Targeted mutations in a highly conserved motif of the nsp1β protein impair the interferon antagonizing activity of porcine reproductive and respiratory syndrome virus

Yanhua Li,1 Longchao Zhu,1 Steven R. Lawson1 and Ying Fang1,2†

1Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD 57007, USA
2Department of Biology/Microbiology, South Dakota State University, Brookings, SD 57007, USA

Non-structural protein 1β (nsp1β) of porcine reproductive and respiratory syndrome virus (PRRSV) contains a papain-like cysteine protease (PLPβ) domain and has been identified as the main viral protein antagonizing the host innate immune response. In this study, nsp1β was determined to suppress the expression of reporter genes as well as to suppress ‘self-expression’ in transfected cells, and this activity appeared to be associated with its interferon (IFN) antagonist function. To knock down the effect of nsp1β on IFN activity, a panel of site-specific mutations in nsp1β was analysed. Double mutations K130A/R134A (type 1 PRRSV) or K124A/R128A (type 2 PRRSV) targeting a highly conserved motif of nsp1β, GKYLQRRLQ (in bold), impaired the ability of nsp1β to suppress IFN-β and reporter gene expression, as well as to suppress ‘self-expression’ in vitro. Subsequently, viable recombinant viruses vSD01-08-K130A/R134A and vSD95-21-K124A/R128A, containing double mutations in the GKYLQRRLQ motif were generated using reverse genetics. In comparison with WT viruses, these nsp1β mutants showed impaired growth ability in infected cells, but the PLPβ cleavage function was not directly affected. The expression of selected innate immune genes was determined in vSD95-21-K124A/R128A mutant-infected cells. The results consistently showed that gene expression levels of IFN-α, IFN-β and IFN-stimulated gene 15 were upregulated in cells that were infected with the vSD95-21-K124A/R128A compared with that of WT virus. These data suggest that PRRSV nsp1β may selectively suppress cellular gene expression, including expression of genes involved in the host innate immune function. Modifying the key residues in the highly conserved GKYLQRRLQ motif could attenuate virus growth and improve the cellular innate immune responses.

INTRODUCTION

Porcine reproductive and respiratory syndrome continues to be a major problem in the swine industry worldwide. It causes late-term reproductive failure in sows and severe pneumonia in neonatal pigs. Since its emergence, this syndrome has been estimated to cost the US swine industry at least US$600 million annually (Miller, 2011). The aetiological agent, porcine reproductive and respiratory syndrome virus (PRRSV), is a small, enveloped, positive-stranded RNA virus that belongs to the order Nidovirales, family Arteriviridae, which also includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Snijder & Meulenberg, 1998). PRRSV can be divided into distinct European (type 1) and North American (type 2) genotypes, sharing about 63% nucleotide identity (Allende et al., 1999; Nelsen et al., 1999; Ropp et al., 2004).

The PRRSV genome is about 15 kb and contains at least ten ORFs. The replicase-associated genes, ORF1a and ORF1b, encode two long non-structural polyproteins, pp1a and pp1ab, with expression of the latter depending on a −1 ribosomal frame shift signal in the ORF1a/ORF1b overlap region. Following their synthesis from the genomic mRNA template, the pp1a and pp1ab replicase polyproteins are processed into 14 non-structural proteins (nsps) by a complex proteolytic cascade directed by four protease domains encoded in ORF1a, which includes two papain-like cysteine proteases (PLPα and PLPβ) located in nsp1α and nsp1β, a papain-like protease (PLP2) domain located...
at the N terminus of nsp2 and a serine protease (SP) located in nsp4. The PLP\(\alpha\) autocleaves between nsp1\(\alpha\) and nsp1\(\beta\), PLP\(\beta\) autocleaves between nsp1\(\beta\) and nsp2, and PLP2 cleaves between nsp2 and nsp3, which mediates the rapid release of nsp1\(\alpha\), nsp1\(\beta\) and nsp2 from the polyproteins. The SP of nsp4 mediates nine cleavages in the nsp3–12 region to generate the other individual nsps (Fang & Snijder, 2010). In addition, a new ORF (TF) was recently discovered in the central region of ORF1a, which expresses a transframe protein, nsp2TF, through a novel -2 ribosomal frame shifting mechanism (Fang et al., 2012).

Viruses depend on the host cellular machinery for survival and replication. Most viral infections are efficiently resolved by the host’s innate and adaptive immune system. The innate immune response provides an important first line of defence against pathogen intruders. It is essential for the initial control of infection and allows time for the establishment of an adaptive immune response. A key aspect of the antiviral innate immune response is the synthesis and secretion of type I interferon (IFN), such as IFN-\(\alpha\) and IFN-\(\beta\). After being secreted, type I IFNs bind to their receptors on adjacent cell surfaces to activate the so-called JAK–STAT signalling pathway. Activation of this pathway induces the activation of IFN-stimulated response elements (ISREs) in the promoter, resulting in the transcription of IFN-stimulated genes (ISGs). Expression of this group of ISGs enables the cell to fight the infection and inhibit virus replication (Weber et al., 2004). As a counteraction to host antiviral responses, viruses have evolved strategies to actively suppress and/or evade the innate immune responses. Some viruses encode proteins that selectively interfere with certain components of the IFN system, whilst others have evolved to target the general host gene expression machinery, resulting in blocking of innate immune responses (Basler et al., 2000; Chen et al., 1999; Foy et al., 2003; Kamitani et al., 2009; Komatsu et al., 2004; Le May et al., 2004; Lokugamage et al., 2012; Wang et al., 2000). Various PRRSV nsps (nsp1\(\alpha\), nsp1\(\beta\), nsp2 and nsp11) appear to be able to suppress the host innate antiviral function (Fang & Snijder, 2010; Yoo et al., 2010). Previous studies from our laboratory and others have demonstrated that individually expressed PRRSV nsp1\(\beta\) showed strong inhibitory effects on IFN-\(\beta\)-promoter activation (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010). The nsp1\(\beta\) was also determined to be able to inhibit the IFN-dependent signalling pathway leading to ISG expression (Beura et al., 2010; Chen et al., 2010). In an effort to attenuate the IFN antagonist function of PRRSV nsp1\(\beta\), in this study we analysed a panel of site-specific mutations in the nsp1\(\beta\) region using both reverse genetics and in vitro expression systems. A highly conserved GKYLQRLQ motif of nsp1\(\beta\) was identified, and mutations in this motif were characterized further to determine their effect on the function of nsp1\(\beta\) in virus replication and cellular immune responses.

## RESULTS

### Generation of PRRSV nsp1\(\beta\) mutants with reduced ability to suppress type I IFN production and signalling

As PRRSV nsp1\(\beta\) was determined to be one of the main IFN antagonists, in an effort to knock down its IFN antagonist function, a panel of site-specific nsp1\(\beta\) mutations was constructed (Table 1). These were designed based on the protein surface accessibility prediction (Emini et al., 1985), hydrophilicity analysis (Hopp & Woods, 1981) and crystal structure of nsp1\(\beta\) (Xue et al., 2010). The conserved hydrophilic amino acids predicted to be exposed on the protein surface were targeted for mutation (Fig. 1). In most of the predicted motifs, double mutations instead of a single mutation gave an effective reduction in the hydrophilic value of the motif; therefore, each of the nsp1\(\beta\) genes carrying double mutations was cloned into the plasmid p3 \(\times\) FLAG-CMV-24, in which gene expression is under the control of the human cytomegalovirus (CMV) promoter and expressed as FLAG-tagged protein. Initially, the panel of nsp1\(\beta\) mutants generated from a type 1 PRRSV strain, SD01-08 (Table 1), was analysed in an IFN-\(\beta\)-promoter-driven luciferase reporter assay. HEK-293T cells were co-transfected with a plasmid expressing WT or mutated nsp1\(\beta\) and a reporter plasmid (p125-Luc) that expresses luciferase reporter gene under the control of IFN-\(\beta\) promoter. As controls, the PCP\(\beta\) catalytic site mutant C96S and empty plasmid (EV) p3 \(\times\) FLAG-CMV-24 were included in the analysis. At 24 h post-transfection, cells were mock infected or infected with Sendai virus (SeV). Cells were harvested to test the luciferase activities at 16 h

<table>
<thead>
<tr>
<th>Construct(^\ast)</th>
<th>Mutation</th>
<th>WT codon</th>
<th>Mutant codon</th>
<th>Virus strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>E28A/D31A</td>
<td>E28→A</td>
<td>GAA</td>
<td>GCA</td>
<td>Type 1</td>
</tr>
<tr>
<td>E41A/E43A</td>
<td>E41→A</td>
<td>GAG</td>
<td>GCC</td>
<td>Type 1</td>
</tr>
<tr>
<td>E68A/E71A</td>
<td>E68→A</td>
<td>GAG</td>
<td>GCG</td>
<td>Type 1</td>
</tr>
<tr>
<td>K130A/R134A</td>
<td>K130→A</td>
<td>AAG</td>
<td>GGC</td>
<td>Type 1</td>
</tr>
<tr>
<td>K124A/R128A</td>
<td>K124→A</td>
<td>AAG</td>
<td>GGC</td>
<td>Type 2</td>
</tr>
<tr>
<td>C96S</td>
<td>C96→A</td>
<td>TGC</td>
<td>AGC</td>
<td>Type 1</td>
</tr>
</tbody>
</table>

\(\ast\)The double mutations targeting the highly conserved motif of nsp1\(\beta\) are indicated in bold.
post-infection (p.i.). As shown in Fig. 2(a), SeV infection induced a high level of luciferase reporter expression in cells transfected with EV, but luciferase expression was about 7–50-fold lower in cells expressing WT nsp1β and the C96S, E28A/D31A, E41A/E43A and E68A/E71A mutants. In contrast, about a 30-fold higher level of reporter signal was detected in cells expressing the K130A/R134A mutant in comparison with that of WT nsp1β. We further determined whether these mutations had an effect on the ability of nsp1β to suppress the IFN-dependent signalling pathway for the ISG expression. The panel of nsp1β mutants was analysed using an ISRE promoter-driven luciferase reporter assay. A similar result was generated as that obtained in Fig. 2(a), with about a
7–14-fold higher level of reporter signal detected in cells expressing the K130A/R134A mutant in comparison with those expressing WT nsp1β and the C96S, E28A/D31A, E41A/E43A and E68A/E71A mutants (Fig. 2b). These results suggested that the K130A and R134A mutations impaired IFN antagonist function of nsp1β.

To further confirm these results, we tested whether the K130A/R134A mutations had an effect on endogenous IFN-β mRNA expression. HEK-293T cells were transfected with EV or plasmid expressing WT nsp1β or the K130A/R134A mutant, and then mock infected or infected with SeV. At 16 h p.i., intracellular RNA was extracted and the amount of IFN-β mRNA was measured by real-time reverse transcription-PCR (RT-PCR). As shown in Fig. 3(a), expression of WT nsp1β strongly inhibited SeV-induced IFN-β mRNA accumulation. In contrast, there was a threefold increase in IFN-β mRNA expression in cells transfected with the K130A/R134A mutant. This result indicated that the K130A and R134A mutations impaired the ability of nsp1β to suppress IFN-β mRNA expression. The K130 and R134 residues in nsp1β of type 1 PRRSV correspond to K124 and R128 in nsp1β of type 2 PRRSV. We repeated our experiment using nsp1β from a type 2 PRRSV strain, SD95-21. The result was consistent with that obtained from type 1 virus. The expression of WT nsp1β strongly suppressed the IFN-β mRNA expression (Fig. 3b), as well as IFN-β activation and signalling (Fig. 4), whilst increased levels of IFN-β mRNA (Fig. 3b) and luciferase reporter expression (Fig. 4) were observed in cells expressing the K124A/R128A mutant.

**Mutation of amino acids K130(124) and R134(128) impairs the ability of nsp1β to suppress 'self-expression' and reporter gene expression in vitro**

Western blot analysis confirmed the expression of nsp1β WT and mutants in luciferase assays (Figs 2 and 4). Interestingly, the data consistently showed that mutations in K130(124) and R134(128) caused an increased amount of nsp1β expression in comparison with that of WT nsp1β and other mutants, which suggested that nsp1β may suppress its ‘self-expression’. To confirm this notion, we repeated luciferase assays and compared the cellular expression level of nsp1β with nsp1α, as nsp1α has also been determined to be an IFN antagonist in previous studies (Beura et al., 2010; Chen et al., 2010; Song et al., 2010). The HEK-293T cells were co-transfected with the reporter plasmid and an equal amount of plasmid DNA expressing nsp1α or nsp1β of type 1 PRRSV. As expected, the result showed that both nsp1α and nsp1β suppressed the expression of IFN-β promoter-driven luciferase (Fig. S1a, available in JGV Online), and nsp1β had stronger inhibition of gene expression under the control of the ISRE promoter (Fig. S1b). Western blot analysis using anti-FLAG antibody detected a higher amount of nsp1β protein, but only a trace amount of nsp1α protein was detected in both luciferase assays.
These data made us speculate that nsp1β may have a general effect on cellular gene expression, including its ‘self-expression’ in vitro. To test this possibility, we examined the effect of nsp1β on reporter gene mRNA expression. HEK-293T cells were co-transfected with a reporter plasmid (pRL-SV40) expressing Renilla luciferase reporter gene under the control of the simian virus 40 (SV40) promoter, and a plasmid expressing type 1 PRRSV WT nsp1β or the K130A/R134A mutant (NA-nsp1β-mt). The EV was used as a control. Intracellular RNA was extracted at 24 h post-transfection, and the amount of mRNA was determined by real-time RT-PCR. In comparison with those cells co-transfected with EV, the level of EGFP expression was observed in cells expressing the K130A/R134A and K124A/R128A mutants. Western blot analysis using anti-nsp1β and anti-EGFP antibodies confirmed the expression of nsp1β and EGFPs (Fig. 5d, f). Due to the difference in sensitivity of different antibodies, we used anti-FLAG antibody to detect and compare the expression levels of different proteins. The result consistently showed that WT nsp1β expression resulted in lower levels of EGFP production, whereas two- or eightfold higher levels of EGFP were detected in cells co-transfected with the plasmid expressing nsp1β mutant from type 1 or type 2 PRRSV. Again, about a two- or threefold increased level of nsp1β protein was detected in cells expressing the K130A/R134A or K124A/R128A mutant in comparison with those cells expressing WT nsp1β, indicating the important role of K130(124)/R134(128) residues involved in nsp1β activities.

**Amino acids K130(124) and R134(128) are located in a highly conserved motif among PRRSV strains from both genotypes**

Based on the crystal structure of nsp1β from a type 2 PRRSV (Xue et al., 2010), the K124 and R128 residues are exposed on the protein surface. We further searched the potential functional motif in this region, and the results showed that these two residues are located in a potential B-cell epitope motif, GKYLQRRLQ (shown in bold) (Kolaskar & Tongaonkar, 1990). This motif had the highest surface accessibility value in comparison with the other regions of nsp1β (Fig. 1a). Amino acid sequence alignment of nsp1β sequences from 212 PRRSV isolates available in GenBank revealed that the GKYLQRRRLQ motif is highly conserved among all available PRRSV strains, including both type 1 and type 2 viruses (Fig. 1b), which suggests an important role of the GKYLQRRRLQ motif in nsp1β function.

**Characterization of recombinant viruses containing mutations in the GKYLQRRRLQ motif**

We investigated further whether specific mutations introduced into the nsp1β region of the virus could improve innate immune responses in PRRSV-infected cells. Initially, two PRRSV nsp1β mutants were generated using reverse genetics: vSD01-08-K130A/R134A (vSD01-08-mt), which contained K130A and R134A mutations in the nsp1β region of type 1 PRRSV SD01-08, and vSD95-21-K124A/R128A (vSD95-21-mt), which contained K124A and...
R128A mutations in the nsp1β region of type 2 PRRSV SD95-21. As a comparison, WT viruses vSD01-08 and vSD95-21 were also recovered by reverse genetics. The stability of the mutations introduced into the virus was determined by serial passaging each virus ten times in MARC-145 cells, and sequence analysis showed that all targeted mutations were stably maintained in the virus. The growth property of these two mutants was compared with their WT parental viruses. Both mutants showed a crippled growth phenotype in MARC-145 cells. The peak viral titre for vSD95-21-mt was \(10^{5.4}\) fluorescent focus forming units (f.f.u.) ml\(^{-1}\) compared with the titre of \(10^{8.3}\) f.f.u. ml\(^{-1}\) for vSD95-21. The vSD01-08-mt virus was found to replicate to very low titres in cell culture (Fig. 6a). The virus titre remained around \(10^{2}\) f.f.u. ml\(^{-1}\) through all passages. Plaque assay results consistently showed that vSD95-21-mt developed smaller plaques than that of WT virus, whilst vSD01-08-mt barely produced plaques (Fig. 6b). In order to stimulate a measurable host IFN response, a high virus dose is required for the initial infection. The extremely low titre of vSD01-08-mt prevented us from obtaining solid data and making an acceptable comparison with the WT virus in terms of type I IFN and ISGs expression. Therefore, vSD95-21-mt was used in subsequent experiments for comparison of innate immune gene expression between cells infected with nsp1β mutant and WT virus (see below and data presented in Fig. 7).

The nsp1β contains a PLPβ protease that is responsible for proteolytic cleavage of the nsp1β/nsp2 site of pp1a and pp1ab polyproteins, a critical step in virus replication (Fang & Snijder, 2010). To investigate whether the crippled growth property of these mutants could be due to an effect on the function of PLPβ in the replicase processing, the nsp1β–nsp2 region was expressed from both type 1 and type 2 PRRSV, designated EU-nsp1β-2 and NA-nsp1β-2, respectively. Subsequently, the K130A/R134A or K124A/R128A mutations were introduced into the nsp1β–nsp2 region to generate the EU-nsp1β-2-mt or NA-nsp1β-2-mt constructs. For a comparison, the PLPβ catalytic site mutant EU-nsp1β-2-C96S was also constructed. These constructs were analysed in a recombinant vaccinia virus/ T7 RNA polymerase expression system. The result showed that the C96S mutant prevented cleavage of the nsp1β/2 site as the nsp1β/2 proteins were detected as an uncleaved precursor form. In contrast, both K130A/R134A and K124A/R128A mutants were efficiently cleaved at the nsp1β/2 site, as individual nsp1β and nsp2 proteins were detected in the amount quite similar to those produced by the WT nsp1β/2 control (Fig. S2). The data indicated that the proteolytic function of PLPβ toward the nsp1β/2 site was not directly affected by mutation of K130(124) and R134(128), suggesting that the reduced growth rate (viral titre) of the vSD01-08-mt and vSD95-21-mt viruses may not directly be caused by a basic defect in replicase.
polyprotein proteolysis but instead may be due to an impaired ability to counteract the cellular innate immune responses (see below).

**Expression of innate immune genes in nsp1β mutant-infected cells**

As mutations introduced in the GKYLQRRLQ motif reduced the ability of nsp1β to suppress IFN-β activity, we further determined whether these mutations could alter the effect of PRRSV on the innate immune response. Initially, we performed a IFN-β promoter–luciferase reporter assay using the full-length cDNA infectious clone of SD95-21 and its K124A/R128A mutant.

HEK-293T cells were co-transfected with p125-Luc reporter plasmid and full-length cDNA of SD95-21 or the K124A/R128A mutant. At 24 h post-transfection, the cells were mock infected or infected with SeV. After 16 h stimulation, cells were assayed for IFN-β activation. As shown in Fig. 7(a), in comparison with that in EV-transfected cells, the replication of WT virus significantly inhibited IFN-β promoter–dependent luciferase expression. The experiment was repeated three times, and the result consistently showed there was approximately a twofold increase in luciferase reporter signal in cells transfected with full-length cDNA of the K124A/R128A mutant compared with cells transfected with full-length cDNA of the parental virus. To further confirm this result, IFN-α expression was examined in nsp1β mutant and WT virus-infected macrophages. Porcine alveolar macrophages were infected with equal m.o.i. of vSD95-21-mt and vSD95-21 WT viruses. At 24 h p.i., culture supernatants were harvested and IFN-α expression level was quantified by a swine cytokine fluorescence microsphere immunoassay (FMIA). As shown in Fig. 7(b), there was a 26-fold increase in IFN-α expression level in the vSD95-21-mt-infected cells (7.33 pg ml⁻¹) in comparison with that in the WT virus-infected cells (0.28 pg ml⁻¹). As a consequence, vSD95-21-mt grew to a lower virus titre (3.71 × 10⁴ f.f.u. ml⁻¹) in comparison with that of the parental vSD95-21 virus (4.31 × 10⁵ f.f.u. ml⁻¹).

We further determined the effect of the K124A and R128A mutations on the expression of ISG15 in mutant or WT virus-infected MARC-145 cells. Initially, cells were mock infected or infected with vSD95-21-mt or vSD95-21 WT virus. At 18 h p.i., cells were treated with IFN-α to activate the expression of ISGs. Western blot analysis showed a fivefold increase in the level of ISG15 protein production in vSD95-21-mt virus-infected cells in comparison with that in WT virus-infected cells (Fig. 7c). The expression level of nsp1β was also analysed by Western blotting. Interestingly,
in contrast to the in vitro expression system, a similar level of nsp1\(\beta\) protein expression was detected in vSD95-21-mt and vSD95-21 WT virus-infected cells.

**DISCUSSION**

Previous studies from our laboratory and from others have identified PRRSV nsp1\(\beta\) as an IFN antagonist, whereby nsp1\(\beta\) inhibits type I IFN activities in expression systems employing mainly reporter gene-based assays (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Patel et al., 2010). It has been suggested that the PRRSV nsp1\(\beta\) protein may act on the IFN-\(\beta\) production and signalling pathways, in which it could have a direct effect on the formation of the transcription enhanceosome on the IFN-\(\beta\) promoter in the nucleus, as well as having an effect on the nuclear translocation of STAT1/STAT2 (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Patel et al., 2010). The data generated from our current study consistently showed that nsp1\(\beta\) inhibited reporter gene mRNA expression, resulting in a strong inhibition in reporter protein synthesis. Expression of nsp1\(\beta\) also suppressed SeV-induced endogenous IFN-\(\beta\) mRNA accumulation and inhibited IFN-\(\beta\) protein synthesis. Another interesting phenomenon is that the level of nsp1\(\beta\) protein expression in nsp1\(\beta\)-transfected cells was lower in comparison with the expression level of another replicase protein, nsp1\(\alpha\), which also has the ability to antagonize IFN function. Initially, we suspected that nsp1\(\beta\) might have a general effect on host gene expression, including its own RNA transcripts in vitro. However, it appeared not to affect 28S and 18S rRNAs and \(\beta\)-actin expression. A similar observation has also been reported for severe acute respiratory syndrome coronavirus (SARS-CoV) nsp1 (Huang et al., 2011; Kamitani et al., 2009; Kamitani et al., 2006; Narayanan et al., 2008). Individual expression of SARS-CoV nsp1 resulted in low expression levels, which was due to its ability to promote cellular mRNA degradation, including its own RNA transcripts. However, during the infection, the virus developed a mechanism to prevent degradation of its own viral mRNAs, and the nsp1 protein only selectively cleaved cellular mRNAs with certain structures. We also noted a difference in PRRSV nsp1\(\beta\) expression levels using different plasmid expression systems. When PRRSV nsp1\(\beta\) was expressed using the p3\(\times\)FLAG plasmid, which is under the control of a CMV promoter, we detected a lower level of nsp1\(\beta\) expression. In contrast, when nsp1\(\beta\) gene was expressed under the control of a T7 RNA polymerase promoter and an internal ribosomal entry site, the WT nsp1\(\beta\) was expressed in a similar level as its mutant (Fig. S2), which could be due to different mRNA structure in comparison to that expressed under the control of a CMV promoter. Similar to that of SARS-CoV nsp1, in infected cells, PRRSV nsp1\(\beta\) did not appear to suppress the expression of its own mRNA (Fig. 7c), suggesting the virus has evolved a mechanism to recognize its own mRNA structure in infected cells. The detailed mechanism of this
activity is still unknown. PRRSV nsp1β may selectively suppress the expression of host cellular genes, including those involved in host innate immune responses. This leaves intact viral mRNA and host translational machineries for viral-specific protein synthesis. Therefore, in addition to promoting the cellular resources beneficial for its replication, PRRSV may also use nsp1β to block host innate immune responses to establish the infection.

In order to knock down the IFN antagonist function of nsp1β, we introduced a panel of site-specific mutations into the hydrophilic regions of the protein. The effect of GKYLQRRLQ motif mutations on the immune suppression function nsp1β highlights the importance of this region in viral pathogenesis. Our results showed that double mutations introduced at the K130(124) and R134(128) residues of the GKYLQRRLQ motif (in bold) impaired the function of nsp1β on the suppression of IFN-β and reporter gene expression, as well as its 'self-suppression' in vitro. The data suggest an association between the function of nsp1β on the suppression of IFN-β and its 'self-suppression', as higher expression levels of the nsp1β-K130A/R134A and nsp1β-K124A/R128A mutant proteins resulted in reduced ability to suppress IFN activities. More importantly, amino acid sequence analysis showed that the GKYLQRRLQ motif is conserved among all known PRRSV strains, implicating its critical role in virus replication and pathogenesis. Crystal structure analysis showed that this motif is exposed on the surface of the PCPβ domain. However, the PCPβ protease cleavage function did not seem to be directly affected by K130(124) and R134(128) mutations in our in vitro protease cleavage assay (Fig. S2). We suspect that these mutations may affect the activity of nsp1β on primary targeting of host mRNA and interaction with host cellular gene expression shut-off factors. A detailed mechanism of this effect needs to be elucidated in the future.

In an effort to reduce the innate immune suppression function of PRRSV, viable recombinant viruses containing mutations in the GKYLQRRLQ motif were generated, but these viruses showed a certain level of impaired growth ability. The vSD95-21-K124A/R128A mutant replication caused a reduced ability to inhibit IFN-α, IFN-β and ISG15 expression in comparison with that of the WT virus. These data further support the notion that PRRSV nsp1β is a key factor to suppress host innate immune response in PRRSV-infected cells. In correlation with the result from in vitro PCPβ cleavage assay, the data suggest that impaired replication of nsp1β mutants may not be caused directly by an effect on PCPβ cleavage but may rather be due to an impaired ability to suppress host
innate immune gene expression. However, we cannot exclude the possibility that these mutations in the GKYLQRRLQ motif may affect other replicative functions of the PCPβ domain, which could have an indirect effect on virus replication.

Identification of viral elements responsible for innate immune evasion is fundamental for the development of a modified live PRRSV vaccine. Our mutagenesis study showed that modifying the key residues in the GKYLQRRLQ motif of nsp1β could attenuate virus growth and improve the host innate immune responses. As the GKYLQRRLQ motif is highly conserved among PRRSV strains, the technology developed in this study would easily be adapted to other viral strains. Future studies are needed to test the ability of nsp1β mutants for induction of protective immune responses in animals.

METHODS

Cells and viruses. HEK-293T cells, BHK-21 cells and MARC-145 cells were maintained in Eagle’s minimum essential medium (Invitrogen) supplemented with 10% FBS and antibiotic (streptomycin, 100 µg ml⁻¹) at 37 °C with 5% CO₂. Porcine alveolar macrophages were obtained by lung lavage of 6-week-old PRRSV-naive piglets using a method described previously (Zeman et al., 1993). SeV Cantell strain was grown in embryonated chicken eggs and designated p361339 of the SD95-21 genome) from the genomic RNA of SD01-08, a eukaryotic expression vector, p361339 or nsp1b (Fang et al., 2006), type 2 PRRSV isolate SD95-21 (GenBank accession no. KC469618) and their nsp1β mutants were used to infect macrophages or MARC-145 cells for subsequent experiments.

Plasmids. The nsp1α or nsp1β-expressing plasmids were constructed by RT-PCR amplification of nsp1α (nt 222–761 of the SD01-08 genome) or nsp1β (nt 762–1376 of the SD01-08 genome or nt 731–1339 of the SD95-21 genome) from the genomic RNA of SD01-08 (EU) or SD95-21 (NA) virus. The PCR products were cloned into a eukaryotic expression vector, p3 × FLAG-CMV-24 (Sigma) and designated p3 × FLAG-EU-nsp1α, p3 × FLAG-EU-nsp1β and p3 × FLAG-NA-nsp1β, respectively. Specific mutations in nsp1β were constructed by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. Table 1 lists each construct and corresponding instructions (Roche Molecular Biochemicals). At 24 h post-transfection, cells were infected with SeV at 100 HAU ml⁻¹ per well for 12–16 h, or treated with IFN-β at 2000 IU ml⁻¹ per well for 16 h. Cells were harvested and subjected to a reporter gene assay using a luciferase reporter system (Promega) according to the manufacturer’s instructions. Firefly or Renilla luciferase activity was measured in a luminometer (Bethyl). Cell lysates were also subjected to SDS-PAGE and Western blot analysis for protein expression.

Quantitative analysis of mRNA. To measure the IFN-β mRNA expression level, HEK-293T cells were transfected with a plasmid expressing nsp1β or its mutant using FuGENE HD transfection reagent following the manufacturer’s instructions. The EV was also used to transfected cells as a control. At 24 h post-transfection, cells were infected with 100 HAU SeV ml⁻¹ or mock infected. At 16 h p.i., total intracellular RNAs were extracted using the TRIzol RNA Isolation Reagents (Invitrogen). The contaminating genomic DNA was digested with DNase I (Invitrogen). Total RNA (1 µg) was used to synthesize first-strand cDNA using a High Capacity RNA-to-cDNA kit (Invitrogen). Subsequently, real-time PCR was performed to quantify the expression of IFN-β mRNA and 18S rRNA using a TaqMan Gene Expression Master Mix kit (Applied Biosystems). A total of 40 cycles were performed on an ABI PRISM 7900 real-time thermocycler (Applied Biosystems). The amount of IFN-β mRNA was normalized to the endogenous 18S rRNA. For quantitative analysis of reporter gene mRNA expression, HEK-293T cells were co-transfected with plasmid expressing nsp1β (or its mutant) and pRL-SV40 expressing Renilla luciferase. At 24 h post-transfection, total intracellular RNAs were extracted and the expression level of Renilla luciferase mRNA was measured by real-time PCR using a TaqMan Gene Expression Master Mix kit (Applied Biosystems).

Western blot analysis. Virus-infected or plasmid DNA-transfected cells were harvested and lysed in lysis buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA] supplemented with Protease Inhibitor Cocktail (Roche). Cell lysates were frozen at –80 °C, thawed and centrifuged to remove the insoluble pellet. Protein concentration in the supernatant was determined by a Bradford assay (Bio-Rad). Equal amounts of protein samples from different treatments were separated by SDS-PAGE. The separated proteins were blotted onto a nitrocellulose membrane and the membrane was blocked with 5% non-fat dry milk in 0.05% Tween 20 in PBS (PBST). The membrane was then incubated with primary antibodies for 1 h at room temperature. For detection of PRRSV nsp1β and nsp2 expression, mouse mAbs against nsp1β and nsp2 were used as described previously (Li et al., 2012). For detection of FLAG-tagged proteins, an anti-FLAG mAb (Sigma) was used. In addition, a mAb to EGFP (Sigma) or IGG (Santa Cruz Biotechnology) was used as a primary antibody. A anti-β-tubulin mAb (Lamda Biotech) was used as a control. After incubation with the primary antibody, the membrane was washed three times with PBST. Dylight 680-labelled goat anti-rabbit antibody or Dylight 800-labelled goat anti-mouse antibody (LI-COR Biosciences) was added and the membrane was incubated for an additional 1 h at room temperature. An image of the membrane was obtained by scanning under an appropriate excitation control of a T7 RNA polymerase promoter and an encephalomyocarditis virus internal ribosomal entry site, followed by a downstream T7 terminator sequence.

Cell transfection and luciferase reporter assay. HEK-293T cells were seeded at 0.5 × 10⁵ cells ml⁻¹ in 24-well plates 1 day prior to the transfection. Cells were transfected with p3 × FLAG-EU-nsp1β or p3 × FLAG-NA-nsp1β (or their mutants) plasmid DNA (0.5 µg) mixed with luciferase reporter plasmid p125-Luc (0.4 µg), pISRE-Luc (0.4 µg) or pRL-SV40 (0.02 µg). Transfection was performed using FuGENE HD transfection reagent following the manufacturer’s instructions (Roche Molecular Biochemicals). At 24 h post-transfection, cells were infected with SeV at 100 HAU ml⁻¹ per well for 12–16 h, or treated with IFN-β at 2000 IU ml⁻¹ per well for 16 h. Cells were harvested and subjected to a reporter gene assay using a luciferase reporter system (Promega) according to the manufacturer’s instructions. Firefly or Renilla luciferase activity was measured in a luminometer (Bethyl). Cell lysates were also subjected to SDS-PAGE and Western blot analysis for protein expression.
Recovery of recombinant virus from infectious cDNA clones. 

The PRRSV full-length cDNA infectious clones pCMV-SD01-08 (type 1 PRRSV) and pCMV-SD95-21 (type 2 PRRSV) and their nsp1β-mutated plasmids were used to transfect BHK-21 cells. Transfection was performed using 2 μg plasmid DNA with Lipofectamine LTX Reagent following the manufacturer’s instructions (Life Technology). To rescue the virus, cell-culture supernatant obtained at 24–48 h post-transfection was passed on MARC-145 cells. Cells were stained with a mAb specific to nsp1β (Li et al., 2012) and FITC-conjugated SOW17 to nucleocapsid protein for monitoring genome replication and subgenomic transcription as described previously (Fang et al., 2006). Passage 3 viruses from the MARC-145 cells were used for further analysis.

Sequencing of the nsp1β mutation regions. To determine the stability of each mutation, cell lysate from recombinant virus-infected cells was harvested and RNA was extracted using a QiaAmp viral RNA kit (Qiagen) following the manufacturer’s instructions. The PRRSV genomic region of nt 1–1377 (type1 PRRSV) or nt 251–1504 (type 2 PRRSV) containing the targeted nsp1β mutations was amplified by RT-PCR, and PCR products were sequenced at the Iowa State University DNA Sequencing Facility (Ames, IA, USA).

Virus titration and plaque assay. MARC-145 cells were infected with nsp1β mutants and WT virus at an m.o.i. of 0.1. Infected cells were harvested at 48 h p.i., and virus titres were determined by a fluorescent focus assay as described previously (Sun et al., 2012). Plaque morphology between the nsp1β mutant and WT virus was compared by plaque assay using a method described previously (Fang et al., 2006).

nsp1β–nsp2 expression in a recombinant vaccinia virus/T7 polymerase system. pl-EU-nsp1β-2-C96S, pl-EU-nsp1β-2, pl-EU-nsp1β-2-mt, pl-NA-nsp1β-2 or pl-NA-nsp1β-2-mt plasmid was transiently expressed in HEK-293T cells using a recombinant vaccinia virus/T7 polymerase expression system (Fuerst et al., 1986) as described previously (Snijder et al., 1994). Cells were harvested at 24 h post-transfection and subjected to Western blot analysis using mAbs specific to nsp1β and the nsp2 PLP2 domain (Li et al., 2012; Fang et al., 2012).

Swine cytokine FMIA. Porcine alveolar macrophages were infected with WT or recombinant viruses at an m.o.i. of 1. At 24 h p.i., culture supernatant was harvested to analyse IFN-α expression using an FMIA as described previously (Lawson et al., 2010). The quantity of IFN-α was determined using mean fluorescent intensity values, and the result was compared with the mean values from mock-infected control cells.

ACKNOWLEDGEMENTS

We thank Dr Takashi Fujita (Kyoto University, Tokyo, Japan) for providing the p125-Luc plasmid. This project was supported by the USDA National Institute of Food and Agriculture (to Y.F., grant number 2011-02925).

REFERENCES


