Intracellular membrane association of the N-terminal domain of classical swine fever virus NS4B determines viral genome replication and virulence

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Classical swine fever virus (CSFV) causes a highly contagious disease in pigs that can range from a severe haemorrhagic fever to a nearly unapparent disease, depending on the virulence of the virus strain. Little is known about the viral molecular determinants of CSFV virulence. The nonstructural protein NS4B is essential for viral replication. However, the roles of CSFV NS4B in viral genome replication and pathogenesis have not yet been elucidated. NS4B of the GPE2 vaccine strain and of the highly virulent Eystrup strain differ by a total of seven amino acid residues, two of which are located in the predicted trans-membrane domains of NS4B and were described previously to relate to virulence, and five residues clustering in the N-terminal part. In the present study, we examined the potential role of these five amino acids in modulating genome replication and determining pathogenicity in pigs. A chimeric low virulent GPE2-derived virus carrying the complete Eystrup NS4B showed enhanced pathogenicity in pigs. The in vitro replication efficiency of the NS4B chimeric GPE2 replicon was significantly higher than that of the replicon carrying only the two Eystrup-specific amino acids in NS4B. In silico and in vitro data suggest that the N-terminal part of NS4B forms an amphipathic α-helix structure. The N-terminal NS4B with these five amino acid residues is associated with the intracellular membranes. Taken together, this is the first gain-of-function study showing that the N-terminal domain of NS4B can determine CSFV genome replication in cell culture and viral pathogenicity in pigs.

INTRODUCTION

Classical swine fever is an economically important and highly contagious disease of pigs caused by classical swine fever virus (CSFV) (Lindenbach et al., 2013). The disease can range from a severe haemorrhagic fever to a nearly unapparent disease, depending on the virulence of the virus strain. CSFV belongs to the genus Pestivirus of the family Flaviviridae together with bovine viral diarrhea virus (BVDV) and border disease virus. CSFV possesses a single-stranded positive-sense RNA genome of approximately 12.3 kb with one large ORF flanked by 5′ and 3′ untranslated regions (UTRs). The genome encodes approximately 4000 amino acids that yield at least 12 cleavage products, Npro, C, Ems, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, through co- and post-translational processing of the polyprotein by cellular and viral proteases (Lamp et al., 2013; Lindenbach et al., 2013). Little is known on the role of the different viral proteins in determining CSFV virulence. Non-structural proteins NS3–NS5B are essential for pestivirus RNA replication (Behrens et al., 1998). Therefore, these non-structural proteins at least consist of the viral replication
complex together with unknown host factors, the replicating viral RNA and intracellular membranes.

The nonstructural protein NS4B is a 38 kDa intracellular-membrane-associated protein of the viral replicase, which is analogous to hepatitis C virus (HCV) NS4B and other related members of the family Flaviviridae (Hügle et al., 2001; Miller et al., 2006; Weiskircher et al., 2009). However, NS4B is a poorly characterized protein and its roles in CSFV replication and pathogenesis are not well understood. A putative Toll/interleukin-1 receptor-like domain was identified in the C-terminal region of NS4B (Fernandez-Sainz et al., 2010). Mutations in this domain of NS4B in the highly virulent Brescia strain resulted in an attenuated phenotype along with enhanced activation of TLR-7-induced genes. In addition, NS4B harbours an NTPase motif (Gladue et al., 2011), but there is no evidence that modulating the NTPase activity may affect virulence. In BVDV, NS4B can act as a modulator of virus cytopathogenicity (Qu et al., 2001). Our previous study demonstrated that two amino acid residues within the predicted trans-membrane domains of NS4B are involved in viral genome replication and that they contribute to the pathogenicity of CSFV in pigs (Tamura et al., 2012). These two residues are a part of the seven amino acids that differ in NS4B between the highly virulent Eystrup strain and the GPE− vaccine strain.

Here, we examined whether the five additional amino acid differences in the N-terminal domain of NS4B would further determine viral genome replication in vitro and the pathogenicity of CSFV in pigs. To this end, a chimeric virus and corresponding replicons carrying the complete NS4B of the highly virulent Eystrup strain in a modified GPE− vaccine strain backbone were generated and analysed for pathogenicity in vivo and for replication efficiency in cell culture, respectively. An in silico approach was used to predict the localizations of these amino acid residues in the N-terminal domain of NS4B. Intracellular membrane localization and the association mediated by the N-terminal domain of NS4B were examined.

RESULTS

The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup confers enhanced pathogenicity to a low virulent GPE−-derived virus

Our previous studies demonstrated the combination of four amino acid residues in Npro (D136), E2 (A830) and NS4B (A2475/V2563) can confer pathogenicity to the CSFV GPE− vaccine strain in pigs (Tamura et al., 2012, 2014) (Fig. 1). These four amino acid residues are conserved in the highly virulent Eystrup strain, although a total of 62 amino acids differ between GPE− and Eystrup strains. The two amino acid residues in the central part of NS4B (A2475/V2563) are part of a total of seven amino acid differences in NS4B. The remaining five amino acid differences (positions at 2377, 2391, 2398, 2399 and 2414) were observed in the N-terminal domain of NS4B. In order to assess whether the five amino acids differing from the GPE− strain in the N-terminal domain of Eystrup NS4B contribute to pathogenicity in pigs, NS4B in the low virulent GPE−-derived virus (vGPE−/N136D; T830A; V2475A; A2563V) was replaced with NS4B from the highly virulent Eystrup strain. This chimeric virus was termed vGPE−/N136D; T830A; Eystrup NS4B. Groups of six 10-week-old to 13-week-old pigs were inoculated intranasally with 10⁶.⁰ TCID₅₀ of these two GPE−-derived viruses. As a control, six pigs were inoculated with the highly virulent Eystrup strain. Infection with the parental virus (vGPE−/N136D; T830A; V2475A; A2563V) did not result in any clinical symptoms in this trial (Fig. 2a, left panel). Nevertheless, the body temperature of these pigs was slightly elevated between days 3 and 7 post-inoculation (p.i.), and low-level viraemia was detected during 2 days (Fig. 2b, c, left panels). In contrast, all six pigs inoculated with the vGPE−/N136D; T830A; Eystrup NS4B virus developed overt clinical manifestations between days 4 and 7 p.i., with five out of six pigs showing fever (Fig. 2a, b, middle panels). Viraemia was detected during 4 days (Fig. 2c, middle panel). Infection with the highly virulent Eystrup strain resulted in severe clinical signs with high and prolonged fever and viraemia in all six pigs. Four pigs were euthanized when they reached a clinical score above 18, between days 6 and 7 p.i. One pig died unexpectedly on day 8 p.i., and one pig recovered by day 14 p.i. These data demonstrate that the N-terminal domain of NS4B contributes to pathogenicity of CSFV in pigs. Nevertheless, NS4B of the highly virulent Eystrup virus is not solely responsible for the high pathogenicity of this virus.
The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup contributes to enhanced RNA replication

Mono-cistronic and bi-cistronic replicons (Fig. 3a, b) were used to determine the effect on viral RNA replication of the amino acids of NS4B differing between the two strains. In SK-6 cells at 24 h post electroporation, the bi-cistronic replicon carrying NS4B of the highly virulent Eystrup strain (rGPE^−/N136D; T830A; Eystrup NS4B) resulted in twofold higher luciferase activity than the replicon carrying only the two amino acid substitutions in the predicted
trans-membrane domains of NS4B (rGPE−Npro-Luc-IRE-NS3/V2475A; A2563V), and in fivefold higher luciferase activity than the parental GPE− virus-derived replicon (Fig. 3c). Consistent with the results obtained with the bi-cistronic replicons, Eystrup NS4B conferred significantly higher Gaussia luciferase production to the GPE− derived mono-cistronic replicon compared with the replicon harbouring only the two V2475A and A2563V substitutions in the GPE− backbone that was also more efficient at expressing the reporter protein than the replicon representing the parental GPE− vaccine strain (Fig. 3b, d). These data indicate that the additional five amino acid differences in the N-terminal domain of Eystrup NS4B contribute to enhanced RNA replication efficiency in cell culture.

The N-terminal domain of NS4B forms a predicted amphipathic α-helix and mediates intracellular membrane association

Secondary-structure prediction was carried out with the NS4B polyprotein sequence of the GPE− and Eystrup strains by using several different software programs. The consensus structures are shown in Fig. 4(a). CSFV NS4B comprises an N-terminal domain (aa 1–81), a central part that harbours four predicted trans-membrane (TM)
Fig. 4. Comparison of the NS4B amino acid sequences of the low virulent GPE– and the highly virulent Eystrup strains. (a) Based on the secondary structure predictions and their consensus, the α-helices and β-strands are depicted by white columns and arrows, respectively. The trans-membrane (TM) domains are shown with black rectangles. Amino acid numbering started with the beginning of NS4B. The amino acid differences are indicated, based on their positions relative to the translation start site of the polyprotein, and they are highlighted in grey. (b) Amphipathic helix wheels in the N-terminal second α-helix (α2, aa 40–81) of the two strains are illustrated, with the amino acid differences indicated by an asterisk. White and black colours indicate hydrophobic and hydrophilic amino acids, respectively. The clustering of hydrophobic, nonpolar residues on one face of the helix suggests an amphipathic configuration. The broken lines separate the two faces of the helix.
segments (aa 82–245) and a C-terminal domain (aa 246–347). The N-terminal domain contains two predicted α-helices from aa 5 to 30 (α1) and from aa 40 to 81 (α2), respectively. Interestingly, five out of the seven amino acid differences between NS4B of the GPE⁻ vaccine strain and of the Eystrup strain are located within the second α-helix, α2. This α-helix (aa 40–81) is predicted to have an amphipathic structure (Fig. 4b). In order to elaborate on this, plasmids were constructed for cytomegalovirus-promoter-driven expression of the N-terminal α1-helix (aa 1–39), of the helix α2 (aa 40–81), of the two α-helices together (aa 1–81) and of the full-length NS4B protein, all tagged with GFP at the C terminus (Fig. 5a). The hydrophobic face of the helix was then disrupted genetically by substitution of charged amino acids for the nonpolar amino acids (Fig. 5b). When these different GPE⁻-derived GFP-tagged NS4B

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**Fig. 5.** Role of the predicted N-terminal amphipathic helix domain for protein localization in porcine cells. (a) Schematic representation of the NS4B-GFP chimeric proteins and of the truncated versions expressed from the pCI vector under the control of a cytomegalovirus promoter. The second α-helix domain (aa 40–81) is shown in black. (b) Amino acid sequences and a helical wheel plot of the N-terminal domain of NS4B amino acids showing the localization of the amino acid substitutions for disruption of its amphipathic character. (c) SK-6 cells were transfected with pCI constructs for expression of the indicated NS4B-GFPs from the GPE⁻ strain. GFP fluorescence (green) was analysed by confocal microscopy after 30 h of culture. A plasmid expressing GFP served as control. Nuclei were stained with DAPI (blue). Bars, 10 μm. (d) Membrane flotation analysis of SK-6 cells expressing the N-terminal domain (aa 40–81) of NS4B. SK-6 cells transfected with the pCI vectors, NS4B(40–81)-GFP and its helix mutant, were disrupted mechanically at 36 h after transfection. Equal amounts of postnuclear supernatants were used for membrane flotation and Western blot analysis with an antibody to GFP, GAPDH or calnexin. For the membrane extraction experiments, the postnuclear supernatants were treated with 100 mM Na₂CO₃ (pH 11.5), with 1 M NaCl or with 1% Triton X-100 and subjected to membrane flotation followed by Western blot analysis.
variants were expressed in SK-6 cells, the fluorescence signal of NS4B(40–81)-GFP, NS4B(1–81)-GFP and NS4B-GFP was located essentially in the cytoplasm, with a tendency towards ‘dot-like’ granular structures, which was clearly different from the pattern observed with NS4B(1–39)-GFP and with GFP alone, which showed diffuse distribution in the cytoplasm and nucleus at the late stage of transfection. The fluorescence of helix mutants (HMT) of NS4B(40–81)-GFP and NS4B(1–81)-GFP was a diffuse distribution and the ‘dot-like’ granular structures were not observed at all (Fig. 5c). These results indicate that the putative amphipathic z2-helix (aa 40–81) is an essential segment for alternation of the fluorescent pattern.

A membrane flotation assay was used to further characterize the predicted membrane association of the z2-helix of NS4B. A representative Western blot from a membrane flotation experiment with extracts from SK-6 cells expressing NS4B(40–81)-GFP from the GPE strain is shown in Fig. 5(d). Low-density membrane fractions are raised towards the top of the gradient, whereas soluble proteins remain with the denser fractions at the bottom. Accordingly, GFP was retained at the bottom of the gradient (fractions 7 and 8) similar to the cytoplasmic soluble protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GFP-tagged z2-helix of NS4B came up in the low-density fractions 3 and 4 (from the top) of the flotation gradient. This was consistent with the accumulation of the membrane-associated calnexin in the gradient fractions 2 and 3 (Fig. 5d, bottom panel). In contrast, the disrupted amphipathic helix mutant of the GFP-tagged z2-helix of NS4B (aa 40–81) was located essentially in the cytoplasm, with a tendency towards ‘dot-like’ granular structures, which was clearly different from the pattern observed with NS4B(40–81HMT)-GFP and NS4B-GFP, NS4B(1–81)-GFP and NS4B-GFP was detected in the soluble fraction. Next, to analyse the membrane association of this segment in more details, membrane flotation was examined after treatments that discriminate between peripheral and integral membrane association. To this end, the postnuclear supernatants from cells expressing NS4B(40–81)-GFP were centrifuged at 100 000 × g, and the pelleted membranes were subjected to different extraction methods. The GFP-tagged NS4B(40–81) remained in the membrane fraction under physiological conditions and after high-salt and alkaline extractions, while detergent extraction resulted in accumulation of NS4B(40–81)-GFP in the higher density soluble fractions. Taken together, the flotation assays confirmed strong intracellular membrane association of the N-terminal amphipathic z2-helix of NS4B (aa 40–81).

Intracellular membrane association mediated by the N-terminal domain of NS4B is a determinant of CSFV RNA replication in cell culture

In order to clarify the roles of intracellular membrane association mediated by the N-terminal amphipathic domain of NS4B for RNA replication, cistronic replicons carrying the same mutations for disruption of the amphipathic z-helix were generated. The luciferase activity of the helix mutant (rGPE−-Npro-Luc-IRES-NS3/NS4B HMT) was significantly low compared with the luciferase activity of the parental GPE− replicon (Fig. 6a). This was similar to that of the replication-deficient replicon,

![Graph](graph.png)

**Fig. 6.** Role of the predicted N-terminal amphipathic helix domain for RNA replication. GPE−-derived bi- and mono-cistronic luciferase reporter replicons carrying the mutations for disruption of its amphipathic character were utilized. Respective firefly (a) and *Gaussia* (b) luciferase activities were analysed as described above. Significance (P<0.05) is indicated with an asterisk.
indicating that the replication capability was completely abolished by the mutations. In the mono-cistronic replicon assay, the helix mutant behaved similarly as the replication-deficient replicon (Fig. 6b). Altogether, these data indicate that intracellular membrane association mediated by the N-terminal domain of NS4B is critical for RNA replication in cell culture.

**The N-terminal domain of NS4B modulates subcellular localization**

In order to determine the subcellular distribution of the N-terminal domain of NS4B, co-localization experiments were performed in SK-6 cells expressing the GFP-tagged fusion proteins. As shown in Fig. 7(a), the fluorescence pattern of the GFP-tagged N-terminal domain of NS4B, NS4B(40-81)-GFP and NS4B(1-81)-GFP, appeared to partially overlap with the endoplasmic reticulum (ER). The co-localization coefficient was the same level over the entire NS4B (Fig. 7b). These data indicate that the N-terminal amphipathic helix determines the subcellular localization of NS4B.

**DISCUSSION**

In CSFV, the nonstructural protein NS4B is essential for viral replication (Behrens et al., 1998). Our previous study demonstrated that the two amino acid residues that differ between the vaccine strain GPE and its parental strain ALD in the predicted trans-membrane domains of NS4B can modulate viral genome replication and influence the pathogenicity of the virus in pigs (Tamura et al., 2012). Here, we generated GPE- derived virus carrying the complete NS4B of the Eystrup strain, in order to determine whether the additional five amino acid differences in the N-terminal domain of NS4B may contribute to the different pathogenicity in pigs. The GPE-derived virus carrying NS4B of the highly virulent Eystrup strain resulted in more severe clinical symptoms, and earlier and prolonged viraemia compared with the virus carrying only the two amino acid substitutions in the predicted trans-membrane domains. These data indicated that the N-terminal domain of NS4B may represent a virulence determinant in vivo. However, as expected, NS4B from the Eystrup strain did not confer a full high virulent phenotype to the GPE-derived virus, demonstrating that additional viral proteins and amino acid residues are involved in determining the virulence of the Eystrup strain. In addition, the specific residues identified in this study as critical for determining virulence in pigs are not general virulence determinants in CSFV (Fig. 8). This suggests that the virulence of CSFV is more likely a multi-genic trait determined by a complex interplay of several viral proteins or genes acting in concert as already postulated in previous studies (Leifer et al., 2013; Tamura et al., 2012). To the best of our knowledge, this is the first study to show that the N-terminal domain of CSFV NS4B can confer enhanced pathogenicity to a low virulent virus, which was related to an enhanced viral replication.

The five amino acid residues differing between the GPE and Eystrup strains in the N-terminal domain of NS4B...
clearly determine the RNA replication efficiency of replicons in vitro. In previous studies, two types of replicons were utilized for studying RNA replication: mono-cistronic (Risager et al., 2013) and bi-cistronic (Behrens et al., 1998). The minimal viral elements required for RNA replication by pestiviruses including CSFV are NS3–NS5B, together with the 5' and 3' UTRs (Behrens et al., 1998). In addition to these minimal elements, the bi-cistronic replicon carries an artificial internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus (EMCV), a luciferase reporter gene, and the viral auto-protease Npro for optimal translation and processing of the reporter protein. The replicative properties of this replicon may, however, be modified by the EMCV IRES. For these reasons, a mono-cistronic replicon was included. Comparable results of NS4B-dependent differences in RNA replication were obtained with the two types of replicons, strengthening the data.

The granular fluorescent signal derived from the N-terminal domain of NS4B was partially co-localized with the ER by confocal fluorescence microscopy. In the membrane flotation assay, the N-terminal domain of NS4B was also bound to the ER in part, suggesting that the ER could be a candidate site of the replication complex in infected cells. However, the major part of the N-terminal domain of NS4B detected the different fractions of the ER marker, calnexin. Thus, further studies will be needed to identify the exact subcellular localization of the replication complex including the natural NS4B in CSFV-infected cells. It was described that BVDV NS4B alone can rearrange the host membrane (Weiskircher et al., 2009) as well as HCV NS4B (Egger et al., 2002), suggesting that pestivirus NS4B might act as a trigger for building replication complex. So far, however, pestivirus replication is considered not to remodel cytoplasmic host cell membranes (Schmeiser et al., 2014) as opposed to HCV, which induces organelle-like structures designated the membranous web where the formation of the viral replication complex is triggered (Romero-Brey et al., 2012), and to other positive-strand RNA viruses (Knoops et al., 2008; Kopek et al., 2007; Spuul et al., 2007; Welsch et al., 2009). Therefore, we hypothesize that the role of pestivirus NS4B may be partially different from that in other members of the Flaviviridae. Further studies on the functions of the pestivirus NS4B will allow us to clarify how the NS4B-dependent modulation of RNA replication may relate to differences in viral replication, morphogenesis and virulence.

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**Fig. 8.** Amino acid alignment of the N-terminal domain (aa 1–81) of CSFV NS4B. The amino acid sequences in the N-terminal domain (aa 1–81) of selected CSFV strains of low, moderate and high virulence were aligned. The hydrophilic amino acids are highlighted in grey. The amino acid differences between the GPE C and Eystrup strains are marked with an asterisk. A dot indicates the same amino acid as for the GPE C strain.
Intracellular membrane association mediated by the N-terminal amphipathic domain of NS4B is essential for RNA replication. This amphipathic structure is conserved among CSFV strains (Fig. 8). Interestingly, the amino acid differences in the N-terminal part of NS4B between the prototype vaccine virus and the highly virulent strain studied here are located on the predicted hydrophilic face of the amphipathic $\alpha$-helix. These residues are not conserved with respect to virulence of CSFV; however, the amino acid differences among CSFV strains were found mostly on the hydrophilic face of the amphipathic $\alpha$-helix, suggesting that the functional differences observed may depend on subtle structure-related differences in association with host proteins and/or other viral nonstructural proteins, modulating replication complex formation, RNA replication and eventually pathogenicity of the virus in pigs. For HCV, the N-terminal amphipathic $\alpha$-helix of NS4B is critical for its polymerization, and thus for its accumulation in organelles (Gouttenoire et al., 2010; Yu et al., 2006). A recent study of HCV demonstrated that hydrophilic amino acids in the N-terminal cytoplasmic NS4B are responsible for virus production (Gouttenoire et al., 2014).

In summary, this is the first study to our knowledge showing the roles of intracellular membrane association mediated by the N-terminal domain of CSFV NS4B in viral RNA replication and pathogenicity in pigs. Further studies are required in order to understand the functionality of the pestivirus replication complex at the molecular level, and how this affects pathogenicity in pigs. Understanding the molecular mechanisms involved in viral replication and pathogenesis will permit design of effective tools for controlling the disease.

**METHODS**

**Ethical statement.** The animal experiments described here were approved by the Animal Welfare Committee of the Canton of Berne with the licence number BE94/12, and conducted in compliance with the Swiss animal protection law and with the national and international animal experimentation guidelines.

**Viruses and cells.** The CSFV strains, vGPE$^{-}$/N136D; T830A; V2475A; A2563V, vGPE$^{-}$/N136D; T830A; Eystrup NS4B and vEy-37, which is identical to Eystrup, were derived from the full-length cDNA clones, i.e. pGPE$^{-}$/N136D; T830A; V2475A; A2563V (Tamura et al., 2014), pGPE$^{-}$/N136D; T830A; Eystrup NS4B and pEy-37 (Mayer et al., 2003), respectively. All of the cDNA-derived viruses were rescued as aforementioned (Moset et al., 1999). The complete genomes of the rescued viruses were verified by nucleotide sequencing to exclude any accidental mutations. SK-6 cells were propagated at 37 °C in the presence of 5 % CO$_2$ in Eagle’s minimum essential medium (Nissui Pharmaceutical) supplemented with 0.3 mg ml$^{-1}$ 1-glutamine and 7 % horse serum (Life Technologies).

**Plasmid constructs.** The cDNA clone of the mono-cistronic replicon carrying the Gaussia luciferase reporter gene, pGPE 2GL, was constructed from rPad2GL (Risager et al., 2013) by replacing the genome of the Paderborn strain with that of the GPE$^{-}$ strain applying the In-Fusion HD Cloning (Clontech) techniques as described previously (Tamura et al., 2012). The cDNA clone of rPad2GL was a gift of T. B. Rasmussen (DTU National Veterinary Institute, Denmark). The infectious cDNA clone (pGPE$^{-}$/N136D; T830A; Eystrup NS4B) and the replicon cDNA clones (pGPE$^{-}$/Npro$^*$-Luc-IRES-N3S/Eystrup NS4B and pGPE 2GL/Eystrup NS4B) were constructed by replacing NS4B in the backbone with the corresponding gene of the highly virulent strain Eystrup as described above. The cDNA clones of the replication-deficient replicons (pGPE$^{-}$/Npro$^*$-Luc-IRES-N3S/GAA and pGPE 2GL/GAA) encoding the amino acid sequence ‘GAA’ instead of ‘GDD’ in the polymerase active site of the nonstructural protein NS5B (Zhong et al., 1998) were constructed from the replicon cDNA clones pGPE$^{-}$/Npro$^*$-Luc-IRES-N3S and pGPE 2GL using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies) and oligonucleotide primers containing the respective mutations based on the aforementioned standard techniques (Tamura et al., 2012). The cDNA clones of the disrupted amphipathic character in the N-terminal domain of GPE$^{-}$ NS4B (pGPE$^{-}$/Npro$^*$-Luc-IRES-N3S/NS4B HMT and pGPE 2GL/NS4B HMT) were accordingly generated from the parental clones (pGPE$^{-}$/Npro$^*$-Luc-IRES-N3S and pGPE 2GL) by mutagenesis described above.

In order to construct the GFP, the NS4B construct with a C-terminal GFP-tag and the truncated versions thereof, the cDNA fragments of the respective regions of the GPE$^{-}$ and Eystrup strains were obtained using reverse transcription and PCR, and the GFP fragments were cloned into the pCI Mammalian Expression Vector (Promega) with In-Fusion HD Cloning and appropriate restriction enzymes. Details of the constructs are available on request.

**Sequencing.** The cDNA clones, *in vitro*-rescued viruses, and expression vectors were completely sequenced as aforementioned (Tamura et al., 2012). In brief, nucleotide sequencing of the cDNA clones, expression vectors and PCR fragments from viral RNA was performed using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies) and a 3500 Genetic Analyzer (Life Technologies). The sequencing data were analysed using Genetyx-Network version 12 (Genetyx).

**Antibodies and markers.** The E2-specific mAb HC/TC26 (Greiser-Wilke et al., 1990) was kindly provided by I. Greiser-Wilke (Hannover Veterinary School, Hannover, Germany). The mAb against viral NS3, 46/1 was generated previously (Kameyama et al., 2006a). The antibodies against cellular organelles, GAPDH and calnexin were purchased from Cell Signaling Technology and Enzo Life Sciences, respectively. The mAb against GFP was obtained from Santa Cruz Biotechnology. A secondary antibody, FITC-conjugated goat anti-mouse IgG, was purchased from MP Biomedicals, and Alexa Fluor 488 F(ab')$_2$ fragment of goat anti-mouse IgG (H + L) was obtained from Life Technologies. Fluorescent probe for detecting the ER (ER-Tracker) was purchased from Life Technologies. For counterstaining of the cell nuclei, DAPI was obtained from Dojindo Molecular Technologies.

**Virus titration.** The virus titres were determined by end-point dilution with SK-6 cells and immunoperoxidase staining using anti-E2 mAb HC/TC 26 or anti-NS3 mAb 46/1 as described previously (Mittelholzer et al., 1997; Sakoda et al., 1998). The titres were calculated using the Reed and Muench formula and expressed as the 50 % TCID$_{50}$ ml$^{-1}$ (Reed & Muench, 1938).

**Experimental infection of pigs.** In order to assess the pathogenicity of the cDNA-derived viruses, groups of six 10-week-old Large White specific-pathogen-free (SPF) pigs obtained from the breeding unit of the Institute of Virology and Immunology (IVI, Switzerland) were inoculated intranasally with $10^{6.5}$ TCID$_{50}$ of the respective viruses. All pigs were kept in separate isolation units in the BSL-4 facility of the IVI. The body temperature and clinical scores
were monitored daily according to a defined scoring system as described previously (Mittelholzer et al., 2000). Whole blood was collected for serum preparation at 3 days before infection and on days 3, 4, 5, 6, 7, 10 and 12 p.i. The pigs that survived the infection were euthanized on day 14 p.i. The virus titres in the serum samples were expressed as the TCID50 ml⁻¹.

**Luciferase assay.** Luciferase assays using bi-cistronic replicons were conducted as previously described (Tamura et al., 2012). In brief, 10⁶ SK-6 cells were electroporated with 1 µg of replicon RNA. Electroporation was performed at 200 V and 500 µF. At different times after transfection, cell extracts were prepared with 200 µl passive lysis buffer, and the firefly luciferase activity was measured using a Luciferase Assay System (Promega) and a Lumat LB9507 luminometer (Berthold).

Luciferase assays with mono-cistronic replicons were performed as aforementioned, with some modifications (Risager et al., 2013). Electroporation was conducted under the conditions described above. At the time indicated, 50 µl of cell medium was harvested from the supernatants of cells transfected with replicons, which was then frozen at −20 °C. After thawing, 20 µl of the cell medium was analysed to determine the Gaussia luciferase activity using Renilla luciferase substrate and a Lumat LB9507 luminometer. The results representing the luciferase activities that were compared with the luciferase activity of the replicon devoid of polymerase activity at the initial time points were measured on the basis of three independent experiments.

**Immunofluorescence assay.** SK-6 cells were transfected with the expression vectors using Lipofectamine 3000 (Life Technologies) and Opti-MEM (Life Technologies), according to the manufacturer’s protocols. At the time indicated, SK-6 cells grown on an eight-well chamber slide (Matsunami) were fixed and stained as described by Sharma et al. (2012) and Yamasaki et al. (2012). Anti-GFP mAb as the primary antibody was used at a concentration of 1 mg ml⁻¹. As the secondary antibodies, FITC-conjugated goat anti-mouse IgG (1: 2500) or Alexa-Fluor-conjugated IgG (1: 1000) were used. In order to counterstain the cell nuclei, the cells were incubated for 30 min with 2 µg DAPI ml⁻¹ in PBS at room temperature. For labelling the cell organelles, the cells were grown on 35 mm glass-based dishes (Iwaki) and washed with PBS, before staining for 30 min with 100 mM ER-Tracker in complete medium at 37 °C with 5 % CO₂. The cells were then washed twice with PBS and pre-warmed PBS was added. The fluorescent cells were analysed with a BZ-9000 (KEYENCE) microscope and a BZ-II Analysery (KEYENCE). The confocal fluorescent images were acquired using Zeiss LSM700 (Carl Zeiss) microscopes (upright and inverted) and ZEN 2012 (Carl Zeiss). Quantitative co-localization analysis was performed as described elsewhere (Dunn et al., 2011). The co-localization ratio of the GFP signals with the organelle marker that represents a co-localization coefficient was quantified using ZEN 2012 software.

**Membrane flotation assay.** SK-6 cells were grown on 10 cm dishes overnight and transfected with the GFP-tagged NS4B and truncated vectors or with the control GFP constructs according to the conditions described above. At 30 h post-transfection, the cells were resuspended in hypotonic buffer (10 mM Tris/HCl, pH 7.5, 10 mM KCl, 5 mM MgCl₂) containing a protease inhibitor (Complete Mini, Roche). The cells were then disrupted by 20 passages through a 25G needle with 1 ml syringe, ensuring approximately 90 % disruption. The samples were spun at 1000 g for 5 min at 4 °C to pellet the cellular debris and nuclei, thereby obtaining postnuclear supernatants. A discontinuous iodixanol (OptiPrep, Alere Technologies) gradient (5 %, 25 % and 30 %) was layered on top of the samples, followed by centrifugation at 230 140 g for 24 h at 4 °C in a P40ST Rotor (Hitachi Koki). In total, eight equal fractions were collected from the top to bottom. Each fraction was precipitated with methanol/chloroform and analysed by SDS-PAGE followed by immunoblotting using anti-GFP, anti-GAPDH and anti-calnexin as described previously (Kameyama et al., 2006b). For the membrane dissociation experiments, the postnuclear supernatants were adjusted to 0.25 M sucrose and centrifuged at 100 000 g for 45 min at 4 °C. The pellet containing the membranes and their associated proteins was resuspended in either high salt buffer (1 M NaCl, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA), 100 mM sodium carbonate (pH 11.5) or 1 % Triton X-100. After incubation at 4 °C for 30 min, the samples were subjected to membrane flotation followed by immunoblotting, as described above. Typically, the membrane-bound proteins were associated with fractions 1–4 whereas the soluble proteins were generally present in fractions 5–8, which were followed by the respective protein markers.

**Structure predictions and sequence analyses.** Multiple sequence alignment and amino acid conservation analyses were performed with the CLUSTALW2 program (Larkin et al., 2007) using the default parameters. The protein secondary structure predictions were generated using DSC (King & Sternberg, 1996), HNN (Guernier, 1997), MLRC (Guernier et al., 1999), PhD (Rost, 1996), Ipred 3 (Cole et al., 2008), Porter (Pollastri & Mclysaght, 2005), PRED (Jones, 1999) and APSSP2 (Raghava, 2002). The trans-membrane domains were predicted using TopPred II (Claros & von Heijne, 1994). HeliQuest (Gautier et al., 2008) was used for z-helix projections.

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**REFERENCES**


