expression. In a near future, further studies will have to be conducted to elucidate the role of the N protein in the nucleus/nucleolus and will certainly give new interesting insight on the cell pathogenesis of PRRSV.

The membrane envelope protein (M)

The M protein is an 18 to 19 kDa class III membrane protein composed of 174 and 173 aa for NA and EU genotypes, respectively, and is encoded by ORF6 viral gene (Figure 2). Its membrane structure consists of a core of three successive membrane-spanning domains preceded by an ectodomain of 13–18 amino acids and followed by a C-terminal endodomain of 81–87 aa (de Vries et al., 1992; Faaberg, Plagemann, 1995; Meulenberg et al., 1995a, b). As demonstrated experimentally for the closely related M protein of coronaviruses (de Haan et al., 1998; Vennema et al., 1996), the arteriviral M protein is likely to play a key role in virus assembly and budding (Table 1). The M protein is unglycosylated and is the most conserved structural protein of arteriviruses and PRRSV (Mardassi et al., 1995a, b; Meulenberg et al., 1993b). In the virion, the GP_5 and M proteins are found as a disulfide-linked heterodimer (Figure 1), which is an essential requirement for virus infectivity for arteriviruses (Delputte et al., 2002; Faaberg et al., 1995a; Mardassi et al., 1996; Snijder et al., 2003). These disulfide-linked complexes are formed in the endoplasmic reticulum (ER) of infected cells preceding or during virus assembly (de Vries et al., 1995; Dobbe et al., 2001; Faaberg et al., 1995a; Mardassi et al., 1996).

The GP_5–M heterodimerization was shown to be essential for virus infectivity and it is a key step in the assembly of EAV virion (Snijder et al., 2003). The disulfide bond between PRRSV GP_5 and M proteins presumably occurs between cysteine residues at positions 50 and 8 (EU isolates), respectively (Verheije et al., 2002). Mutating either one of these residues resulted in a complete block of particle production (Verheije et al., 2002), indicating that the covalent association of GP_5 and M is crucial for virus assembly. Using a reverse genetics approach it was shown that the two major envelope proteins, the GP_5 and M proteins are essential for the

419 formation of EAV particles (Wieringa et al., 2004). Noteworthy, it is interesting to point out that
420 the disulfide-linked ectodomains of the LDV heterodimer VP-3P-M protein (VP-3P is the GP_5
421 homolog protein of LDV) have been proposed to stabilize the virus attachment site for
422 interaction with a host cell receptor (Faaberg et al., 1995b).

423 The development of neutralizing antibodies was reported in mice inoculated with a
424 *Mycobacterium bovis* bacille Calmette-Guérin (BCG) expressing the M protein (Bastos et al.,
425 2002). More recently, three replication-defective recombinant adenoviruses were developed as
426 potential vaccine against PRRSV and evaluated in a mouse model (Jiang et al., 2006a). Three
427 groups of BALB/c mice were inoculated subcutaneously twice at 2-week intervals with the
428 recombinants adenovirus expressing PRRSV GP_5 (rAd-GP_5), M (rAd-M), and M-GP_5 fusion
429 protein (rAd-M-GP_5). The results showed that the mice inoculated with recombinant
430 adenoviruses developed PRRSV-specific antibodies and a cellular immune response at 2 weeks
431 post second inoculation (Jiang et al., 2006a). However, only mice immunized with rAd-M-GP_5
432 developed significantly higher titers of neutralizing antibodies to PRRSV and produced stronger
433 lymphocyte proliferation responses compared to mice immunized with rAd-M or rAd-GP_5 (Jiang
434 et al., 2006a). In conclusion, the presence of M protein increases the immune response against
435 GP_5 by increasing the cellular immune response and the productions of neutralizing antibodies
436 (Abs).

437 Using Pepscan technology and sera of 15 experimentally NA PRRSV infected pigs, the
438 B-cell linear epitopes were identified in some of the non-structural proteins and all of the
439 structural proteins (de Lima et al., 2006). Overall, the highest degree of immunogenicity and
440 conservation was exhibited by two epitopes identified in the C-terminal end of the M protein
441 (ORF6) (de Lima et al., 2006). These immunoreactive peptides are located at position 151–174

442 which corresponds to the C-terminus end of the endodomain of M protein. In order to confirm
443 the results demonstrated by the sequence alignment, the reactivity of both synthetic peptides was
444 further tested with sera from pigs experimentally infected with several other NA PRRSV strains
445 and all sera samples were confirmed positive. It was found that the peptide containing the
446 residues A^{161}VKQGVVNLVKYAK^{174} can be particularly useful for diagnostic purposes and one
447 attractive candidate as a negative serological marker in a PRRSV vaccine (de Lima et al., 2006).
448 Earlier study (Oleksiewicz et al., 2002), using phage-displayed technology, also reported the
449 identification of one epitope localized in the large putative endodomain of the M protein (aa
450 138–159) of a EU PRRSV strain. This epitope was recognized by sera collected very late in
451 infection (Oleksiewicz et al., 2002).
452

The major glycosylated envelope viral protein (GP_5)

One of the most variable regions of the PRRSV genome is the ORF5 gene (Figure 2) which encodes the GP_5 glycoprotein (Andreyev et al., 1997; Mardassi et al., 1995a; Meng et al., 1995b). The GP_5 is a transmembrane protein of approximately 25 kDa (Dea et al., 2000a; Gagnon et al., 2003; Meulenberg et al., 1995a). The GP_5 protein contains an N-terminal putative signal sequence which is assumed to be cleaved (Mardassi et al., 1995a; Meng et al., 1995b; Meulenberg et al., 1995a; Murtaugh et al., 1995) of approximately 30 aa, followed by an ectodomain of approximately 35 residues with a variable number of potential N-glycosylation sites, a long hydrophobic region of about 60 residues that is presumed to span the membrane one to three times, and a hydrophilic C terminus of approximately 70 aa (Meulenberg et al., 1995a). Some authors have suggested that it possesses two membrane-spanning motifs between residues 65–130 and 170–190 (Dea et al., 2000a) while others have proposed that the protein may span the membrane three times (Meulenberg, 2000). GP_5 is one of the most variable (Table 1) structural protein in the PRRSV genome (Mardassi et al., 1995a; Meng et al., 1995b) with the highest degree of diversity within one genotype: 89–94% identity among NA isolates and 87.1–99.25% identity among EU isolates (Andreyev et al., 1997; Suarez et al., 1996b). It is important to mention that some other parts of the genome also possess considerable degree of variability such as ORF3 gene and the nsp2 protein (Fang et al., 2004; Oleksiewicz et al., 2000). Interestingly, it was hypothesized that PRRSV exists during natural infection as a quasispecies distribution of related genotypes and that this variation could account for the inability of traditional approaches such as vaccination to control PRRS adequately. It was also found that multiple variants of PRRSV can exist simultaneously on farms and within individual animals

476 during natural infection (Goldberg et al., 2003). Nonetheless, the existence of quasispecies could
477 be responsible for the emergence of viruses with novel clinical and/or antigenic properties and
478 reduce the efficiency of vaccination.

479 The GP_5 protein of PRRSV is one of the most important structural proteins (Table 1)
480 exposed on the surface of the virion and contains epitopes involved in virus neutralisation and
481 protection (Ansari et al., 2006; Gonin et al., 1999; Pirzadeh, Dea, 1997, 1998; Wissink et al.,
482 2003). Monoclonal Abs (mAbs) against GP_5 are able to neutralize virus infectivity in cell culture
483 (Gonin et al., 1999; Pirzadeh, Dea, 1997; Weiland et al., 1999; Zhang et al., 1998). *In vivo*, most
484 of the neutralizing antibodies that could be found in infected animals are predominantly directed
485 against GP_5 (Gonin et al., 1999). Pigs vaccinated with genetically engineered vaccines that
486 express the GP_5 protein could produce neutralizing antibodies against PRRSV (Jiang et al.,
487 2006b; Kheyar et al., 2005; Pirzadeh, Dea, 1998) and the pigs could be partially protected
488 against generalized viremia and lung lesions development following PRRSV challenge
489 (Pirzadeh, Dea, 1998; Qiu et al., 2005; Xue et al., 2004). Pregnant gilts treated with
490 hyperimmune serum from convalescent pigs are fully protected from reproductive failure upon
491 homologous challenge which suggests that IgG can provide a protective immunity *in vivo*
492 (Osorio et al., 2002). On the other hand, other experiments have shown that GP_5 antibodies alone
493 do not provide full protection against PRRS disease even after homologous challenge (Pirzadeh,
494 Dea, 1998; Plana-Duran et al., 1997a). Consequently, GP_5 is not the only determinant of
495 protective immunity, and is particularly unlikely to be the determinant of cross protection in case
496 of heterologous challenge since it is highly variable.

497 Hydropathy profiles of the GP_5 protein predict that it possesses a signal sequence (aa 1–
498 32), an ectodomain (aa 33–63), a transmembrane region (aa 64–134), and an endodomain (aa

135–200), although there are some differences in the length of the predicted signal sequence between the NA and EU strains of PRRSV (Figure 4) (Andreyev et al., 1997; Dea et al., 2000a; Meng et al., 1995b; Meulenberg et al., 1995a; Stadejek et al., 2002; Yang et al., 1998). Comparison of the nt and aa sequences of the GP_5 protein of a large number of PRRSV strains from NA and EU strains identified a hypervariable region (aa 32–40), two variable regions (aa 57–70 and 121–130) and three conserved regions (aa 41–56, 71–120 and 131–200), and multiple potential N-glycosylation sites (Andreyev et al., 1997; Faaberg, Plagemann, 1995; Key et al., 2001; Meng et al., 1995b; Plagemann, 2004; Plagemann, 1996; Stadejek et al., 2002; Yang et al., 1998). The first potential N-glycosylation site is located in the hypervariable region (N32 or N33 or N34) of the GP_5 ectodomain, and it is present in many but certainly not all NA and EU strains of PRRSV (Figure 4). The second and third potential N-glycosylation sites are located at either aa residues N44 and N51 (NA strains of PRRSV) or N46 and N53 (EU strains of PRRSV), and are highly conserved among field strains of PRRSV. In addition to these three N-glycosylation sites some NA isolates (U.S. ATCC VR-2332 and Quebec IAF-Klop reference strains) have an additional (fourth) potential N-glycosylation site at aa residue N30 (Dea et al., 2000a; Key et al., 2001; Plagemann, 2004). Using peptide mapping it was shown that the major neutralization epitope of PRRSV is located into the middle of the GP_5 ectodomain (aa 36–52) (Plagemann et al., 2002). Using swine polyclonal antisera and mouse mAbs, the non-neutralizing (epitope A) and neutralizing epitopes (epitope B) in the ectodomain of the GP_5 protein of PRRSV were identified (Ostrowski et al., 2002). Epitope B was recognized by a neutralizing mAb and high SN-titered swine antisera. Neutralizing epitope B includes aa 37–45 of GP_5 and is conserved among PRRSV isolates, but it is not immunodominant (Figure 4). In contrast, the non-neutralizing epitope A includes aa residues 27–30, and is hypervarible and immunodominant

(Figure 4). Interestingly, PRRSV-infected pigs first develop antibodies against epitope A and neutralizing antibodies against epitope B only appear later. Thus, the immunodominant epitope A may act as a decoy and so delay the neutralizing antibody response against epitope B, although the true significance of this potential mechanism of immune evasion of field strains of PRRSV remains uncertain (Plagemann, 2004). The presence of N-glycans in and around epitope B may also reduce the immunogenicity of this critical neutralization determinant in the GP_5 protein, consistent with the low immunogenicity of PRRSV virions into mice and their failure to develop a strong neutralizing antibody response to the virus. Using the site-directed mutagenesis and pepscan analysis it was shown that the aa residue at position 24 of the GP_5 signal sequence of an EU strain of PRRSV was critical for a neutralizing mAb recognition; specifically, the presence of a proline residue at position 24 promotes cleavage of the signal peptide at residues 28 and 29 rather than 32 and 33 (Wissink et al., 2003). They also demonstrated a second neutralization epitope recognized by mAbs that includes aa 29–35 of GP_5. Together these data suggest that the major neutralization determinants of PRRSV are located in the N-terminal portion of the GP_5 protein, and that inherent properties of the protein such as "decoy" epitopes and heterogeneous glycosylation that obscures the critical neutralization site(s) can impede and decrease the humoral immune response against PRRSV GP_5.

Several studies provide evidence that glycosylation of GP_5 of PRRSV play an important role in escaping or minimizing virus-neutralizing antibody response by a N-glycan-shielding mechanism (Johnson et al., 2003; Wei et al., 2003). This N-glycan neutralisation shielding mechanism is also observed in LDV (Chen et al., 2000) and others viruses such as hepatitis B virus (HBV) (Lee et al., 2003), simian immuno deficiency virus (Reitter, Desrosiers, 1998), Influenza virus (Skehel et al., 1984), and human immuno deficiency virus (HIV) (Wei et al.,

2003). Biochemical studies indicate that the mature GP_5 contains high-mannose-type sugar moieties at all three N-glycosylation sites (N34, N44, and N51) (Figure 4). The mutation involving residue N44 did not result in infectious progeny production, indicating that N44 is the most critical aa residue for infectivity. Viruses carrying mutations at N34, N51, and N34/51 grew to lower titers than the wild-type (wt) PRRSV (Ansari et al., 2006). All mutated viruses exhibited enhanced sensitivity to neutralization by swine sera. Furthermore, inoculation of pigs with the mutant viruses induced significantly higher levels of neutralizing antibodies against the mutants as well as the wt PRRSV, suggesting that the loss of N-glycan residues in the ectodomain of GP_5 enhances both the sensitivity of these viruses to *in vitro* neutralization and the immunogenicity of the nearby neutralization epitope (Ansari et al., 2006). Similar results have been obtained in regards to GP_5 of the EU reference strain LV and demonstrate that the N-glycan moitie of the N46 glycosylation site (which corresponds to the N44 in the case of NA strains) is required for virus particle production (Wissink et al., 2004). Interestingly, the authors also found that the N-linked glycans normally located at the N53 of GP_5 (which corresponds to the N51 for NA strains) and present on the GP_{2a} protein are not essential for particle formation. Furthermore, the lack of N-linked oligosaccharides on GP_{2a} and at N53 of GP_5 did not significantly affect the infectivity of the viruses compared to GP_5 N46 mutant (Wissink et al., 2004). In conclusion, preventing the glycosylation of the GP_5 protein at position N44 and N46, respectively for NA and EU strains, had dramatic effects on virion production and virus infectivity (Wissink et al., 2004). The N-linked glycosylation, in general, is important for correct folding, targeting, and biological activity of proteins (Helenius, 1994; Helenius, Aebi, 2001, 2004). In many enveloped viruses, the envelope proteins are modified by the addition of sugar moieties and the N-linked glycosylation of envelope protein plays diverse functions such as receptor binding, membrane

568 fusion, penetration into cells, and virus budding (Braakman, van Anken, 2000; Doms et al.,
569 1993). Furthermore, it has become evident that glycosylation of viral envelope proteins is a
570 major mechanism for viral immune evasion and virus persistence which has been used by several
571 different enveloped viruses to escape, block, or minimize the virus-neutralizing antibody
572 response. Examples of this effect have now been reported for PRRSV and several other viruses
573 such as arterivirus LDV (Chen et al., 2000), simian immunodeficiency virus (Reitter, Desrosiers,
574 1998), hepatitis B virus (Lee et al., 2003) influenza virus (Skehel et al., 1984).

575 GP_5 forms an heterodimeric complex with the M protein (Figure 1) via disulfide bound
576 (Mardassi et al., 1996; Snijder et al., 2003). The disulphide bond between GP_5 and M occurs
577 between Cys50 and Cys8 (EU isolates) of the respective proteins (Verheije et al., 2002).
578 Mutating either one of these residues resulted in a complete block of particle production
579 (Verheije et al., 2002), indicating that the covalent association of GP_5 and M is crucial for virus
580 assembly (Verheije et al., 2002). The PRRSV GP_5–M complexes were shown to interact with
581 heparin (Delputte et al., 2002), suggesting a role in regards to attachment of virus to alveolar
582 macrophages. Thereafter, it has been proposed that the GP_5 is involved in the entry of virus into
583 the host cells, presumably by interacting with the macrophages host cell receptor sialoadhesin
584 (Delputte, Nauwynck, 2004). The role of GP_5 in receptor recognition is supported by the
585 presence of a major neutralization epitope in the N-terminal ectodomain (Ostrowski et al., 2002),
586 implying a central role for the GP_5 ectodomain in the infection process.

587 The GP_5 protein is involved in the apoptosis phenomenon induce by PRRSV (Gagnon et
588 al., 2003; Suarez et al., 1996a). The apoptotic inducing region of GP_5 has been mapped to the
589 first N-terminal 119 aa (Fernandez et al., 2002)**.** During apoptosis in PRRSV infected MARC-
590 145 cells and in MARC-145 cells expressing the GP_5 protein, specific apoptotic enzymes, are

591 activated, like caspase-3 which is a hallmark of apoptosis pathways induction (Gagnon et al.,
592 2003).

593 The MARC-145 and HeLa cells were transfected with ORF4 and ORF5 of PRRSV and
594 differential gene expressions were studied using microarray chips embedded with 1718 human-
595 expressed sequence tags (Lee et al., 2004). Genes associated to protein degradation, protein
596 synthesis and transport, and various other biochemical pathways were found to be regulated. In
597 GP_5-expressing HeLa cells, actin-related protein 1 (ARP1) homologs A and B were identified to
598 be up-regulated (Lee et al., 2004). These genes encode a subunit of dynactin that binds to both
599 microtubules and cytoplasmic dynein, and is involved in ER-to-Golgi transport (Lees-Miller et
600 al., 1992). Then it is possible that the GP_5 may play a role in the intracellular transport of viral
601 and cellular components. The bisphosphoglycerate mutase (BPGM) gene was down-regulated by
602 GP_5 by more than six-folds (Lee et al., 2004). In humans, deficiency of BPGM has been shown
603 to be associated with anemia (Jacobasch, Rapoport, 1996). RASSF2 was also down-regulated by
604 GP_5. RASSF2 is a new member of the RASSF1 family and shares the properties of Ras
605 effector/tumor suppressors (Vos et al., 2003). Similarly, cyclin D3 gene expression was found to
606 be suppressed. D-type cyclins are the key regulators along with cyclin E for cell cycle
607 progression from G1 to S phase. Complexes formed between cyclin D or cyclin E and their
608 kinase partners are involved in phosphorylation of retinoblastoma protein, which ultimately leads
609 to activation of E2F transcription factor and progression to S phase of the cell cycle. Other study
610 demonstrates a clear reduction of cyclin D3 and cell cycle arrest in G0/G1 phase in cells infected
611 with mouse hepatitis coronavirus, a member of the nidoviruses (Chen, Makino, 2004). In
612 addition, a consistent increase in the expression of cathepsin genes was observed in GP_5
613 expressing cells (Lee et al., 2004). Cathepsin is involved in protein degradation. Despite the fact

614 that a specific role of cathepsin during virus replication remains to be determined, the up-
615 regulation of genes encoding proteases may represent a cellular defence against expression of
616 foreign proteins.

617 In resume, the GP_5 protein is one of the most variable proteins of PRRSV and is an
618 important virus structural protein exposed on the surface of the virion that is involved in 1) cells
619 death by apoptosis, 2) cells recognition and binding, 3) antibodies-dependent virus neutralisation
620 and 4) immune protection in immunized or infected animals. Noteworthy, GP_5 is not the only
621 determinant of a protective immunity and is particularly unlikely to be an important determinant
622 in regards to the cross protection immunity which is needed because of the broad spectrum of
623 PRRSV strains heterogeneity. The N-glycosylation of GP_5 decreases significantly the
624 immunogenicity of the nearby enclosed neutralizing epitope and provides a way to evade the
625 neutralizing antibody immune response. Other viral epitopes, which are able to induce
626 neutralizing antibodies but with a significantly lesser extent compared to GP_5, appear to reside
627 on the other PRRSV proteins like M, GP_{2a}, GP_3, and GP_4. GP_5 immunized animals develop a
628 partial protective immune response. GP5 fusion proteins (such as M-GP_5 and GP_3–GP_5, GP_4–
629 GP_5, or GP_3–GP_4–GP_5) are able to increase the protective immune response in vaccinated
630 animals compared to GP_5 alone. Thus, it is easy to conclude that for vaccine development, GP_5
631 should be optimized in its ability to induce neutralizing antibodies by allowing its fusion with
632 other viral proteins or by reducing its N-glycosylation state. Nonetheless, all data suggest that
633 other viral proteins should be further investigated, such as the nsp proteins (see the nsp proteins
634 section bellow), if we hope to develop an efficient method to increase significantly the protection
635 of swine against PRRSV infection.

Minor PRRSV structural proteins

Three N-glycosylated minor envelope proteins (GP_{2a}, GP_3, and GP_4) are translated from ORF2a, 3, and 4 and are found in virions as an heterotrimers form by disulfide linkage (Figure 1 and 2) (Wissink et al., 2005). The ORF2b gene, which is completely embedded in ORF2a, encodes another non-glycosylated minor protein named E protein (Figure 2) (Wu et al., 2001).

The ORF2 gene (Figure 2) encodes two minor structural proteins (GP_{2a} and E) (Snijder et al., 1999). It was shown that NA PRRSV ORF2 contains a small 219 nt ‘‘ORF2b’’ gene and its translation is initiated five nucleotides downstream of the initiation codon of the large ORF2, now named ‘‘ORF2a’’. Using a baculovirus expression construct, it was shown that the full-length ORF2 gene expressed both GP_{2a} and E in insect cells and that ORF2b is in a better context for translation and may be preferentially expressed compared to ORF2a (Wu et al., 2001).

The minor glycosylated envelope viral protein GP_{2a}

The 29 to 30 kDa GP_{2a} is a putative class I integral membrane protein with an N-terminal signal sequence and a C-terminal membrane anchor domain (Meulenberg et al., 1995a). The GP_{2a} of the NA and EU strains possesses (Table 1) 256 and 249 aa, respectively (Meulenberg et al., 1995a). The GP_{2a} of EU and NA strains of PRRSV contain 2 distinctive hydrophobic peaks and share two highly conserved putative N-linked glycosylation sites (Meng et al., 1995b; Meulenberg et al., 1995a; Meulenberg et al., 1997; Morozov et al., 1995; Wissink et al., 2004). The significance of the N-glycosylation of the GP_{2a} protein for virus production and viral infectivity has been investigated in regards to the EU LV strain (Wissink et al., 2004). After

analysing the GP_{2a} protein of virus mutants, the results showed that the GP_{2a} N-linked glycans are not essential for virus particle formation (Wissink et al., 2004). The antigenicity of the GP_{2a} is largely unexplored and there is no data in regards to the NA strains. Two GP_{2a} linear epitopes were found to be immunoreactive with 60% (9/15) of swine sera (de Lima et al., 2006). The reactive peptides comprise regions at aa positions 41–55 and 121–135. Using phage-displayed peptides, three weakly antigenic B-cell epitopes were identified in the GP_{2a} at positions 36–51, 117–139 and 120–142 (Oleksiewicz et al., 2002). When ORF2 and ORF4 genes (which encode for minor structural glycoproteins GP_{2a}/E and GP_4, respectively) were individually deleted from the viral genome of a NA strain, both PRRSV deletion mutants were non-viable in MARC-145 cells and PAM, indicating that both genes are essential for virus replication (Welch et al., 2004).

The minor non-glycosylated envelope viral protein E

The E protein is an unglycosylated small hydrophobic protein which is membrane-associated and believed to be present in all arteriviruses (Snijder et al., 1999; Wu et al., 2001). The PRRSV E protein, is translated from the internal ORF2b starting from the +6 nucleotide position in mRNA2 (Figure 2) (Snijder et al., 1999). The E protein of the NA and EU strains possesses (Table 1) 73 and 70 aa, respectively (Wu et al., 2001). The E protein is a small 10 kDa protein with a central hydrophobic domain, and an hydrophilic C terminus containing a cluster of basic residues (Snijder et al., 1999). In addition, the E protein possesses a potential N-terminal N-myristoylation site and a potential casein kinase II phosphorylation site (Snijder et al., 1999). The E protein of PRRSV has been identified to be incorporated into the envelope virion structure such as its EAV homologous protein (Wu et al., 2005) (Figure 1).

682 Besides its structural function, the E protein function is currently unknown. Reverse
683 genetics, using an infectious EAV clone, demonstrated that the E protein is required for the
684 production of infectious virions (Snijder et al., 1999). It was shown that a covalent association of
685 the E protein with the GP_{2b}-GP_3-GP_4 heterotrimers exists in EAV, suggesting a possible role of
686 the heteromultimeric complex in the virus entry process. The absence of the E protein entirely
687 prevented the incorporation of the GP_{2b}-GP_3-GP_4 proteins into viral particles (Wieringa et al.,
688 2004). Several studies using a EU PRRSV isolate showed that the E protein is incorporated into
689 the virions and associated covalently with GP_{2a}–GP_3–GP_4 heterotrimers (Figure 1), suggesting a
690 critical role for the heteromultimeric complex (Wissink et al., 2005). The PRRSV E protein
691 possesses two cysteines at positions 49 and 54 that are highly conserved among NA isolates. The
692 importance of the cysteine residues of the E protein was determined using a NA PRRSV
693 infectious cDNA clone. Each cysteine was substituted by a serine. When the mutated PRRSV
694 infectious cDNA clone were transfected into MARC-145 cells, all cysteine mutant clones
695 induced PRRSV-specific cytopathic effects and produced infectious progeny virus. The data
696 indicates that cysteine residues in the E protein are not essential for PRRSV replication (Lee,
697 Yoo, 2005). In addition, the results obtained show that the N and E proteins of PRRSV do not
698 form cysteine-linked covalent linkages but that they do form cysteine-independent non-covalent
699 associations (Lee, Yoo, 2005). Therefore, the authors postulated that the interaction between N
700 and E may be initiated following the binding of viral RNA to the N protein (Yoo et al., 2003).
701 Subsequently, the N–RNA interaction may promote the N protein association with E, providing
702 stable assembly of the core structure in the virion. Noteworthy, the cysteines of E protein are not
703 well conserved among arteriviruses (Snijder et al., 1999). In comparison to PRRSV NA strains,
704 the EU strains contain only a single cysteine at position 51, while E proteins of LDV, EAV and

SHFV contain a single cysteine residue at different positions (Snijder et al., 1999). Interestingly, it was found that a single NA PRRSV mutation at arginine 51 in the basic region of E is lethal for PRRSV, supporting the premise that the N–E non-covalent interaction may be a requirement for PRRSV replication (Lee, Yoo, 2005). On the other hand, an infectious cDNA clone was used to generate an E gene-knockout PRRSV mutant, and this study report that the E protein is essential for virus infectivity but dispensable for virus particle formation (Lee, Yoo, 2006). The E deleted non-infectious virus particles were able to enter cells but unable to continue further steps of replication (Lee, Yoo, 2006). It is therefore tempting to speculate that PRRSV may contain a viral ion channel protein to promote the uncoating process in the endosome during the early stage of infection. Furthermore, two ion channel blockers were shown to greatly affect PRRSV replication at early stages of infection, suggesting that ion channel activity was essential for virus uncoating (Lee, Yoo, 2006). In addition it was found that expression of the E protein enhances membrane permeability of hygromycin B in bacterial cells. Interestingly, the coronavirus E protein has been shown to modify membrane permeability (Liao et al., 2004; Madan et al., 2005) as well as to form cation-selective ion channels in an artificial membrane (Wilson et al., 2004). Results obtained in a previous study support the hypothesis that the PRRSV E protein may function as an ion channel for virus uncoating (Lee, Yoo, 2006).

Recently, the E protein myristoylation was explored using the reverse genetics approach (Du et al., 2010). The E protein contains the consensus 1MGxxxS6 motif for its myristoylation, and in the presence of 2-hydroxymyristic acid, the virus $TCID_{50}$ titer decreased by 2.5 log10 and the level of viral RNA was reduced significantly. Thus, the E protein myristoylation is non-essential for PRRSV infectivity but promotes the growth of the virus (Du et al., 2010).

The minor glycosylated envelope viral protein GP_3

The GP_3 protein is encoded by the ORF3 (Figure 2) gene and is one of the most variable PRRSV proteins, showing approximately 54 to 60% aa identity between the NA and EU genotypes (Dea et al., 2000a). The GP_3 is comprised (Table 1) of 254 and 265 aa for NA and EU isolates, respectively (Mardassi et al., 1995a; Meng et al., 1995b; Meulenberg et al., 1995a; Murtaugh et al., 1995). Several studies have reported that the most variable region of GP_3 is located at the first 35 aa, which is 29% identical among EU and NA isolates (Katz et al., 1995; Mardassi et al., 1995a; Murtaugh et al., 1995). The 45 to 50 kDa molecular weight GP_3 protein is highly glycosylated (Table 1) and possesses seven potential N-linked oligosaccharides and has a single N terminal hydrophobic domain (Meulenberg et al., 1995a). The glycosylation sites are highly conserved among NA and EU PRRSV stains (Gonin et al., 1998; Mardassi et al., 1995a; Meulenberg et al., 1995a; Wissink et al., 2004). The GP_3 protein seems to take a special position among the arterivirus glycoproteins. Since its membrane topology has not been resolved and seems to be strain dependent (Hedges et al., 1999; Mardassi et al., 1998; Wieringa et al., 2002), its virion structural nature is also controversial (Figure 1). For LDV, the GP_3 protein was reported to be a non-structural, soluble glycoprotein that is secreted from infected cells (Faaberg, Plagemann, 1997). In contrast, the GP_3 protein of EAV was clearly demonstrated to be incorporated into virions (Wieringa et al., 2002). For PRRSV, both situations have been described (de Lima et al., 2009; Gonin et al., 1998; Mardassi et al., 1998; Meulenberg et al., 1995a; van Nieuwstadt et al., 1996; Wissink et al., 2005). Thus, the GP_3 protein of the prototype EU LV strain was found to be incorporated into virions (Meulenberg et al., 1995a; van Nieuwstadt et al., 1996) and membrane-associated as heterotrimers with GP_{2a} and GP_4 (Wissink

et al., 2005). However, the GP_3 protein of the IAF-Klop reference strain (a typical NA isolate) was found to be non-structural and secreted from PRRSV infected cells (Gonin et al., 1998; Mardassi et al., 1998). This dual nature of GP_3 is a reminiscence of viral glycoproteins which could be found in several other viruses such as the glycoproteins G (gG) of alphaherpesviruses (Bryant et al., 2003; Murata et al., 2002) and the E^{rns} glycoprotein of bovine viral diarrhea virus (BVDV) (Iqbal et al., 2004). The gG protein of alphaherpesviruses is found in the virus envelope and, in some alphaherpesviruses, is also secreted after proteolytic processing. In addition, these secreted forms of gG were reported to have chemokine binding activity, thereby inhibiting the biological activity of these chemokines *in vitro* (Bryant et al., 2003). The BVDV E^{rns} protein, in addition to being a virion protein, is also secreted from infected cells into the extracellular environment. This secreted form was recently shown to block the double-stranded RNA interferon-inducing signal (Iqbal et al., 2004).

According to several reports, GP_3 is highly antigenic (Faaberg, Plagemann, 1997; Gonin et al., 1998; Hedges et al., 1999; Katz et al., 1995). Although the GP_3 antibody is present at a very low level in pigs, it reportedly plays a role in clearing the viral infection (Plana Duran et al., 1997b) and may be involved in viral neutralization along with the GP_5 and M proteins (Cancel-Tirado et al., 2004). Using a phage display library, two epitopes spanning aa regions 60–85 and 243–250 of a EU GP_3 protein were identified (Oleksiewicz et al., 2002; Oleksiewicz et al., 2001b). Two other GP_3 epitopes of a NA strain were found and located at aa 73–87 and 66–81 (Zhou et al., 2006). Further analyses revealed that the two epitopes are not conserved among all NA-type isolates. After analyzing the degree of antigenic variability in a series of EU isolates using monoclonal antibodies, it was determined that the C-terminus antigenicity of GP_3 varied a lot among the different EU isolates (Drew et al., 1995). Two replication-defective recombinant

adenoviruses expressing GP_3 (rAd-GP_3) or truncated GP_3 (rAd-tGP_3, a hydrophobic region of aa 2–64 was deleted) were constructed and their immunogenicity was tested in mice (Jiang et al., 2007). The results showed that the mice immunized with recombinant adenoviruses developed PRRSV-specific neutralizing antibodies and cellular immune response, including T-cell proliferation responses and cytotoxic T-cell responses, by 2 weeks post-primary immunization. Moreover, the levels of immune responses of mice immunized with rAd-tGP_3 were significantly higher than that of mice with rAd-GP_3.

The minor glycosylated envelope viral protein GP_4

The 31 to 35 kDa molecular weight GP_4 protein is a putative class I integral membrane protein with an N-terminal signal sequence and a C-terminal membrane anchor (Meulenberg et al., 1995a). The GP_4 protein is highly glycosylated during transport through the ER–Golgi complex (van Nieuwstadt et al., 1996). Four putative N-linked glycosylation sites which are conserved among the NA and EU PRRSV strains are found within the protein (Meulenberg et al., 1995a; van Nieuwstadt et al., 1996; Wissink et al., 2004). The aa sequence shows that GP_4 contains a putative N-terminal signal sequence at positions 1–22, and an additional hydrophobic sequence at positions 162–178 at the C-terminus. The GP_4 protein is a minor structural protein consisting of 178 and 183 aa (Table 1) for NA and EU strains, respectively (Murtaugh et al., 1995) and like GP_5, is also able to induce neutralizing antibodies with lesser extent (Meulenberg et al., 1997; Weiland et al., 1999). The GP_4 protein of LV possesses neutralizing epitopes which are not conserved among NA and EU strains (Meulenberg et al., 1997; Weiland et al., 1999). The neutralizing domain of the GP_4 of the LV strain has been mapped to a hydrophilic exposed

region, adjacent to its N-terminal region (aa position 40–79) which appears to be highly variable among the EU strains (Meulenberg et al., 1997; Weiland et al., 1999). Approximately 65% of PRRSV-positive sera obtained from affected pig farms in the US and Canada react positively by immunoblotting against the *E. coli*-expressed recombinant ORF4 protein (Gonin et al., 1999; Kwang et al., 1994). However, no correlation has been demonstrated between virus neutralization titers of convalescent pig sera and the presence of anti-GP_4 antibodies (Gonin et al., 1999). Using Pepscan technology Lima et al. (2006) detected the B-cell linear epitope in NA GP_4 (aa 51-65) and using phage display libraries Oleksiewicz et al. (2001 and 2005) detected a single linear epitope in EU GP_4 (aa 59-71).

Transfection of PRRSV permissive cells with an ORF4 deleted infectious cDNA clone revealed that the ORF4 gene is essential for virus replication (Welch et al., 2004). The experiments conducted by Lee and collaborators (Lee et al., 2004) demonstrated that like GP_5, the GP_4 is able to alter cellular mRNA synthesis. In cell expressing GP_4, the majority of differentially expressed genes were involved in synthesis, transport, and biochemical pathways (Lee et al., 2004). This observation implicates that the GP_4 protein may utilize or change host cell machinery to transport viral or cellular components to the cell surface.

In resume, the minor structural proteins are essential for virus infectivity and they are incorporated into virions as a multimeric complex. Recently they became the point of interest of several PRRSV investigators. Therefore, new interesting findings in regards to the function of the minor viral structural proteins should be expected. The minor structural proteins multimeric complex was shown to interact with the cell receptor CD163 with or without the involvement of

819 M-GP_5 heterodimers. Thus, this latest finding could lead us to formulate new important
820 questions such as: 1) can minor structural proteins multimeric complex target the virus to a
821 certain cell type and organ?; or 2) is the multimeric complex involved in the receptor-mediated
822 endocytosis virus entry? Other questions could certainly be formulated. Nonetheless, new
823 investigations in regards to the minor structural proteins will certainly help to increase our
824 understanding of PRRSV cell pathogenesis.
825
826
827 **Viral structural proteins interactions**
828
829 While the major viral structural proteins are essential for particle formation, the minor
830 viral structural proteins are essential for virus infectivity (Wissink et al., 2005). Furthermore, the
831 minor structural proteins are incorporated into virions as a multimeric complex (Figure 1).
832 Interestingly, the absences of any of the four proteins appear to affect the incorporation of all
833 three others. Thus, no GP_3 was detected in viral particles when the expression of either GP_{2a}, or
834 E, or GP_4 was prevented, and no detectable GP_4 was incorporated in the absence of GP_{2a}, E, and
835 GP_3. Similar observations were made with EAV (Wieringa et al., 2004). From these results, three
836 main conclusions could be draw. First of all, each of the membrane viral proteins is essential for
837 the production of infectious virus. Second, the GP_5 and M protein are both crucial for particle
838 formation, consistent with a role as the fundamental building blocks of the viral envelope. Third,
839 while not required for particle assembly, the minor glycoproteins GP_{2a}, GP_3, and GP_4, and
840 probably also the minor envelope protein E, interact with each other. This interaction is likely to
841 be of critical importance for their incorporation as heteromultimeric complexes into PRRSV

842 particles and essential for rendering these particles infectious (Wissink et al., 2005).
843 Noteworthy, the contradictory report in regards to the presence or the absence of GP_3 in viral
844 particles seems to be a strain dependant phenomenon and its absence does not seem to abolish
845 the formation of infectious viruses (Wissink et al., 2005). Recently, a strong interaction was
846 found between GP_4 and GP_5 proteins, although weak interactions among the other minor
847 envelope glycoproteins and GP_5 have been detected (Das et al., 2010). Both GP_{2a} and GP_4
848 proteins were found to interact with all the other viral glycoproteins resulting in the formation of
849 multiprotein complex. In addition, it was found that the multimeric complexes of the minor
850 glycoproteins, with or without the M and GP_5 proteins, are involved in direct interaction with the
851 cell surface receptor (Das et al., 2010). It appears that the GP_4 is a critical viral envelope protein
852 that not only mediates interactions with other viral envelope glycoproteins, but also along with
853 GP_{2a}, mediates interactions with the CD163 for virus entry. More specifically, it was show that
854 only the GP_{2a} and GP_4 proteins interact with CD163. Since the minor envelope proteins are not
855 required for particle formation (Wissink et al., 2005) and there are only few large multimeric
856 glycoprotein complexes present on the virion envelope (Spilman et al., 2009), these results
857 suggest that the major function of the minor glycoprotein complex on the virion envelope is to
858 interact specifically with the cell surface receptor for virus entry (Das et al., 2010).

859 Several research groups have been involved in the study of the immune response
860 triggered by each of the major structural proteins (GP_5 and M) of PRRSV (Bastos et al., 2002;
861 Bastos et al., 2004; Jiang et al., 2006b; Pirzadeh, Dea, 1998; Plana-Duran et al., 1997a, b; Qiu et
862 al., 2005) but only few information is yet available on the characterization of the minor structural
863 proteins, such as GP_3 and GP_4. A recent study (Jiang et al., 2008) reports the tandem cloning and
864 expression of two or three proteins of PRRSV, the GP_3, GP_4, or GP_5, in replication-defective

865 human adenovirus type 5 (Ad5) vectors. Mice inoculated with the recombinant Ad5 expressing
866 the GP_3–GP_5, GP_4–GP_5, or GP_3–GP_4–GP_5 of PRRSV developed a specific immune response
867 against the viral proteins, and this response was significantly increased against GP_5 when GP_3
868 and/or GP_4 were present in the adenovirus vector compared to GP_5 alone (Jiang et al., 2008).
869 These results indicated that fusion expression GP_3 and/or GP_4 with GP_5 may be an alternative
870 strategy to design more effective vaccines.

Non-structural PRRSV proteins

RNA synthesis of the positive-stranded RNA viruses that belong to the order *Nidovirales* involves the replication of the genome-length RNA (RNA1) and the synthesis of subgenomic mRNAs (sg mRNAs). Both processes are mediated by a 'replication/transcription complex' (RTC) composed of virus-encoded non-structural proteins (nsp) and presumably also host factors (Gorbalenya et al., 2006). Following virus entry and release of the genome into the cytoplasm, the nidovirus life cycle starts with the expression of the large replicase gene that consists of ORF1a and ORF1b (Figure 2). The replicase-associated genes, ORF1a and ORF1b, are located on the 5' end of the genome (representing almost 75% of the viral genome) and encode for the polyproteins pp1a and pp1ab (Figure 2). The pp1a is encoded by ORF1a, and the synthesis of pp1ab occurs through a ribosomal frame shift at the ORF1a-1b junction (Brierley, 1995; Snijder, Meulenberg, 1998). The pp1a is predicted to be cleaved at eight (Figure 2) sites to form nine nsp: nsp1α, nsp1β, and nsp2 to nsp8 (den Boon et al., 1995; Snijder, Meulenberg, 1998). Proteolytic cleavage of the ORF1b gene portion of the pp1ab generates (Figure 2) products nsp9 through nsp12 (van Dinten, 1996). The products derived from pp1a possess proteolytic activities (Table 1) and are responsible for processing the other nsp cleavage products; whereas nsp9 to nsp12 are involved in virus transcription and replication (Snijder, Meulenberg, 1998; van Dinten, 1996). These polyproteins are immediately translated upon virus entry and then proteolytically processed by viral encoded proteinases into intermediate precursors and at least 12 mature nsp, which appear to be responsible for forming membrane-bound replication complexes, called virus-induced double-membrane vesicles and which are sites for viral RNA synthesis (Snijder, Meulenberg, 1998). The processing of pp1a and pp1ab is believed to be mediated by accessory

894 proteinases (Figure 2), located in nsp1 and nsp2, and the main serine proteinase located in nsp4,
895 the 3C-like proteinase (3CLpro) (Ziebuhr et al., 2000). The catalytic sites of these enzymes and
896 the corresponding cleavage sites of pp1a are well conserved among arteriviruses (Ziebuhr et al.,
897 2000). Processing of pp1a in PRRSV begins with the N-terminal nsp1, which is automatically
898 cleaved by papain-like cysteine proteases (PCPα and PCPβ) which are contained in nsp1 (Figure
899 2) (Kroese et al., 2008), while the PCPα domain is inactive in EAV (den Boon et al., 1995;
900 Snijder et al., 1993). Arterivirus nsp2 contains a predicted cysteine proteinase (PL2pro) in its N
901 terminus (Figure 2) that was shown to cleave the pp1a at the nsp2-nsp3 junction in EAV (Snijder
902 et al., 1995b; Snijder, 1998). The 3CLpro (Figure 2) is responsible for processing the remainder
903 of ORF1a and ORF1ab into several subunits (nsp3 to 12) (van Dinten et al., 1999; Ziebuhr et al.,
904 2000).

905 The non-structural polypeptides encoded in *Nidovirales* ORF1b regions have been
906 predicted to have essential roles (Table 1) in virus replication and gene expression (Allende et
907 al., 1999; Gorbalenya, 1989; Meulenberg et al., 1993a; Nelsen et al., 1999; van Dinten, 1996).
908 Evidence for the significant role of these proteins in arteriviruses has been derived using an
909 infectious clone of EAV (van Dinten et al., 1997; van Dinten et al., 1999; van Dinten, 2000; van
910 Marle et al., 1999). Putative RNA-dependent RNA polymerase and NTPase/RNA helicase
911 motifs are associated with nsp9 and nsp10, respectively. The nsp10 also contains a putative zinc
912 finger region, which is implicated in subgenomic mRNA synthesis and genome replication.
913 Thus, it can be assumed that the reduction of ORF1ab transcripts and nsp10 expression might
914 affect negatively expression of the other viral proteins. The nsp11 contains a region with
915 unknown function, but is conserved in all nidoviruses which suggests a crucial role for this viral

protein (den Boon et al., 1991; Godeny et al., 1993; Gorbalenya, 1989; Meulenberg et al., 1993a; van Dinten, 1996).

The nsp1 is a multifunctional protein (Table 1) containing two papain-like cysteine proteases and a zinc-finger motif required for sg mRNA transcription (den Boon et al., 1995; Kroese et al., 2008; Oleksiewicz et al., 2004; Tijms, Snijder, 2003; Tijms et al., 2001). Intracellular concentrations of nsp1 may be higher than other nsp, due to translation from heteroclite RNAs (Yuan et al., 2000; Yuan et al., 2004). Its papain-like autoprotease domain releases nsp1 from the replicase polyproteins, a cleavage essential for viral RNA synthesis. Several mutations in the putative N-terminal zinc finger domain of nsp1 selectively abolished transcription, while replication was either not affected or even increased. Other nsp1 mutations did not significantly affect either replication or transcription but still dramatically reduced the production of infectious progeny. Thus, nsp1 is involved in at least three consecutive key processes in the EAV life cycle: ORF1ab polyprotein processing, transcription, and virion biogenesis (Tijms et al., 2007). Both N-terminal cleavage products of nsp1, the nsp1α and the nsp1β, of the replicase polyproteins of PRRSV contain a papain-like autoproteinase domain, which have been named PCPα and PCPβ, respectively. To assess their role in the PRRSV life cycle, substitutions and deletions of the presumed catalytic cysteine and histidine residues of PCPα and PCPβ were made into a PRRSV infectious cDNA clone. Mutations that inactivated PCPα activity completely blocked sg mRNA synthesis, but did not affect genome replication. In contrast, mutants in which PCPβ activity was blocked proved to be non-viable and no sign of viral RNA synthesis could be detected, indicating that the correct processing of the nsp1β/nsp2 cleavage site is essential for PRRSV genome replication. In conclusion, the data presented here shows that a productive PRRSV life cycle depends on the correct processing of both the nsp1α

/nsp1β and nsp1β/nsp2 junctions (Kroese et al., 2008). Cleavage sites between nsp1α/nsp1β and nsp1β/nsp2 were identified by protein microsequencing analysis (Chen et al., 2010). The cleavage site between nsp1α/nsp1β is located at position 180M/181A (Figure 2) and is conserved among different NA PRRSV strains (Chen et al., 2010). The cleavage site between nsp1β/nsp2 is located at position 383G/384A (Figure 2) and is well conserved among different PRRSV isolates (Chen et al., 2010). The replicase polyproteins of PRRSV are predicted to be cleaved into 13 nsp by the nsp4 main proteinase and three accessory proteinases residing in nsp1α, nsp1β and nsp2 (van Aken et al., 2006a; van Aken et al., 2006b; van Aken et al., 2006c; Ziebuhr et al., 2000). PCPα directs the release of nsp1α, whereas the liberation of nsp1β depends on the activities of PCPα and a second proteinase, PCPβ. A third cysteine proteinase, residing in the N-terminal domain of nsp2, is responsible for the cleavage of the nsp2/nsp3 site (den Boon et al., 1995; Snijder et al., 1995a, b; Snijder, 1998). Viral papain-like proteinases are characterized by a catalytic dyad that consists of a nucleophilic cysteine residue and a downstream histidine (Figure 2).

Among the nsp, the nsp2 multidomain is the largest PRRSV replicative protein and is predicted to possess approximately 980 aa (Allende et al., 1999; Ziebuhr et al., 2000). Three major domains could be discerned through the alignment of arterivirus nsp2 proteins: 1) an N-terminal cysteine proteinase domain (PL2), 2) a functionally unspecified middle region, and 3) a hydrophobic transmembrane (TM) region near the C terminus (Han et al., 2006; Ziebuhr et al., 2000). The C-terminal end of nsp2 forms a non-covalent interaction with nsp3, which together form a scaffolding complex that supports the formation of double membrane vesicles and virus replication complexes (Snijder, Meulenberg, 1998; Snijder et al., 2001; Snijder et al., 1994; Ziebuhr et al., 2000). The PL2 of PRRSV nsp2 (Figure 2) is predicted to act on the potential

substrates at the nsp2-nsp3 junction and two potential cleavage sites have been proposed: 981G/G and 1196G/G/G (Allende et al., 1999; Ziebuhr et al., 2000), according to the knowledge obtained from nsp2 of EAV, which prefers G/G dipeptides (Snijder et al., 1995b, Snijder, 1998). The function of PRRSV nsp2 in the virus life cycle is poorly understood, while EAV nsp2 has been shown to be involved in the generation of double-membrane vesicles together with nsp3, also functions as a co-factor with nsp4 serine protease to process the other cleavage products (Snijder et al., 1994; Snijder, 1995a, b; Wassenaar et al., 1997). Although PRRSV nsp2 possesses potential enzymatic function, it has been shown to be highly heterogeneous and variable (Table 1). It is well documented that PRRSV nsp2 accounts for the major genetic differences (with GP_5 major structural protein) between NA and EU PRRSV strains, sharing less than 40% similarity at the aa level (Allende et al., 1999; Nelsen et al., 1999). In addition, nsp2 is also the key region for genomic differentiation between PRRSV genotypes NA and EU strains. Natural mutations, insertions, or, most notably, deletions are seen in the middle region or near the N-terminal region of the nsp2 protein in field strains, while the putative PL2 domain, predicted TM domain, as well as the predicted cleavage sites of nsp2 remain well conserved (Allende et al., 1999; Fang et al., 2004; Gao et al., 2004; Han et al., 2006; Nelsen et al., 1999; Ropp et al., 2004; Shen et al., 2000).

Areas in nsp2 with deletions and aa hypervariability are predicted to be immunologically important (Fang et al., 2007). During the past 2 years, an atypical clinical outbreak, caused by a highly pathogenic PRRSV strain with a unique 30 aa deletion in its nsp2 coding region, was implicated in China (Li et al., 2007; Tian et al., 2007; Zhou et al., 2008). In the field, the virus was associated with high mortality/morbidity in pigs of all ages, with mortality as high as 90% in some herds. The mechanism for enhanced virulence was not determined but seems to be

associated to nsp2 30 aa deletion (Li et al., 2007). Recently, few full-length infectious cDNA clones were generated, in which the nsp2 region containing the 30 aa deletion was replaced by the corresponding region of the low virulence PRRSV strain (Zhou et al., 2009). The researchers concluded that the 30 aa deletion is not related to the virulence of the highly pathogenic PRRSV that recently emerged in China (Zhou et al., 2009).

The nsp2 gene may be an ideal marker for monitoring the genetic variation and for developing differential diagnostic tests. Since this region tolerates mutations, including insertions and deletions, nsp2 may represent the ideal site for the development of recombinant marker vaccines derived from infectious clones (Fang et al., 2004). In that regards a number of studies have indicated that PRRSV nsp2 is able to tolerate aa deletion and foreign gene insertion (Fang et al., 2004; Fang et al., 2006; Gao et al., 2004; Han et al., 2007; Kim et al., 2007; Ran et al., 2008; Tian et al., 2007) and is then an excellent gene candidate for modifications (de Lima et al., 2008; Fang et al., 2006; Kim et al., 2007).

PRRSV non-structural proteins play a key role in processing and maturation of the repertoire of structural and nsp's of the virion, but little is known about the anti-nsp immune response. In one recent study (Johnson et al., 2007), it was hypothesized that pronounced antibody responses are generated against PRRSV nsp1 and nsp2. Accordingly, nsp1 and nsp2 were cloned and expressed, and antibody responses in the sera of infected and vaccinated pigs were determined (Johnson et al., 2007). Pigs mounted significant cross-reactive antibody responses against nsp1 and nsp2 that appeared equivalent to or greater than the response to N protein. Antibody reactivity to nsp1 and N was highly dependent on refolding of denatured proteins, suggesting that the porcine antibody response is directed primarily to conformational epitopes. The proteins were recognized with sera from pigs infected with PRRSV heterogeneous

strains, indicating that multiple epitopes are conserved. Antibody responses against nsp1 and nsp2 were much higher than those directed against nsp4 (Johnson et al., 2007).

PRRSV has been shown to be a poor inducer of interferon (IFN) *in vitro* and *in vivo*. Recently, several nsp (nsp1, nsp2, nsp4 and nsp11) were identified to downregulate the immune response (Beura et al., 2010). They were found to possess an inhibitory effect on interferon-beta (IFN-β) promoter activation for which the strongest inhibitory effect was exhibited by nsp1 followed by, nsp2, nsp11, and nsp4 (Beura et al., 2010). In addition, it was found that nsp1α, nsp1β and nsp11 strongly inhibit dsRNA signaling pathways. The nsp1β was found to inhibit both IFN regulatory factor 3 (IRF3) and NF-kB-dependent gene induction by dsRNA and Sendai virus. Investigation of IRF3 phosphorylation status after dsRNA stimulation showed that nsp1β inhibits IRF3 (Ser396) phosphorylation and subsequently its nuclear translocation (Beura et al., 2010). Another recent report has revealed that nsp1α and nsp1β dramatically inhibit IFN-β expression (Chen et al., 2010). This study indicates that nsp1β possesses the ability to inhibit both IFN synthesis and its signaling pathway, while nsp1α alone strongly inhibits IFN synthesis.

The four predicted ORF1b non-structural polypeptides comprise a putative RNA dependent RNA polymerase (POL or nsp9), a nsp10 protein (nsp10) which contains a putative metal-binding domain (MBD) and nucleoside triphosphate binding or helicase motif (HEL), and two proteins of unknown functions (nsp11 and nsp12) (Allende et al., 2000; Allende et al., 1999; Bautista et al., 2002; den Boon et al., 1991; Godeny et al., 1993; Gorbalenya, 1989; Herold et al., 1996; Meulenberg et al., 1993a; Nelsen et al., 1999; Shen et al., 2000; Snijder, Meulenberg, 1998; van Dinten et al., 1999; van Dinten, 1996; Wootton et al., 2000). PRRSV nsp10 was found to possess a thermolabile and pH-sensitive NTPase activity that was modulated by polynucleotides and was able to unwind dsRNA in a 5' to 3' polarity (Bautista et al., 2002).

These results provide the first evidence of the functional properties of PRRSV helicase and further support the finding that nidovirus helicases possess properties that distinguish them from other viral helicases (Bautista et al., 2002). Based on significant homology in the catalytic domains and in predicted cleavage sites of PRRSV strains it was suggested that PRRSV polymerase may undergo similar proteolytic processing as that of EAV (Snijder, Meulenberg, 1998; van Dinten et al., 1999; Wassenaar et al., 1997; Ziebuhr et al., 2000).

Following the first identification of PRRSV in the early 90's, most of the research was oriented toward the study of the viral structural proteins. During the last five years, an increasingly significant number of reports were oriented toward the acquisition of new knowledge in regards to the non-structural viral proteins. In accordance with these recent reports, it became evident that the nsp play a tremendous role in regards to viral pathogenesis such as the immune response down regulation. The nsp ability to inhibit the IFN indicate that they are important virulence determinants. Thus, we are now entering a new exciting PRRSV research era that will focus on a better understanding of how PRRSV take control of the host immune response to its advantage.

CONCLUSION

PRRS continues to be an economically devastating disease affecting swine industry worldwide. Herein, an overview of current knowledge on virus structural and non-structural proteins in regard to viral pathogenesis was given.

To date, little is known about the biological functions of the individual nsp of PRRSV, except that they exhibit replicase, protease and polymerase activities. With the continuing emergence of viral strains, the nsp2 replicase protein with is noted mutations, insertions, and deletions has become a vital region for monitoring the rapid viral genomic evolution and for molecular epidemiology research on PRRSV (Allende et al., 1999; Fang et al., 2004; Fang et al., 2006; Fang et al., 2007; Gao et al., 2004; Han et al., 2007; Han et al., 2006; Kim et al., 2007; Ropp et al., 2004; Yoshii et al., 2008). The PRRSV nsp anti-IFN activity is certainly involved in the paradoxical immune response observed in PRRSV infected swine and would explain the inability of the host to clear PRRSV. The capability of the PRRSV nsp to interfere with the establishment of the innate and cellular antiviral state suggests that nsp are critical virulence determinants. In a near future, we could expect that research program involving nsp immune regulation of the infected host will provide important insight into the mechanism of PRRSV pathogenesis which could lead to new and innovative approaches for the control of PRRSV. In regards to viral structural proteins, it has been demonstrated that N, M and GP_5 are essential and sufficient for particle formation, but that the minor structural proteins are also required for virus infectivity (Wieringa et al., 2003; Wieringa et al., 2004; Wissink et al., 2005). The N protein seems to be the most immunogenic viral protein and an ideal target for the serological detection of infected pigs (Dea et al., 2000b; Denac et al., 1997; Ferrin et al., 2004; Seuberlich et al.,

2002). Nonetheless, some non-structural viral proteins, such as nsp2, are also highly immunogenic (Fang et al., 2004; Johnson et al., 2007; Oleksiewicz et al., 2001a, 2002; Oleksiewicz et al., 2001b). In addition, nuclear and/or nucleolar localization of N protein could have an important role in cell genes expression regulation (Hiscox, 2002, 2003, 2007; Rowland, Yoo, 2003; Uchil et al., 2006; Weidman et al., 2003).

It is further evident that the current vaccines based on a single PRRSV strain and on expression of GP_5 alone, are not or only partially effective (Kimman et al., 2009). Viral epitopes that are capable of inducing neutralizing antibodies appear to reside on the M, GP_{2a}, GP_3, GP_4, and GP_5 proteins (Ansari et al., 2006; Kim, Yoon, 2008; Plagemann, 2006; Yang et al., 2000). Of these, neutralizing antibodies to GP_5 appear to be most relevant for protection since most of the neutralizing Abs found in infected animals are directed against the GP_5. Unfortunately, it is also one of the most variable proteins between viral strains. Several recent studies using expression of the fusion proteins M-GP_5 and GP_3–GP_5, GP_4–GP_5, or GP_3–GP_4–GP_5 showed that specific immune response against GP_5 was significantly increased by fusion of proteins compared to animal immunized with GP_5 alone (Jiang et al., 2006a; Jiang et al., 2008). In addition, it was found that glycosylation of GP_5 of PRRSV play an important role in escaping or minimizing virus-neutralizing antibody response by a N-glycan-shielding mechanism (Kimman et al., 2009). Therefore, the glycosylation of GP_5 diminishes the immunogenicity of the nearby enclosed neutralizing epitope, and are thus critical for the induction or evasion of a neutralizing antibody response. Taken these results together, we can conclude that for vaccine development, GP_5 should be optimized in its ability to induce neutralizing antibodies by allowing its fusion with other viral proteins or by reducing its glycosylation state.

The virulence of PRRSV is considered to be associated with multiple factors such as, host genetic, viral genetic, environment, concomitant infections etc., and consequently it is not easy to identify the determinant regions in the genome of PRRSV involved in the viral virulence (Wang et al., 2008). However, searching any viral genomic regions or viral genes related to PRRSV virulence is an essential step toward making a contribution to the understanding of the pathogenicity of PRRSV. In order to determine virulence associated genes in PRRSV, reverse genetic has been abundantly used (Kwon et al., 2008; Wang et al., 2008). Using this experimental approach, it was found that PRRSV virulence is multigenic and resides in both the non-structural and structural viral proteins (Kwon et al., 2008; Wang et al., 2008).

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FIGURES LEGENDS

Figure 1. Schematic representation of the PRRSV particle. The location of the structural proteins: GP_{2a}, E, GP_3, GP_4, GP_5, M and N (encoded by ORFs 2-7) are shown. The virion possesses a non-segmented single-strand RNA genome, enclosed into a nucleocapsid protein (N), yielding a helicoidal capsid structure. The N protein is the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions (homodimer). The major envelope viral protein (GP_5) forms a heterodimer structure with the membrane non-glycosylated protein (M) which dominates the virion surface. The minor structural proteins (GP_{2a}, E, GP_3 and GP_4) are incorporated into virions as a multimeric complex. The minor structural viral proteins multimeric complex also interacts with the GP_5-M heterodimer (not illustrated). Although the GP_3 is a structural viral protein, it has been reported once that the GP_3 is a non-structural and secreted viral protein (which suggest that it could be a strain dependent phenomenon).

Figure 2. Schematic representation of the PRRSV genome organization and replication. The replicase 1a and 1ab polyproteins (ppla and pp1ab) are expressed from the genomic viral RNA. The filled circle indicates the ribosomal frameshift site, and the filled box indicates the leader sequence. The structural proteins are expressed from a nested set of subgenomic mRNAs, RNAs 2-7, that contain a common 5' leader sequence. The pp1a is predicted to be cleaved at eight sites to form nine non-structural proteins (nsp): nsp1α, nsp1β, and nsp2 to nsp8. Proteolytic cleavage of the ORF1b gene portion of the pp1ab generates products nsp9 through nsp12. The processing of pp1a and pp1ab is mediated by accessory proteinases located in nsp1 (papain-like cysteine proteinases: PCPα and PCPβ) and nsp2 (chymotrypsin-like cysteine protease: PL2pro);

and the main serine proteinase located in nsp4 (the serine/3C-like proteinase: 3CLpro). PCPα and PCPβ are responsible for the autoproteolytic release of the nsp1α and nsp1β, respectively. In addition nsp1 contain zinc-finger motif (ZF) required for subgenomic mRNA transcription. Predicted catalytic dyads of both PCP domains and the estimated positions of their respective nsp1α/nsp1β and nsp1β/nsp2 cleavage site are illustrated.

Figure 3. PRRSV N protein schematic model of the North American isolates. The numbers identify the amino acids positions covered by each domain. **I-V:** five domains of antigenic importance (30-52; 37-52; 52-69; 69-112 and 112-123); 23-Cys: identifies the location of the conserved cysteine residue involved in the intermolecular disulfide bonds N protein homodimer formation; 120-Ser: identifies the location of a serine residue that is a phosphorylation target; NLS-1: Nuclear localisation signal- 1 or cryptic NLS; NLS-2: Nuclear localisation signal -2 or fonctional NLS; NoLS: Nuclear localisation signal sequence. This figure is adapted from Rowland and Yoo, 2003.

Figure 4. Functional and structural domains of the PRRSV GP_5 protein of North American isolates. The numbers identify the amino acids positions covered by each domain. Conserved (black) and non-conserved (grey) glycolysation sites: N30, N34, N44 and N51; the conserved cysteine residue involved in the formation the disulfide bond between GP_5 and M is illustrated: 48-Cys; signal sequence: 1-32; ectodomain: 33-63; transmembrane region: 64-134; endodomain: 135-200; Major neutralizing epitope: 36-52; Hypervariable immunodominant non-neutralizing epitope A (decoy epitope): 27-30; Conserved non-immunodominant neutralizing

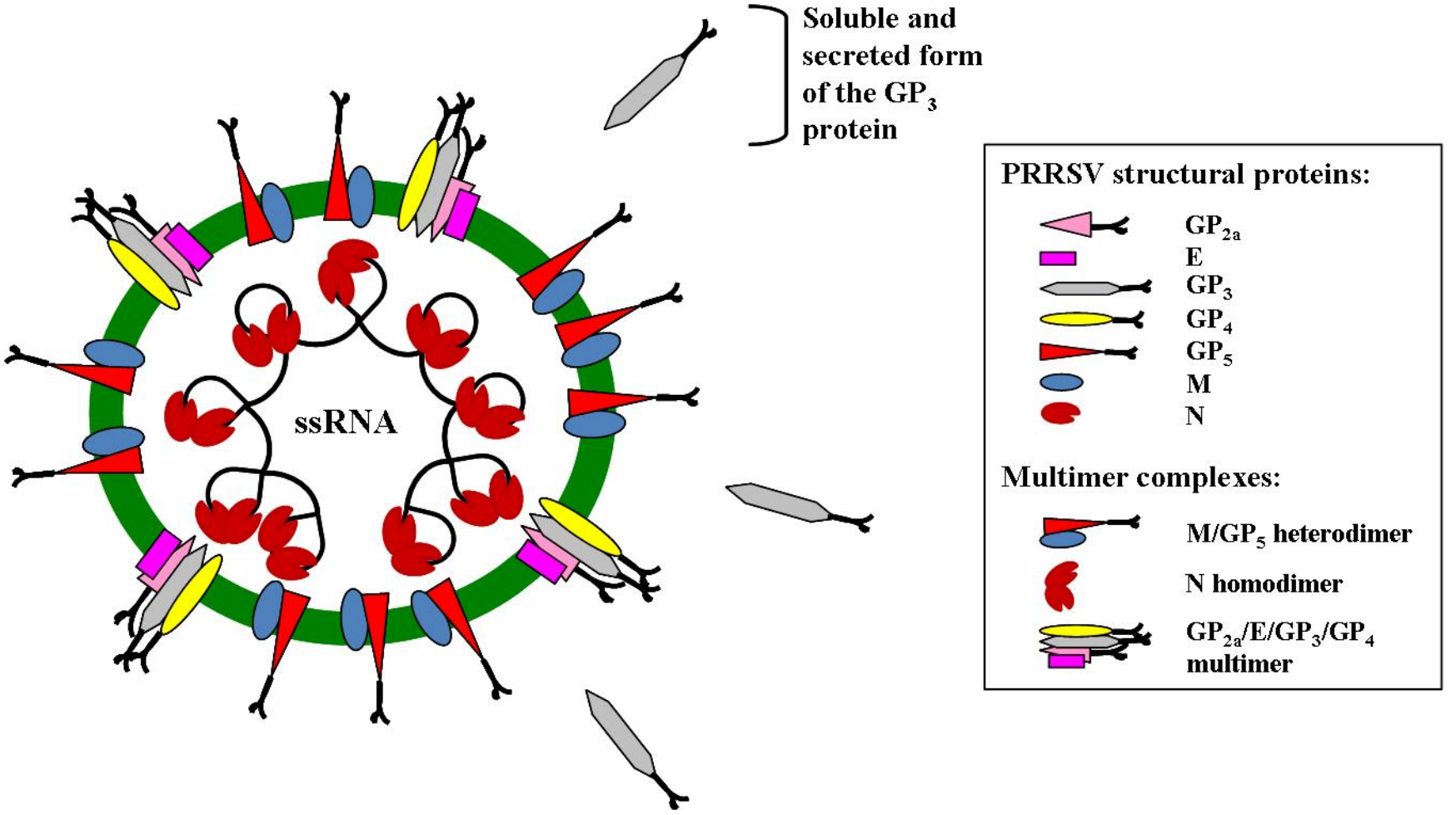
epitope B: 37-45; Variable regions 57-70 and 121-130; Conserved regions: 41-56, 71-120 and 131-200.

Table 1. PRRSV structural and non-structural proteins characteristics and functions.

Proteins	Genes	Amino acids lengths		Characteristics and functions
		EU strains	NA strains	
nsp1	**ORF1a**	**385**	**383**	**Non-structural multifunctional regulatory protein (processing, transcription, virion biogenesis); proteolytic activities; IFN inhibition.**
nsp2		**861**	**980**	**Non-structural, the largest PRRSV replicative protein; the major genetic differences between NA and EU PRRSV strains; ideal marker for monitoring the genetic variation and for developing differential diagnostic tests; proteolytic activities; IFN inhibition.**
nsp3		**447**	**446**	**Non-structural proteins; proteolytic activities; nsp4: IFN inhibition.**
nsp4		**203**	**204**	
nsp5		**170**	**170**	
nsp6		**16**	**16**	
nsp7		**269**	**259**	
nsp8		**45**	**45**	
nsp9	**ORF1b**	**645**	**640**	**Non-structural proteins; virus transcription and replication; nsp9: RNA-dependent RNA polymerase and NTPase; nsp10: helicase; nsp11: IFN inhibition;.**
nsp10		**442**	**441**	
nsp11		**224**	**223**	
nsp12		**152**	**153**	
GP_{2a}	**ORF2a**	**249**	**256**	**Minor structural protein; contain 2 two highly conserved putative N-linked glycosylation sites; essential for virus infectivity; incorporated into virions as a multimeric complex; viral attachment protein.**
E	**ORF2b**	**70**	**73**	**Minor unglycosylated and myristoylated structural protein; essential for virus infectivity; incorporated into virions as a multimeric complex; possesses ion-channel like properties and may function as a viroporin in the envelope.**
GP_3	**ORF3**	**265**	**254**	**Minor structural protein, one of the most variable PRRSV proteins; highly glycosylated with potentially seven N-linked oligosaccharides; its membrane topology seems to be strains dependent; highly antigenic and may be involved in viral neutralization; essential for virus infectivity; incorporated into virions as a multimeric complex.**
GP_4	**ORF4**	**183**	**178**	**Minor structural highly glycosylated protein (four N-linked glycosylation sites); essential for virus infectivity; key glycoprotein for formation of the multiprotein complex incorporated into virions; mediates interaction between multiprotein complex of the minor viral glycoproteins and GP_5; viral attachment protein and may be involved in viral neutralization.**
GP_5	**ORF5**	**201**	**200**	**Major structural, transmembrane protein with a variable number of potential N-glycosylation sites; the most variable structural proteins in the PRRSV genome with GP_3; involved in virus neutralisation and protection; the covalent association of GP_5 and M is crucial for virus assembly; involved in the entry of virus into the host cells and in the apoptosis phenomenon.**
M	**ORF6**	**173**	**174**	**Major unglycosylated structural protein which is the most conserved; play a key role in virus assembly and budding; GP_5-M heterodimerization is crucial for virus infectivity.**
N	**ORF7**	**128**	**123**	**Major unglycosylated, phosphorylated and structural protein; highly immunogenic and a suitable candidate for the detection of virus-specific antibodies and diagnosis of the disease; the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions; able to localize in the nucleus/nucleolus and interact with cellular transcription factor.**

ORF: Open reading frames; EU: European PRRSV strains; NA: North American PRRSV strains.

Fig. 1


Soluble and secreted form of the GP3 protein
ssRNA
PRRSV structural proteins:
GP2a
E
GP3
GP4
GP5
M
N
Multimer complexes:
M/GP5 heterodimer
N homodimer
GP2a/E/GP3/GP4 multimer

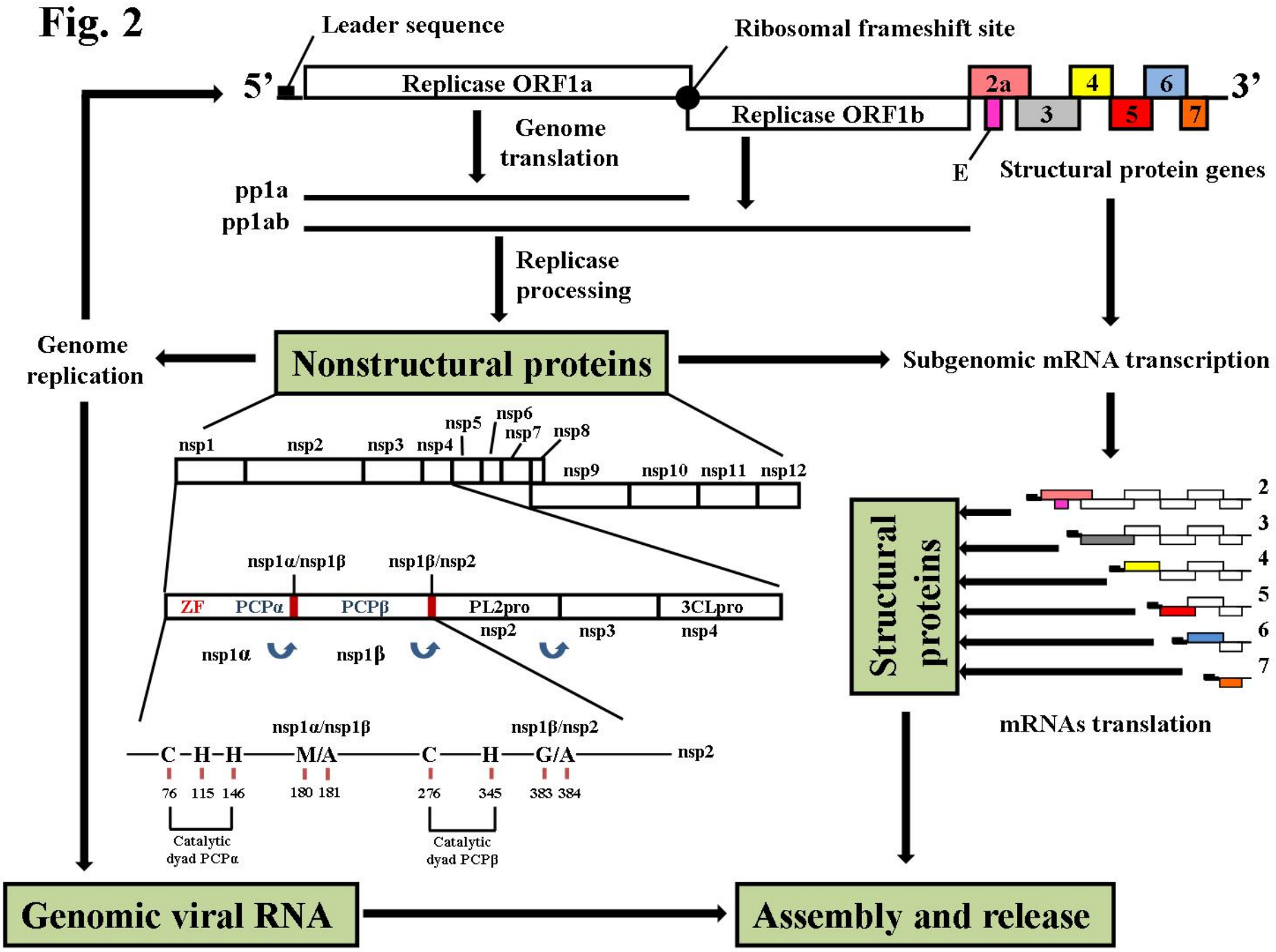


Fig. 2
Leader sequence
Ribosomal frameshift site
5'
Replicase ORF1a
Replicase ORF1b
2a
4
6
3'
3
5
7
E
Structural protein genes
Genome translation
pp1a
pp1ab
Replicase processing
Genome replication
Nonstructural proteins
Subgenomic mRNA transcription
nsp1
nsp2
nsp3
nsp4
nsp5
nsp6
nsp7
nsp8
nsp9
nsp10
nsp11
nsp12
nsp1α/nsp1β
nsp1β/nsp2
ZF
PCPα
PCPβ
PL2pro
3CLpro
nsp2
nsp3
nsp4
nsp1α
nsp1β
Structural proteins
2
3
4
5
6
7
mRNAs translation
nsp1α/nsp1β
nsp1β/nsp2
C–H–H
M/A
C
H
G/A
nsp2
76 115 146
180 181
276 345
383 384
Catalytic dyad PCPα
Catalytic dyad PCPβ
Genomic viral RNA
Assembly and release

Fig. 3

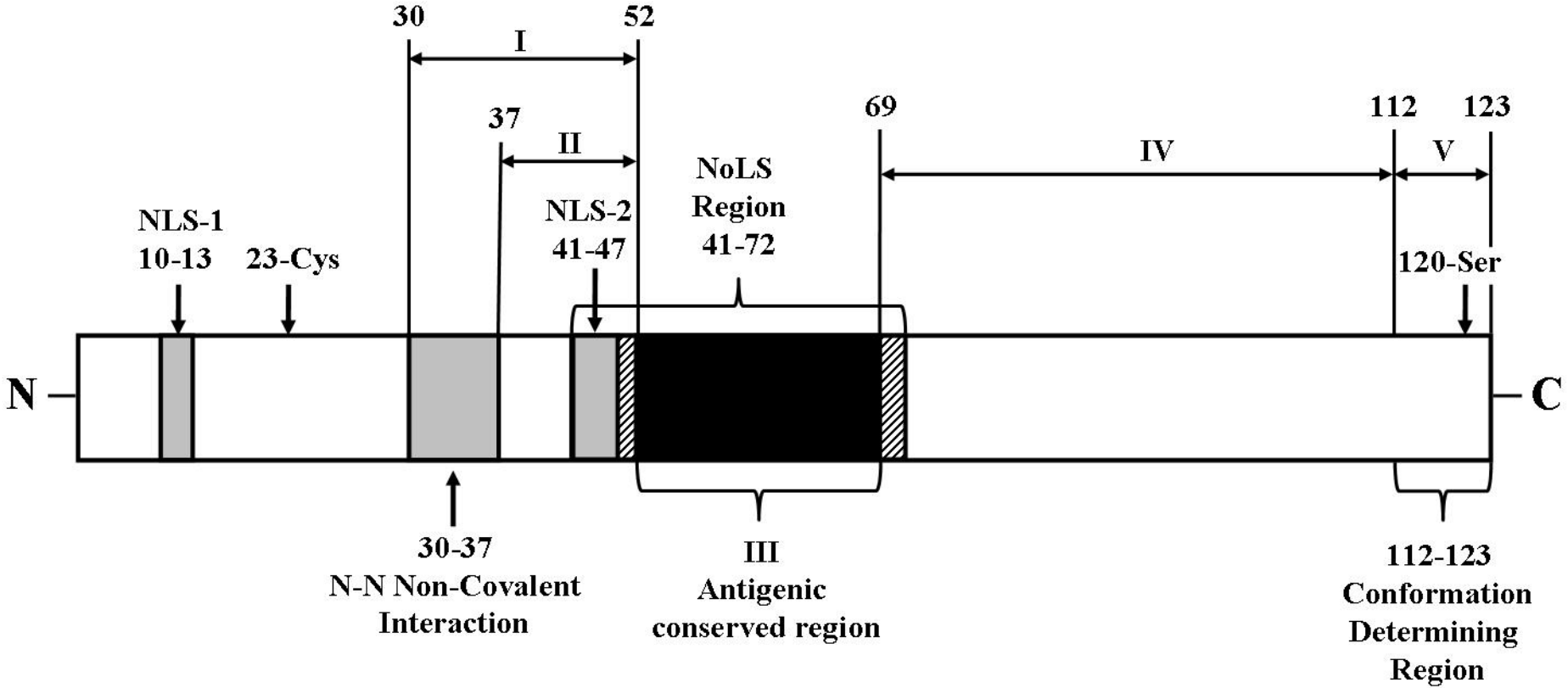
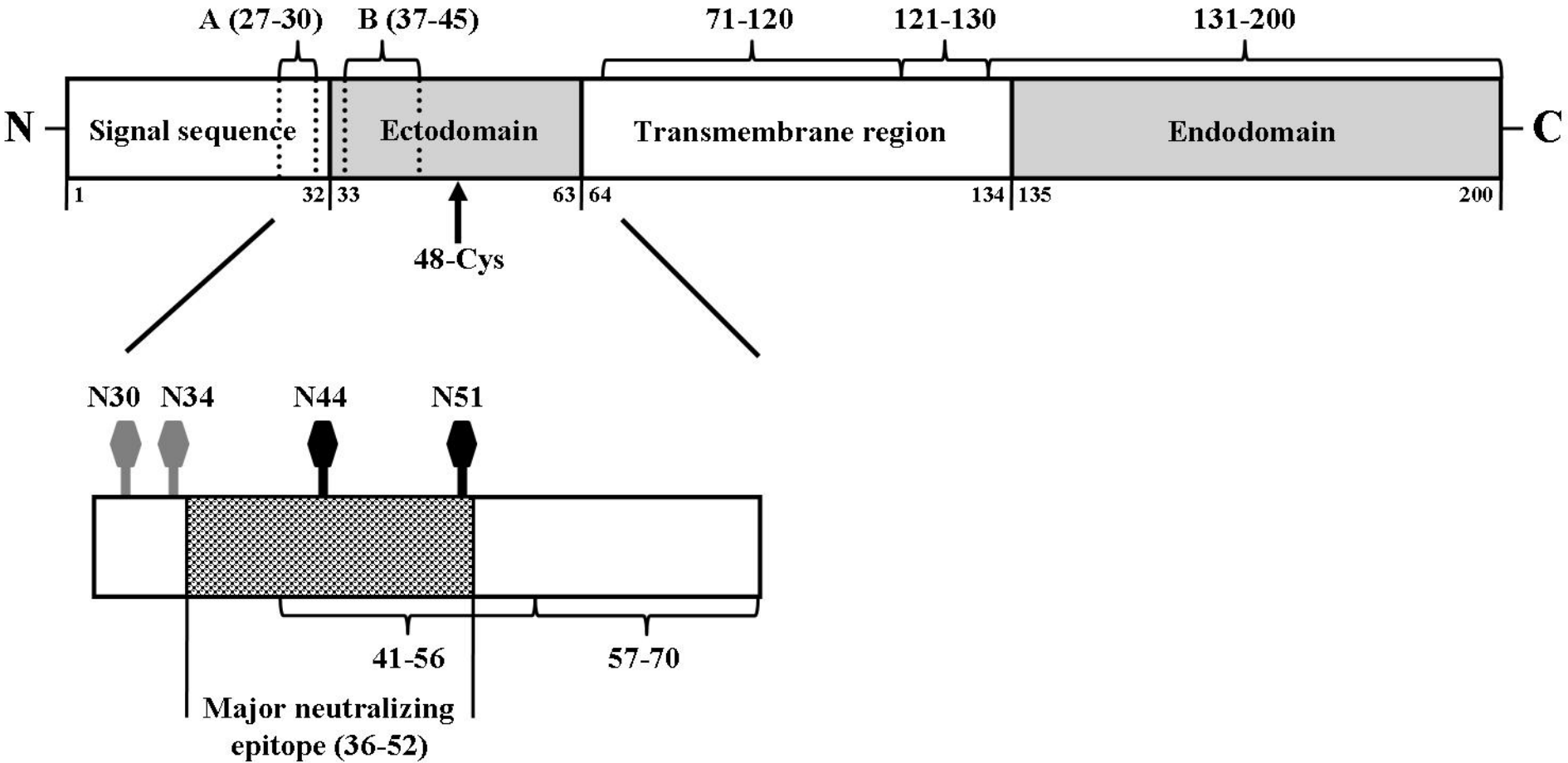

30
52
I
37
II
69
112
123
IV
V
NoLS
Region
41-72
NLS-1
10-13
23-Cys
NLS-2
41-47
120-Ser
N
C
30-37
N-N Non-Covalent
Interaction
III
Antigenic
conserved region
112-123
Conformation
Determining
Region

Fig. 4


A (27-30)
B (37-45)
71-120
121-130
131-200
N
Signal sequence
Ectodomain
Transmembrane region
Endodomain
C
1
32
33
63
64
134
135
200
48-Cys
N30
N34
N44
N51
41-56
57-70
Major neutralizing
epitope (36-52)