Identification of porcine reproductive and respiratory syndrome virus ORF1a-encoded non-structural proteins in virus-infected cells

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The porcine reproductive and respiratory syndrome virus (PRRSV) replicase gene consists of two large ORFs, ORF1a and ORF1b, the latter of which is expressed by ribosomal frameshifting. The ORF1a-encoded part of the resulting replicase polyproteins (pp1a and pp1ab) is predicted to be processed proteolytically into ten non-structural proteins (nsps), known as nsp1–8, with both the nsp1 and nsp7 regions being cleaved internally (yielding nsp1a and nsp1b, and nsp7a and nsp7b, respectively). The experimental verification of these predictions depends strongly on the ability to identify individual cleavage products with specific antibodies. In this study, a panel of monoclonal and polyclonal antibodies was generated, which together were able to recognize eight ORF1a-encoded PRRSV nsps. Using these reagents, replicase cleavage products were detected in PRRSV-infected MARC-145 cells using a variety of immunoassays. By immunofluorescence microscopy, most nsps could be detected by 6 h post-infection. During the early stages of infection, nsp1a, nsp2, nsp4, nsp7a, nsp7b, and nsp8 co-localized in distinct punctate foci in the perinuclear region of the cell, which were determined to be the site of viral RNA synthesis by in situ labelling. Western blot and immunoprecipitation analysis identified most individual nsps and several long-lived processing intermediates (nsp3–4, nsp5–7, and nsp8). The identification and subcellular localization of PRRSV nsps in virus-infected cells documented here provides a basis for the further structure–function studies. Thus, this PRRSV antibody panel will be an important tool for future studies on the replication and pathogenesis of this major swine pathogen.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), a disease first described in the USA in 1987 (Keffaber, 1989) and in Europe in 1991 (Wensvoort et al., 1991), has caused tremendous economic losses to the swine industry since its appearance, with recent annual costs estimated to be at least US$600 million in the USA alone (Neumann et al., 2005). Hallmark symptoms of PRRS are mild to severe respiratory disease in infected newborn and growing pigs, and reproductive failure in pregnant sows. The aetiological agent, PRRS virus (PRRSV), was first identified in the Netherlands in 1991, and is represented by the European prototypic strain, Lelystad virus (Wensvoort et al., 1991). In the USA, PRRSV was first isolated in 1992, and the North American prototypic strain was designated VR-2332 (Benfield et al., 1992; Collins et al., 1992). At the genome level, these two genotypes, European (type I) and North American (type II), share ~60% sequence identity (Allende et al., 1999; Nelsen et al., 1999). PRRSV is classified in the family Arteriviridae, which also includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus and simian hemorrhagic fever virus (Snijder & Meulenberg, 1998).

PRRSV is a small, enveloped virus containing a single-stranded, positive-sense RNA genome. The genome is ~15 kb and contains at least ten ORFs. The 3′-terminal quarter of the genome encodes structural proteins, including four envelope glycoproteins (GP2a, GP3, GP4 and GP5), three non-glycosylated membrane proteins [2b, ORF5a and matrix (M)] and the nucleocapsid protein (N) (Meulenberg et al., 1995a, b; Mournier et al., 1995; Bautista et al., 1996; Mardassi et al., 1996; Meng et al., 1996; Meulenberg & Petersen-den Besten, 1996; Snijder et al., 1999; Wu et al., 2001; Firth et al., 2011; Johnson et al., 2011). The PRRSV replicase gene consists of two ORFs, ORF1a and ORF1b, which occupy the 5′-proximal three-quarters of the genome. Both are expressed from the viral genome, with expression...
of ORF1b depending on a conserved ribosomal frameshifting mechanism. Subsequently, extensive proteolytic processing of the resulting pp1a and pp1ab polyproteins yields the functional non-structural proteins (nsps), most of which assemble into a membrane-associated replication and transcription complex. This enzyme complex not only mediates genome replication but also controls the synthesis of a nested set of subgenomic mRNAs for expression of the structural proteins (Snijder et al., 1994; van Dinten et al., 1996; van der Meer et al., 1998; Ziebuhr et al., 2000; Fang & Snijder, 2010).

The proteolytic maturation of the arterivirus pp1a and pp1ab replicase polyproteins has been studied in detail only for EAV, the family prototype. A complex proteolytic cascade was uncovered, involving three ORF1a-encoded proteases, at least 11 cleavage events (Snijder et al., 1992, 1995; Ziebuhr et al., 2000) and alternative processing pathways (Wassenaar et al., 1997). Although, based on sequence comparisons, many of these features appear to be conserved in other arteriviruses, a number of striking differences complicate a straightforward comparison (Fig. 1). First, the currently known arteriviruses are only distantly related. For example, the overall sequence identity in the pp1ab replicase polyproteins of EAV and PRRSV is about 30–45%, depending on the genome region and specific strains analysed (Nelsen et al., 1999). Secondly, there are considerable size differences, in particular in the N-terminal region of ORF1a, leading to a pp1a of only 1727 aa in EAV, whereas the PRRSV counterpart can be up to ∼2500 aa. Finally, an additional protease domain is known to be functional in the case of PRRSV, and probably all other arteriviruses, which has lost its proteolytic activity in the case of EAV (den Boon et al., 1995). Thus, the PRRSV polyproteins are assumed to be cleaved by three papain-like proteases (PLPα, PLPβ and PLP2) located in the nsp1–nsp2 region, and the viral main protease, SP, residing in nsp4. A comparison with the EAV sequence and data would suggest that the PRRSV replicase polyproteins are being cleaved into at least 14 nsps, specifically nsp1 to nsp12, with both the nsp1 and nsp7 parts being subject to an internal cleavage (yielding nsp1α and nsp1β, and nsp7α and nsp7β, respectively). Although nsp1α and nsp1β have been detected previously in PRRSV-infected cells (Chen et al., 2010), a comprehensive analysis of the processing steps and products in PRRSV replicase maturation clearly depends on the availability of specific antibodies that can recognize individual cleavage products in a variety of arteriviruses.

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**Fig. 1.** Comparison of EAV and PRRSV replicase polyprotein size, domain organization and (tentative) proteolytic processing schemes. Top: PRRSV genome organization, showing the 5'-proximal replicase ORF1a and ORF1b, as well as the downstream ORFs encoding the viral structural proteins: envelope (E), M, N, ORF5a (5a), GP2 to GP5 and the 3' poly(A) tail. Bottom: overview of the PRRSV and EAV pp1ab replicase polyproteins derived from genome translation when ORF1a/1b ribosomal frameshifting occurs. Arrowheads indicate the sites cleaved by the three or four virus-encoded proteases [the cysteine proteases PLPα (Pα), PLPβ (Pβ), PLP2 (P2) and the ‘main protease’, the nsp4 serine protease (SP)]. The numbering/nomenclature of the resulting nsps is indicated. In addition to the protease domains, three ORF1b-encoded enzymic domains are depicted: the nsp9 RNA-dependent RNA polymerase (RdRp), the nsp10 helicase (Hel) and the nsp11 endoribonuclease (Ne). Domains labelled T are putative transmembrane regions that are thought to be involved in membrane anchoring of the viral replicase complex. Note the presence of an uncleaved nsp1 in EAV and nsp1α and nsp1β subunits in PRRSV, and the remarkable size difference between the nsp2 subunits of the two viruses. Amino acid numbering is based on PRRSV strain SD01-08 (Fang et al., 2006a) and EAV strain Bucyrus (den Boon et al., 1991). ZF, Zinc finger; HVR, hypervariable region; C, cluster of conserved Cys residues; Z, predicted zinc-binding domain; RFS, –1 ORF1a/1b ribosomal frameshift site. Adapted from Nedialkova et al. (2010) and Fang & Snijder (2010).
immunoassays. Since the identification of PRRSV more than 20 years ago, and despite the importance of the virus as a veterinary pathogen, such a panel of reagents has not yet been described. Therefore, as a first and essential step towards understanding the structure and function of PRRSV nsps, we have generated a panel of antibodies against the PRRSV ORF1a-encoded nsps and used it to detect the expression of these proteins and analyse their subcellular localization in virus-infected cells.

RESULTS

Generation of antibodies to ORF1a-encoded replicase subunits

Based on comparative sequence analysis and the established EAV replicase cleavage sites, the predicted cleavage sites for PRRSV replicase polyprotein processing have been inferred previously (Ziebuhr et al., 2000; Fang & Snijder, 2010). In this study, these putative cleavage sites were used as borders for cloning and expression in Escherichia coli of sequences representing individual PRRSV replicase subunits (nsp1, nsp2, nsp4, nsp7 and nsp8) or synthesis of peptide antigens (nsp2, nsp3, nsp5 and nsp6) for production of nsp-specific antibodies (Table 1). Previous studies identified the nsp1 production of nsp-specific antibodies (Table 1). Previous studies identified the nsp1 production of nsp-specific antibodies (Table 1). Previous studies identified the nsp1 production of nsp-specific antibodies (Table 1). Previous studies identified the nsp1 production of nsp-specific antibodies (Table 1).

Table 1. Generation of antibodies to PRRSV SD01-08 ORF1a-encoded replicase proteins

<table>
<thead>
<tr>
<th>Non-structural protein</th>
<th>Predicted molecular mass (kDa)</th>
<th>Antibody name (replicase pp1a aa)*</th>
<th>mAb isotype</th>
<th>Detection assay(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsp1 (nsp1x+nsp1β)</td>
<td>44.0</td>
<td>pAb Eu-nsp1x (nsp1x)</td>
<td>–</td>
<td>RIP</td>
</tr>
<tr>
<td></td>
<td>(18.9 + 25.1)</td>
<td>mAb Eu22-28 (nsp1β)</td>
<td>IgG1</td>
<td>IFA, WB, RIP</td>
</tr>
<tr>
<td>nsp2</td>
<td>114.1</td>
<td>pAb Eu-nsp2</td>
<td>–</td>
<td>IFA, RIP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mAb Eu36-19</td>
<td>IgG1</td>
<td>IFA, RIP</td>
</tr>
<tr>
<td>nsp2 + 3</td>
<td>24.6</td>
<td>pAb Eu-nsp2 + 3</td>
<td>–</td>
<td>IFA, WB, RIP</td>
</tr>
<tr>
<td>nsp4</td>
<td>21.1</td>
<td>mAb Eu54-19</td>
<td>IgG1</td>
<td>IFA, WB, RIP</td>
</tr>
<tr>
<td>nsp7α</td>
<td>15.7</td>
<td>mAb Eu9-31</td>
<td>IgG1</td>
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</tr>
<tr>
<td>nsp7β</td>
<td>13.8</td>
<td>mAb Eu24-40</td>
<td>IgG1</td>
<td>IFA, RIP</td>
</tr>
<tr>
<td>nsp8</td>
<td>4.8</td>
<td>mAb Eu12-40</td>
<td>IgG1</td>
<td>IFA, WB, RIP</td>
</tr>
</tbody>
</table>

*The nsp2 + 3 antigen was prepared from the PRRSV Lelystad isolate (GenBank accession no. M96262), and the rest of the antigens were prepared from PRRSV SD01-08 isolate (GenBank accession no. DQ489311).

Mice immunized with nsp1 produced a strong antibody response to nsp1β. All of the mAbs obtained from mice immunized with nsp1 recognized nsp1β. Additionally, we obtained mAbs against nsp2, nsp4, nsp7 and nsp8, but not against nsp1x, nsp3, nsp5 or nsp6. The isotypes of the various mAbs are listed in Table 1. To facilitate dual-labelling experiments, we immunized rabbits with recombinant proteins representing nsp1x or nsp2, or a combination of synthetic peptides derived from the nsp2 N terminus and the nsp3 C terminus (Table 1), and obtained a set of rabbit polyclonal antibodies (pAbs).

Immunofluorescence microscopy was performed on cells infected with strain SD01-08 (genotype I virus) or strain VR2332 (genotype II virus), which revealed that this panel of mAbs is highly genotype specific, as cross-reactivity between our type I-specific mAbs and type II virus-infected cells was not observed. The pAb against nsp1x was also genotype specific, but the Eu-nsp2 and nsp2+3 pAbs generated with type I-specific antigens also reacted with type II virus (data not shown).

mAb reactivity to nsp7α and nsp7β

In EAV, the nsp7 subunit was found previously to contain an internal cleavage site (van Aken et al., 2006), which is used with a relatively low efficiency by the nsp4 SP to generate the nsp7α and nsp7β subunits. Sequence alignment showed that this cleavage site is conserved in PRRSV nsp7 (*2214E*N2215*). To determine which part of nsp7 was recognized by the anti-nsp7 mAbs that we obtained (Table 1; mAbs Eu9-31/Eu24-40), nsp7, nsp7α and nsp7β were expressed from cytomegalovirus promoter constructs (vector pCAGGS) and a similar construct was used to co-express nsp4. Initially, HEK293T cells were transfected with pCAGGS-nsp7α or pCAGGS-nsp7β, and IFA was performed using mAb Eu9-31 or Eu24-40. The result showed...
that mAb Eu9-31 recognized nsp7α, whilst mAb Eu24-40 recognized nsp7β (Fig. 2a). To further confirm that PRRSV nsp7 could actually be cleaved into α and β subunits, nsp7 and nsp4 were co-expressed in HEK293T cells and nsp7 processing was analysed by Western blotting. As shown in Fig. 2(b), nsp7 was detected as a single protein of ~30 kDa in lysates from cells transfected with pCAGGS-nsp7 only. In contrast, in cells co-transfected with pCAGGS-nsp7 and pCAGGS-nsp4, the level of nsp7 was much lower than in cells transfected with pCAGGS-nsp7 only, and both nsp7 and nsp7α were detected by mAb Eu9-31, demonstrating that nsp7 was indeed cleaved by nsp4. In three independent experiments, mAb Eu24-40 appeared not to react with nsp7β in the Western blot, but it did recognize the full-length nsp7 (Fig. 2b). In our previous studies (Brown et al., 2009; Langenhorst et al., 2012), we noted that recombinant nsp7β was sensitive to degradation after purification from E. coli. This observation may relate to the result of the current study, as most of the cleaved nsp7β could be rapidly degraded during sample preparation for Western blot analysis, and/or our detection method may not have been sensitive enough to detect the remaining part of nsp7β if it were present.

Identification of ORF1a-encoded replicase proteins in virus-infected cells by Western blotting and RIP

To investigate the expression and processing of the 2379 aa encoded by PRRSV SD01-08 ORF1a, we used the panel of antibodies raised against nsp1 to nsp8 of the SD01-08 virus in Western blot and RIP analysis. Rabbit pre-immunization sera, mouse ascites containing non-PRRSV-specific mAb and uninfected cell lysates were used as negative controls.

In the Western blot, pAb Eu-nsp1α detected a single protein band just below the 22 kDa molecular marker, which corresponded to the predicted size of nsp1α (18.9 kDa; Fig. 3a), whilst mAb Eu22-28 strongly detected a protein band above the 22 kDa molecular marker, corresponding to the predicted size of nsp1β (25.1 kDa; Fig. 3b). These two proteins, nsp1α and nsp1β, were also consistently detected in RIP experiments (Fig. 4a, b).

The mAb against nsp2 (Eu36-19) generated a strong background signal in the Western blot analysis (data not shown), but in RIP this mAb brought down a specific protein band migrating substantially slower than the 97 kDa molecular mass marker (Fig. 4c). Several additional weak protein bands were detected in the nsp2 RIP, but it remains to be established whether these represent nsp2-related processing or degradation products.

Rabbit antiserum pAb Eu-nsp2+3 was raised using a combination of nsp2 and nsp3 epitopes (Table 1) and in Western blot analysis a specific protein of ~22 kDa (nsp3) and a large protein in the size range predicted for nsp2 were both detected (Fig. 3c). However, in immunoprecipitation analysis of infected cell lysates, pAb Eu-nsp2+3 mainly recognized nsp3 (Fig. 4d). The apparent size of the presumed nsp2 subunit detected by both Western blotting and RIP appeared to be larger than the predicted size (114 kDa), but this may have been due to aberrant migration of this product, which contains a sizeable hydrophobic domain. In contrast, we could not formally exclude the possibility that the high-molecular-mass band

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**Fig. 2.** Reactivity of mAbs to PRRSV nsp7α and nsp7β. (a) HEK293T cells were transfected with plasmids expressing PRRSV nsp7α or nsp7β protein and analysed by immunofluorescence microscopy following staining with the specific antibodies indicated. Bar, 100 μm. (b) HEK293T cells were transfected with a plasmid expressing PRRSV nsp7 or co-transfected with plasmids expressing nsp7 and nsp4. At 24 h post-transfection, cells were harvested and Western blot analysis was performed using nsp7- or nsp4-specific antibodies as indicated. The arrows indicate specific PRRSV proteins.
in Fig. 3(c) was the nsp2–3 precursor, as serum pAb Eu-
nsp2+3 only recognized the cleaved nsp3, and not nsp2, in
RIP analysis.

mAb Eu54-19 against nsp4, the viral main protease,
detected a distinct protein band around 24 kDa in both
Western blotting (Fig. 3d) and RIP (Fig. 4e). In the case of
EAV, nsp3–4 and nsp3–8 were observed as relatively long-
lived intermediate cleavage products (Snijder et al., 1994).
The predicted sizes of the PRRSV counterparts of these
products are 46 kDa (nsp3–4) and 100 kDa (nsp3–8), and
in RIP analysis, products nicely matching these sizes were
specifically recognized by mAb Eu54-19 (Fig. 4e). This
observation suggests important parallels between the two
arteriviruses with regard to the sequence and timing of
replicase processing events.

In virus-infected cell lysates, the nsp7α-specific mAb Eu9-
31 recognized a doublet around the 50 kDa size marker
(Fig. 3e), which appeared to match the calculated sizes of

![Fig. 3. Western blot detection of ORF1a-encoded nsps in PRRSV-infected cell lysates using a panel of ORF1a protein-
specific antibodies. Cell lysates from PRRSV SD01-08-infected or mock-infected cells were separated by SDS-PAGE and
transferred to a nitrocellulose membrane. Each membrane was probed with an nsp-specific mAb or pAb as indicated. The
position of the molecular mass markers and various PRRSV nsps are indicated. M, Mock-infected cell lysate; V, PRRSV-
infected cell lysate.](http://vir.sgmjournals.org)

![Fig. 4. RIP analysis of PRRSV-infected or mock-infected MARC-145 cell lysates with ORF1a protein-specific antibodies.
For each antibody indicated, a set of two lanes is shown: M, immunoprecipitation with mock-infected cell lysate; V,
immunoprecipitation with PRRSV-infected cell lysate. The position of the molecular mass markers and various PRRSV nsps are
indicated. Cells were infected at an m.o.i. of 5 and cellular protein synthesis was labelled with 35S from 24 to 30 h p.i.](http://vir.sgmjournals.org)
the prominent nsp5–7 (49.7 kDa) and nsp5–8 (54.7 kDa) processing intermediates observed in the EAV system (Snijder et al., 1994; Wassenaar et al., 1997). In addition, a product of ~15 kDa was observed that corresponded to nsp7x (see also Fig. 2b). The identification of nsp5–7 and nsp5–8 was further supported by the fact that only the upper of the two bands was recognized by the nsp8-specific antibody Eu12-40 (Fig. 3f). The last subunit upstream of the ribosomal frameshift site is nsp8, which is identical to the N-terminal domain of the RdRp-containing nsp9 subunit, the N terminus of which is released by cleavage of the nsp7/8 site. On the Western blot, we noted a weak protein band around 85 kDa, which is the predicted size of nsp9 (Fig. 3f), but further studies are needed to confirm the identity of this product.

The antibody against nsp7β (mAb Eu24-40) did not specifically recognize any PRRSV proteins in the Western blot (data not shown). However, when used in RIP, this antibody did bring down the presumed nsp5–7 and nsp5–8 bands (Fig. 4f). In addition, the nsp8-specific mAb Eu12-40 brought down the same doublet (Fig. 4g), which can be explained by co-immunoprecipitation of the two products under these conditions, as nsp5–7 should not be recognized by this antibody and only the nsp5–8 band was recognized in the Western blot (Fig. 3f). The nsp8-specific RIP also revealed a very small product that may correspond to fully cleaved nsp8 (predicted size ~5 kDa). This product was not detected in Western blot analysis (Fig. 3f), which may be due to a lower abundance of the protein and/or the sensitivity of the assay, as nsp5–8 was detected in the Western blot using mAb Eu12-40. Finally, the RIP experiments targeting nsp4, nsp7 and nsp8 revealed a number of larger precursors, including nsp5–8 (Fig. 4e–g).

Intracellular localization of PRRSV ORF1a replicase proteins

Previous immunofluorescence microscopy studies of EAV-infected cells have shown that replicase processing products assemble in the perinuclear region where they form a membrane-associated complex that is thought to direct virus replication and transcription (van der Meer et al., 1998). Here, we used our newly obtained antibody panel to study whether PRRSV nsps behave similarly, and to visualize the site of viral RNA synthesis.

To investigate the intracellular localization of PRRSV ORF1a-encoded replicase subunits, the panel of antibodies was used in immunofluorescence microscopy of infected cells. To assess the timing of replicase expression, MARC-145 cells were infected at an m.o.i. of 0.1, and cells were fixed at 4, 5, 6, 7, 8, 10 and 12 h post-infection (p.i.) and stained with the different antibodies. No convincing nsp-specific labelling was detected before 6 h p.i., suggesting that until that time point replicase expression levels were too low to be detected by our microscopy methods. By 6 h p.i., the first signals for nsp1β, nsp2, nsp4, nsp7x, nsp7β and nsp8 could be detected in the perinuclear area of infected cells (data not shown). At 7 h p.i., small but bright fluorescent foci were clearly observed, mostly concentrated on one side of the nucleus (Fig. 5). By 12 h p.i., a more intense and diffuse labelling pattern was observed. For nsp1β, a predominantly nuclear and perinuclear staining pattern was observed in ~80% of the cells at 10 and 12 h p.i. (Fig. 5). The pAb directed against nsp1x (pAb Eu-nsp1x) could not be used in IFA as it produced a high background signal. To verify co-localization of the different nsps, PRRSV-infected MARC-145 cells were doubly stained with anti-nsp mAbs and a rabbit antiserum to nsp2. At 8 h p.i., the labelling for nsp1β, nsp4, nsp7x, nsp7β and nsp8 was largely co-localized with nsp2 in punctate and perinuclear spots (Fig. 6), although some additional nsp2 labelling was commonly found in the periphery of the cell. Furthermore, as described above, part of nsp1β localized to the nucleus.

In EAV-infected cells, viral RNA synthesis was found previously to be associated with the nsp-containing membrane structures in the perinuclear region (van der Meer et al., 1998; Pedersen et al., 1999). To determine whether the perinuclear foci that labelled for PRRSV nsps were indeed the site of viral RNA synthesis, we treated infected cells with actinomycin D to block host-cell mRNA synthesis and transfected them with 5-bromouridine 5’-triphosphate (BrUTP) to label the newly synthesized viral RNA for 1 h. Transfected cells were doubly stained with antibodies that recognized BrUTP-labelled RNA and PRRSV nsp2. The result showed co-localization of PRRSV nsp2 with de novo-synthesized viral RNA (Fig. 7). Overall, these data support the findings of previous studies on EAV (van der Meer et al., 1998), and suggest that PRRSV nsps also assemble into a membrane-bound replication and transcription complex.

DISCUSSION

Since the first isolation of PRRSV, a wealth of information has been obtained on the structural proteins of this pathogen. However, little is known about the structure and function of PRRSV nsps, whose coding sequences occupy ~75% of the viral genome (Snijder & Meulenberg, 1998; Fang & Snijder, 2010). So far, the existence of individual PRRSV nsps and their involvement in genome replication and expression have mainly been deduced from studies of EAV. As underlined by these EAV studies, specific antisera are key reagents in studies on nsp structure and function (Snijder et al., 1995; Dea et al., 1995; Pedersen et al., 1996; Dea et al., 1996; Yang et al., 1999, 2000), few mAbs were raised against nsps. This study is the first to describe the development of a panel of antibodies against PRRSV ORF1a-encoded nsps and their use to detect these proteins in PRRSV-infected cells. For nsp1, we detected two products, nsp1x and -1β, in PRRSV-infected cells, in contrast to one mature nsp1
product from EAV. PRRSV nsp1β showed two different intracellular localization patterns, an early punctate perinuclear localization and a predominantly nuclear labelling late in infection. As our anti-nsp1α antiserum did not specifically recognize nsp1α of type I PRRSV by IFA, its intracellular localization could not be determined. However, our previous study using type II PRRSV demonstrated that nsp1α also localizes to the nucleus.

Fig. 5. Detection of PRRSV ORF1a-encoded nsp expression in infected cells by immunofluorescence microscopy. MARC-145 cells were infected with PRRSV SD01-08 and fixed at 7, 8, 10 or 12 h p.i. Cells were stained with the nsp-specific mAbs listed in Table 1. Specimens were visualized on a Zeiss LSM510 confocal microscope. A 0.8 μm slice through the nucleus is shown in each image. Bar, 20 μm.

Fig. 6. Co-localization of PRRSV replicase proteins. MARC-145 cells were fixed at 8 h p.i. and doubly stained with an nsp2-specific rabbit antiserum and a panel of mAbs recognizing other nsps. Note that each individual nsp localized to the perinuclear region. Bar, 20 μm.
during the course of infection (Chen et al., 2010), and similar observations were made for EAV nsp1. This protein was also found to interact with the host protein p100, a transcriptional co-activator, suggesting that nsp1 might modulate transcription in the infected cell (Tijms & Snijder, 2003). Recent studies demonstrated that PRRSV nsp1α and nsp1β are probably involved in modulating the interferon response; in particular, the nuclear localization of PRRSV nsp1 was determined to be associated with the degradation of interferon transcription factor CREB-binding protein (Kim et al., 2010). These studies suggest that arterivirus nsp1 proteins not only function in virus replication but also play an important role in host metabolism and immune modulation.

Comparative sequence analysis showed that the PRRSV nsp2 was predicted to be cleaved at \( ^{385}\text{G}/^{386}\text{A} \) of SD01-08 at its N terminus. The C-terminal cleavage site is most probably located at \( ^{1446}\text{G}/^{1447}\text{A} \) of EAV, 1 aa downstream from the previously predicted site at \( ^{1445}\text{G}/^{1446}\text{G} \) (Ziebuhr et al., 2000), as the nsp2-encoded cysteine protease is now known to have a preference to cleave after GG instead of between GG (Frias-Staheli et al., 2007). The predicted size for nsp2 (replicase residues 386–1446 of SD01-08) is 114 kDa. However, in our analysis, the nsp2-specific antibody recognized a product with a considerably larger apparent molecular mass. Whether this was caused by aberrant migration or post-translational modifications could not be resolved in this study. In addition, several smaller and less abundant products were also detected by nsp2-specific antibodies in RIP experiments. A similar phenomenon was also observed in nsp2 immunoprecipitation experiments using a type II PRRSV, VR2332 (Han et al., 2010). At this time, we cannot exclude the possibility of the existence of other nsp2-related processing or degradation products. Furthermore, we also cannot exclude the involvement of host-cell proteases that could generate smaller nsp2-related products. In EAV, the 61 kDa nsp2 was found to be cleaved internally by a host-cell-specific protease, yielding 18 and 44 kDa cleavage products in Vero cells, but not in BHK-21 and RK-13 cells (Snijder et al., 2001). Identification of other smaller protein products is in progress in our laboratory.

Protein sequence analysis showed that nsp3 and nsp5 are largely composed of hydrophobic domains, as in other arterviruses. The generally hydrophobic nature of these proteins is a probable explanation for our failure to generate specific mAbs against these replicase subunits. It is predicted that nsp6 consists of only 11 aa, but this peptide seemed to be poorly immunogenic and we could not generate antibodies by immunizing mice with an nsp6 synthetic peptide.

Based on the EAV replicase processing model of major versus minor proteolytic processing pathways (Wassenaar et al., 1997), nsp5 and nsp5–6 may not be produced at all, as cleavage of the nsp4/5 site seems to prevent cleavage of the nsp5/6 and nsp6/7 sites and vice versa. Consequently, nsp5 and nsp6 were only detected in the form of processing intermediates with other nsps. Using the PRRSV nsp7- and nsp8-specific antibodies, we detected a protein doublet with a molecular mass around 50 kDa. Based on the size estimates, we assume that this protein doublet is formed by the processing intermediates nsp5–7 and nsp5–8 (predicted sizes of ~50 and ~55 kDa). Further studies, in particular mutagenesis of specific cleavage sites, are needed to determine the actual protein components of these products. Unlike the N-terminal portion of pp1a/pp1ab, no functional assignments have been made for the C-terminal portion nsp6–nsp8 region. We believe that antibodies generated from this study will prove to be valuable tools for future studies on the structure and function of these proteins.

In IFAs, PRRSV ORF1a-encoded nsps could be detected by 6 h p.i. This implies that nsp-specific mAbs can also be useful tools for the early detection of PRRSV infection. Furthermore, as replicase proteins contain both highly variable domains and regions that are conserved within/ between genotypes, it would be useful to identify a panel of nsp-specific mAbs that is both broadly reactive to various field strains and is able to differentiate between certain specific strains or (sub)genotypes.

In conclusion, we have developed a panel of antibodies against PRRSV ORF1a-encoded nsps, and confirmed the existence of individual mature nsps in virus-infected cells. This study provides a basis for both basic and applied research on the role of PRRSV nsps in virus replication and pathogenesis.

**METHODS**

**Cells and viruses.** HEK293T cells and MARC-145 cells (a PRRSV-permissive cell line) were maintained in appropriate medium and...
under appropriate incubation conditions, as described previously (Kim et al., 1993). The type I PRRSV strain SD01-08 (GenBank accession no. DQ489311; Fang et al., 2006a) was propagated in MARC-145 cells.

**Antigen expression.** Recombinant proteins, including nsp1 (nsp1x and nsp1f together), nsp2, nsp4, nsp7 and nsp8, were generated based on the sequence of the PRRSV SD01-08 strain. For nsp2 antigen expression, the conserved PLP2 domain region was expressed as a recombinant protein. The antigens for production of antibodies to nsp1, nsp4, nsp7 and nsp8 were expressed as recombinant proteins based on the predicted cleavage sites (Table 1). These nsp-coding regions were amplified by RT-PCR, and the PCR products were cloned into the pET-28a (+) vector (Novagen). Recombinant proteins were expressed and purified as described previously (Johnson et al., 2007; Brown et al., 2009). Sequence analysis (Hopp–Woodwards hydrophilicity analysis) showed that nsp3 and nsp5 contained mostly hydrophobic regions but included several predicted B-cell linear epitopes. Therefore, instead of expressing the entire nsp3 and nsp5 regions as recombinant proteins, predicted B-cell linear epitope regions were synthesized as synthetic peptides for mouse immunization. The predicted nsp6 subunit, consisting of only 11 aa, was also synthesized. A rabbit antiserum recognizing both nsp2 and nsp3 was raised previously (Kroese et al., 2008) using synthetic peptides. These were based on the sequences of the PRRSV type I Lelystad isolate, with the N-terminal nsp2 peptide containing four point mutations compared with SD01-08, and the nsp3 C-terminal peptide being identical for both isolates.

**Antibody production.** mAbs were produced by immunizing BALB/c mice with 50 μg antigen mixed with Freund’s incomplete adjuvant at 2-week intervals for 8 weeks. Mouse splenocytes were fused with NS-1 myeloma cells. Specific anti-PRRSV mAbs were screened by ELISA and IFA as described previously (Fang et al., 2006b). Hybridomas secreting PRRSV-specific mAbs were subcloned. The mAbs were produced by intraperitoneal injection of pristine–primed mice with hybridoma cells (5 x 10⁶ cells), and the mAb-containing mouse ascites fluids were collected about 2 weeks after injection. mAbs were isotyped using an IsoStrip kit (Serotech) following the manufacturer’s instructions.

pAbs were raised in New Zealand white rabbits. For primary immunizations, 100 μg antigen was mixed with an equal volume of Freund’s incomplete adjuvant and injected subcutaneously at six different locations. Rabbits were boosted twice at 2-week intervals. The immune response was monitored using IFA and ELISA.

**Expression plasmids and transfection.** The nsp4-, nsp7-, nsp7x- and nsp7f-coding regions of the SD01-08 virus were RT-PCR amplified from genomic RNA, and the PCR products were cloned into the eukaryotic expression vector pCAGGS (a generous gift from Dr Adolfo Garcia-Sastre, Mount Sinai School of Medicine, NY, USA), designated pCAGGS-nsp4, pCAGGS-nsp7, pCAGGS-nsp7x and pCAGGS-nsp7f, respectively. HEK293T cells were seeded in 24-well plates and co-transfected with pCAGGS-nsp4 and pCAGGS-nsp7 DNA, or transfected with individual pCAGGS-nsp4, pCAGGS-nsp7, pCAGGS-nsp7x or pCAGGS-nsp7f expression plasmids. Transfection was performed using FuGENE HD transfection reagent following the manufacturer’s instructions (Roche Molecular Biochemicals). At 24 h post-transfection, cells were fixed with 80 % acetone for immunofluorescence microscopy. For Western blot detection of nsp expression, transfected cells were harvested and lysed in Laemmli sample buffer (Laemmli, 1970).

**Immunofluorescence microscopy.** For detection of nsp expression, MARC-145 cells were infected with PRRSV at an m.o.i. of 0.1, and the cells were fixed with 3.7 % formaldehyde in PBS (pH 7.4) at 4, 5, 6, 7, 8, 10 and 12 h p.i. Following permeabilization with 0.1–0.2 % Triton X-100 in PBS for 15 min at room temperature, cells were incubated with nsp-specific mAbs or pAbs at dilutions of 1:1000 or 1:200, respectively, for 1 h. Subsequently, FITC-conjugated goat anti-mouse IgG (ICN Biomedicals) or FITC-conjugated goat anti-rabbit IgG (Invitrogen) was used as secondary antibody. For nsp co-localization studies, pAb Eu-nsp2 was used to stain nsp2, and DyLight 549-labelled goat anti-rabbit IgG (H+L) (KPL; red fluorescence) was used as the secondary antibody. The same infected cell was doubly stained with mAbs against nsp1b, nsp4, nsp7x, nsp7f or nsp8, as indicated in Fig. 6, and FITC-conjugated goat anti-mouse antibody (ICN Biomedicals) was added as the secondary antibody. Nuclear staining with DAPI was performed as recommended by the manufacturer (Molecular Probes). Specimens were imaged using a Zeiss LSM510 confocal microscope with a 63 x objective, and images were processed with NIH ImageJ and Adobe Photoshop 6.0 software.

**BrUTP labelling of viral RNA synthesis.** Metabolic labelling of viral RNA synthesis with BrUTP (Sigma) was performed as described previously (van der Meer et al., 1998). Briefly, the newly synthesized viral RNA in PRRSV-infected MARC-145 cells was labelled at 9 h.p.i., and 30 min before labelling cells were given 10 μg actinomycin D (Sigma) ml⁻¹ to block host-cell transcription. BrUTP was introduced into the cells by liposome-mediated transfection using X-tremeGENE 9 (Roche) following the manufacturer’s instructions. Viral RNA synthesis was labelled for 1 h, after which cells were fixed with 3.7 % formaldehyde for 15 min at room temperature and blocked with 0.1–0.2 % Triton X-100 and 3 % BSA in PBS for 15 min at room temperature. BrUTP-labelled viral RNA was visualized using anti-BrDU mAb clone BU-33 (Sigma) and DyLight 549-labelled goat anti-rabbit IgG (H+L) as secondary antibody. Cells were also stained for nsp2 using the polyclonal rabbit antisera and FITC-conjugated goat anti-rabbit antibody (ICN Biomedicals).

**Western blotting.** PRRSV-infected MARC-145 cell lysates were heated at 96 °C in Laemmli sample buffer for 6 min and the proteins were separated by SDS-PAGE. Protein bands were blotted onto a nitrocellulose membrane as described previously (Wu et al., 2001). After blotting, membranes were blocked with PBS (with 0.05 % Tween 20) and 5 % non-fat dried milk. The membranes were then incubated with nsp-specific antibodies (1:500 dilution of mAbs and 1:50 dilution of antibody sera) for 1 h at room temperature. After washing with PBS, HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit antibodies (MP Biomedicals) were added and the samples were incubated for 45 min. Protein bands were visualized using a chemiluminescence detection kit (Pierce) following the manufacturer’s instructions.

**RIP and SDS-PAGE.** MARC-145 cells were infected with PRRSV SD01-08 at an m.o.i. of 5 for 24 h in Dulbecco’s modified Eagle’s medium containing 2 % FCS. Cells were washed with PBS, followed by starvation for 30 min in methionine- and cysteine-free medium ( Gibco). Subsequently, protein synthesis in the infected cells was metabolically labelled for 6 h in methionine- and cysteine-free medium containing 200 μCi [³⁵S]methionine/cysteine mixture (Perkin-Elmer) ml⁻¹. After labelling, the cells were harvested in lysis buffer [20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 % NP-40, 0.1 % sodium deoxycholate, 0.1 % SDS] and nuclei were removed by centrifugation. Routinely, 5 μl mouse ascites or rabbit antisera was used for the immunoprecipitation analysis of a 200 μl aliquot of cell lysate, equivalent to 4 x 10⁶ cells. Binding was carried out overnight at 4 °C in lysis buffer containing from 0.1 to 0.5 % SDS, depending on the antibody used. Subsequently, immune complexes were pulled down using a 1:1 mixture of protein A–Sepharose and protein G–Sepharose beads (GE Healthcare). Beads were washed three times in NET buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 837

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0.5% NP-40] before complexes were dissolved in Laemmli sample buffer. Prior to loading onto 12% polyacrylamide gels, samples were heated at 96°C for 6 min. Gels were exposed to phosphorimager screens, which were subsequently scanned using a Typhoon Variable Mode Imager (GE Healthcare). Image analysis and quantification of band intensities were performed using ImageQuant TL software (GE Healthcare).

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REFERENCES


Identification of PRRSV non-structural proteins


