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Alteration of the *N*-linked glycosylation condition in E1 glycoprotein of Classical Swine Fever Virus strain Brescia alters virulence in swine

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ABSTRACT

E1, along with E^{rns} and E2 is one of the three envelope glycoproteins of Classical Swine Fever Virus (CSFV). Previously we showed that glycosylation status of virulent CSFV strain Brescia E2 or E^{rns} affects virus virulence. Here, the three putative glycosylation sites of E1 were serially removed by means of site directed mutagenesis of a CSFV Brescia infectious clone (BICv) and their effect on virulence assessed in swine. Removal of all three putative glycosylation sites in E1, at CSFV positions N500, N513 and N594, yielded nonviable progeny, while single or dual site mutants excluding N594 were viable. Individual N594A (E1.N3 virus) or combined N500A/N513A (E1.N1N2 virus) substitutions resulted in BICv attenuation. Furthermore infection with E1.N3 or E1.N1N2 viruses efficiently protected swine from challenge with virulent BICv at 3 and 28 days post-infection. As previously observed with E^{rns} and E2 and here with E1 data suggest that modification of glycosylation patterns could be used for developing CSFV live-attenuated vaccines.

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Introduction

Classical Swine Fever (CSF) is a highly contagious disease of swine. The etiological agent, CSF virus (CSFV), is a small, enveloped virus with a positive, single-stranded RNA genome and, along with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) is classified as a member of the genus *Pestivirus* within the family Flaviridae (Fauquet et al., 2005). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898-amino-acid polyprotein and ultimately yields 11 to 12 final cleavage products (NH_2–Npro–C–E^{rns}–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Lindenbach et al., 2007). Structural components of the CSFV virion include the capsid (C) protein and glycoproteins E^{rns}, E1, and E2. E1 and E2 are anchored to the envelope at their carboxyl termini and E^{rns} loosely associates with the viral envelope (Weiland et al., 1990 and 1999). E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (Thiel et al., 1991). E1 has been implicated (Wang et al., 2004), along with E^{rns} and E2 (Huslt and Moormann, 1997), in viral adsorption to host cells. Importantly, modifications introduced into these glycoproteins appear to have an important effect on CSFV virulence (Meyers et al., 1999; Risatti et al., 2005a, 2005b, and 2006; Van Gennip et al., 2004).

Glycosylation is one of the most common types of protein modifications. *N*-linked oligosaccharides are added to specific asparagine residues in the context of the consensus sequence Asn-X-Ser/Thr (Kornfeld and Kornfeld, 1985). According to a glycosylation analysis algorithm (http://www.cbs.dtu.dk/services/), E1 of the CSFV strain Brescia has three putative *N*-linked glycosylation sites although this prediction has not been experimentally confirmed. Predicted E1 glycosylation sites (at CSFV amino acid residue position N500, N513 and N594) are highly conserved among CSFV isolates and two of them (N513 and N594) also conserved among other Pestiviruses. However, the significance of viral envelope protein glycosylation in virus replication, pathogenesis, and virulence in the natural host is not completely defined. We have recently described that removal of specific putative glycosylation sites in E^{rns} and E2 results in attenuation of highly virulent CSFV strain Brescia in swine (Fernandez Sainz et al., 2008; Risatti et al., 2007).

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Table 1
Set of CSFV E1 glycosylation mutant viruses constructed in this study

E1 position	Wild type sequence	Mutant sequence	Codon change	Mutant
500	NVTS	**A**VTS	AAT → **GC**T	E1.N1
513	NCTP	**A**CTP	AAC → **GC**C	E1.N2
594	NLTV	**A**LTV	AAT → **GC**T	E1.N3
500/513	NVTS/NCTP	**A**VTS/**A**CTP	AAT → **GC**T/AAC → **GC**C	E1.N1N2
513/594	NCTP/NLTV	**A**CTP/**A**LTV	AAC → **GC**C/AAT → **GC**T	E1.N2N3
500/594	NVTS/NLTV	**A**VTS/**A**LTV	AAT → **GC**T/AAT → **GC**T	E1.N1N3
500/513/594	NVTS/NCTP/NLTV	**A**VTS/**A**CTP/**A**LTV	AAT → **GC**T/AAC → **GC**C/AAT → **GC**T	E1.N1N2N3

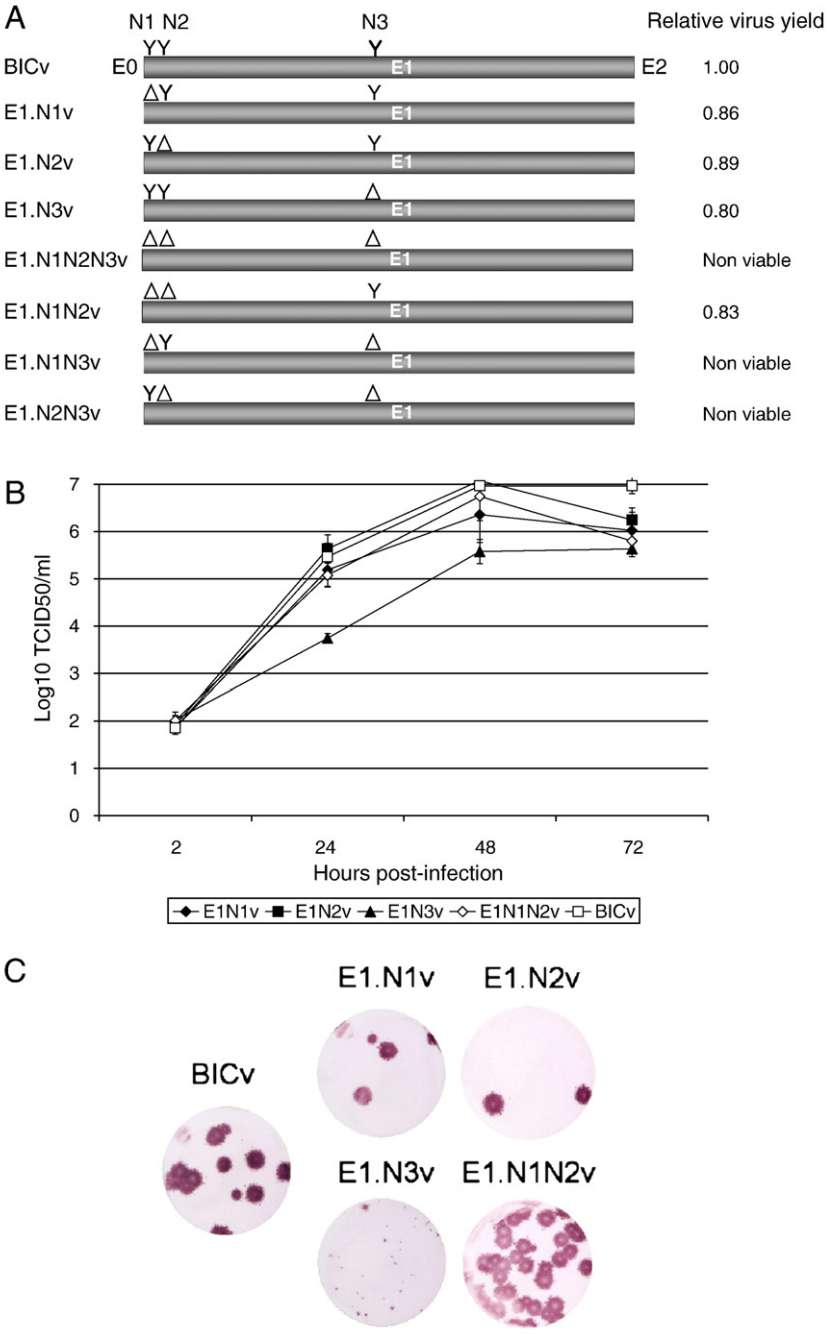

Fig. 1. (A) Schematic representation of glycosylation mutants of Classical Swine Fever Virus E1 protein, generated by site-directed mutagenesis of a cDNA full-length clone pBIC (Risatti et al., 2005b)). Wild type E1 glycoprotein shown at the top. Y: putative glycosylation sites. Mutants were named with an N (*N*-linked glycosylation) followed by a number that represents the relative position of putative glycosylation sites within E1 amino acid sequence (N1, N2 and N3). Relative virus yield is final point virus yield as proportion of final end point (72 h post-infection) virus yield of parental BICv. (B) *In vitro* growth characteristics of E1 glycosylation mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI=0.01) with each of the mutants or BICv and virus yield titrated at times post-infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥1.8 $TCID_{50}$/ml (log_{10}). (C) Plaque formation of E1 glycosylation mutants and BICv. SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37 °C for 3 days. Plates were fixed with 50% (vol/vol) ethanol–acetone and stained by immunohistochemistry with mAb WH303.

In this study, we have used oligonucleotide site-directed mutagenesis to construct a panel of glycosylation mutants by modifying predicted *N*-glycosylation sites within CSFV glycoprotein E1 using as target a full-length cDNA clone of highly virulent CSFV strain Brescia. These mutants were applied to investigate whether the removal of each of these glycosylation sites in the E1 glycoprotein could affect viral infectivity and virulence in swine. We found that rescue of viable virus was completely impaired by removal of all putative glycosylation sites in E1, and, interestingly, removal of glycosylation sites at amino acid N594 (E1.N3 virus), as well as the combined removal of those sites at residues N500 and N513 (E1.N1N2 virus) renders attenuated viruses with decreased virus replication and shedding in infected swine.

Results

Construction of CSFV glycosylation mutant viruses

Infectious RNA was *in vitro* transcribed from full-length ICs of the CSFV Brescia strain (pBIC) or a set of glycosylation mutants (Table 1, Fig. 1) and used to transfect SK6 cells. Mutants referred to as E1.N1v, E1.N2v, E1.N3v represent each of three putative glycosylation sites starting from the N terminus of E1 (Table 1), whereas multiple mutants are represented by combinations of indicated sites (Table 1, Fig. 1A). Viruses were rescued from transfected cells by day 4 post-transfection. Nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted glycosylation sites were reflected in rescued viruses.

Replication of glycosylation mutants in vitro

In vitro growth characteristics of viable mutant viruses E1.N1v, E1.N2v, E1.N3v and E1.N1N2v (Fig 1A), were evaluated relative to parental pBIC-derived virus (BICv) in a multistep growth curve (Fig. 1B). Primary swine macrophage cell cultures were infected at a MOI of 0.01 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at times post-infection through 72 h. All single glycosylation site mutants exhibited titers approximately an order of magnitude lower than those corresponding to BICv. Additionally, when viruses were tested for their plaque size in SK6 cells E1.N3v exhibited a noticeable reduction in plaque size relative to BICv (Fig. 1C). Interestingly, some viruses were not rescued from SK-6 cells transfected with RNA transcribed from full-length cDNA clones carrying multiple glycosylation site mutations (E1.N1N2N3, E1.N1N3 and E1.N2N3) that included N to A substitution at E1.N3 position (N594) (Fig 1A).

Relative electrophoretic mobility of mutant E1 glycoprotein

Relative electrophoretic mobility of E1 was analyzed by Western immunoblot in lysates of SK-6 cells infected with different E1mutant and parental viruses using a rabbit anti-E1 serum raised against CSFV Brescia E1 glycoprotein expressed in *E. coli* BL21 (DE3). Assuming that differences in E1 mobility among mutants and parental viruses are likely due to the number of carbohydrate moieties attached to the protein, we observed that E1 from single mutants E1.N1v, and E1.N3v migrated further than E1 from mutants E1.N2v, or parental BICv (Fig. 2). Analysis of E1 mobility in lysates from cells infected with mutant virus missing N1 and N2 glycosylation sites (E1.N1N2v) demonstrated a further migration than in E1.N1v suggesting the glycosylation of N2 site although this is not evident in the mobility of E1 from E1.N2 virus (Fig. 2). Overall, data suggests that, at least, CSFV sites N1 and N3 of the E1 glycoprotein are targeted for glycosylation in swine SK-6 cells.

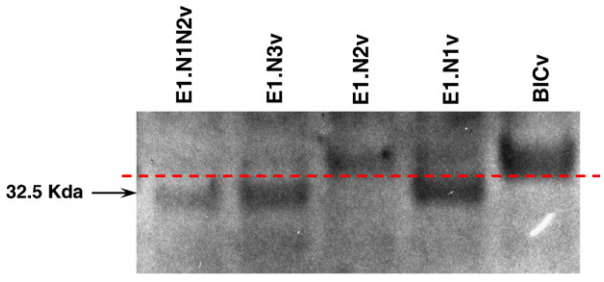

Fig. 2. Analysis of E1 glycosylation mutants was done by Western immunoblotting. SK6 monolayers were infected (MOI = 1) with each of the mutants or parental BICv and harvested 48 h post-infection. Cell lysates were run under reducing conditions in 12% sodium dodecyl sulfate-polyacrylamide gels. CSFV E1 was detected with CSFV E1 rabbit polyclonal antibody.

Table 2
Swine survival and fever response following infection with CSFV E1 glycosylation mutants and parental BICv

Virus	No. of survivors/ total no.	Mean time to death (days ± SD[a])	Fever	
			No. of days to onset (days ± SD)	Duration (days ± SD)
BICv	0/6[b]	8.2 ± 0.9	3.4 ± 1.1	6.5 ± 0.9
E1.N1v	0/2	5.5 ± 0.7	2 ± 0.0	3.5 ± 0.7
E1.N2v	0/2	9.5 ± 2.1	3 ± 0.0	6.5 ± 2.1
E1.N3v	6/6[b]	–	–	–
E1.N1N2v	6/6[b]	–	–	–

[a] SD: Standard Deviation.
[b] The original experiment performed with 2 animals was repeated with 4 more pigs. Presented results represent data from both experiments.

Mutants E1.N3v and E1.N1N2v lack determinants necessary for CSFV virulence in swine

To examine the effect of E1 glycosylation on CSFV virulence, and to establish the impact of mutations at individual glycosylation sites in swine virulence, individual mutant viruses were intranasally inoculated in naïve animals, at doses of 10^5 $TCID_{50}$, and monitored for clinical disease relative to the parental virus. BICv exhibited a characteristic virulent phenotype (Table 2). Animals infected with E1.N3v survived the infection and remained normal throughout the observation period (21 days). All animals infected with E1.N1v and E1.N2v presented clinical signs of CSF starting 5 to 8 DPI, with clinical presentation and severity similar to those observed in animals inoculated with BICv. White blood cell and platelet counts dropped by 4 to 6 DPI in animals inoculated with E1.N1v, E1.N2v or BICv and kept declining until death, while a transient decrease in cell counts was observed in animals inoculated with E1.N3v (Fig. 3).

Since E1.N1v and E1.N2v were as virulent as wild type BICv it was interesting to assess the effect on viral virulence caused by the simultaneous substitutions (N to A) of both N500 and N514 in E1 resulting in mutant E1.N1N2v. Two animals were then infected with 10^5 $TCID_{50}$ of E1.N1N2v under the same conditions described above. Infected animals remained normal without signs of the disease throughout the observation period (21 days) showing only a transient decrease in their blood cell counts (Table 2 and Fig. 3).

Virus shedding and viremia in E1.N3v and E1.N1N2v inoculated animals were almost undetectable, while viral loads in E1.N1v, and E1.N2v were in general 1.5–2.5 logs below of those observed in BICv infected swine varying with the time elapsed after infection (Fig. 3). In all cases partial nucleotide sequences of E1 protein from viruses recovered from infected animals were identical to those of stock viruses used for inoculation (data not shown).

The capability of E1.N3v and E1.N1N2v to establish a systemic infection in intranasally inoculated animals was compared with that of virulent parental virus BICv. Randomly selected animals were euthanized at 2, 4, 6, 8, and 12 DPI (one animal/time point/group) and virus titration was performed in collected tissues (tonsils, mandibular lymph nodes, kidney and spleen). Titers measured in those tissue

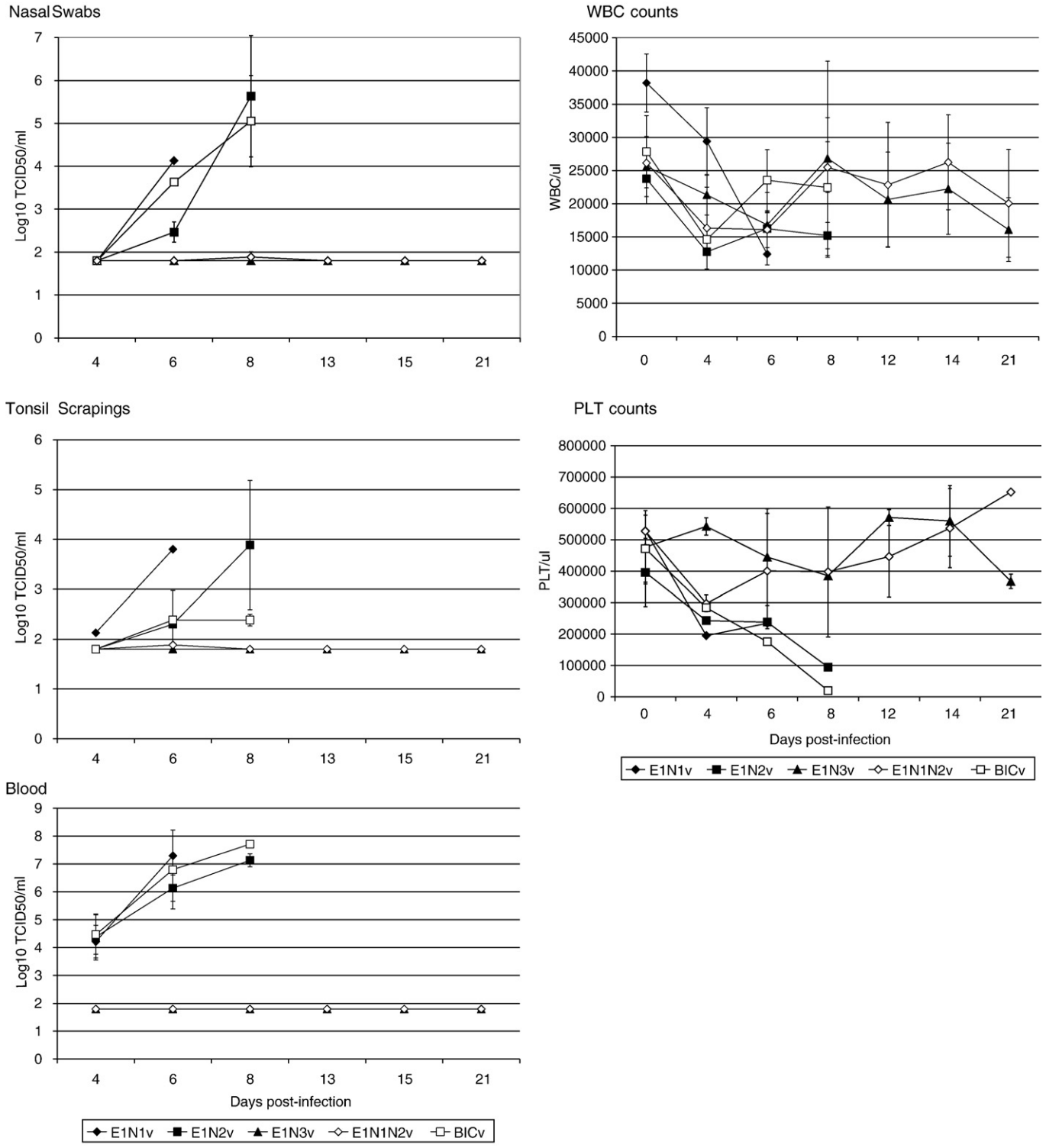

Fig. 3. Hematological data (panels on the right) and virus titers (panels on the left) in clinical samples (nasal swabs, tonsil scrapings, and blood) from animals infected with CSFV E1 glycosylation mutants or parental BICv. Peripheral white blood cell and platelet counts are expressed as numbers/μl of blood. Sensitivity of virus detection: ≥1.8 $TCID_{50}$/ml. Data represent means and standard deviations from at least two animals.

samples are shown in Table 3. *In vivo* replication of E1.N3v and E1.N1N2v was transient in tonsils with titers reduced up to 10^2 to 10^5, depending on the time post-infection, relative to viral loads observed in animals infected with BICv. Differences in viral loads between E1.N3v or E1.N1N2v and BICv were also observed in mandibular lymph nodes, spleen, and kidney, indicating a limited capability of E1.N3v and E1.N1N2v to spread within the host.

E1.N3v and E1.N1N2v mutants protect pigs against lethal CSFV challenge

The limited *in vivo* replication kinetics shown by E1.N3v and E1.N1N2v is similar to that observed with CSICv (Risatti et al., 2005a), a CSFV live-attenuated vaccine strain. However, restricted viral replication *in vivo* could also impair protection against wild-type virus infections. Thus, the ability of E1.N3v and E1.N1N2v to induce

Table 3
Virus titers in target organs after intranasal inoculation with mutant E1.N1N2v, E1.N3v or parental BICv

Virus	DPI	Log_{10} $TCID_{50}$/g in			
		Tonsil	Mandibular lymph node	Spleen	Kidney
E1.N1N2v	2	nd[a]	2.63	1.97	nd
	4	4.47	nd	nd	1.97
	6	3.63	nd	2.13	2.47
	8	2.8	nd	nd	nd
	12	nd	nd	nd	nd
E1.N3v	2	2.3	nd	2.13	2.8
	4	1.97	nd	1.97	nd
	6	2.3	nd	2.47	2.47
	8	7.8	nd	1.97	nd
	12	nd	nd	1.97	2.13
BICv	2	3.12	1.97	nd	nd
	4	7.13	3.8	2.97	2.8
	6	6.8	6.13	6.13	5.13
	8	7.13	4.97	7.13	5.47
	12	D[b]	D	D	D

[a] nd (not detectable): virus titers <1.8 $TCID_{50}$/ml (log_{10}).
[b] D: Animals in this group were all dead by this time point.

protection against virulent BICv was assessed in early and late vaccination-exposure experiments. Groups of pigs were intranasally inoculated with 10^5 $TCID_{50}$ of E1.N3v or E1.N1N2v and challenged at 3 or 28 DPI. Mock-vaccinated control pig groups receiving 10^5 $TCID_{50}$ of BICv developed anorexia, depression, and fever by 4 days post-challenge (DPC), and a marked reduction of circulating leukocytes and platelets by 4 DPC (data not shown), and died or were euthanized *in extremis* by 9–11 DPC (Table 4). Notably, E1.N3v and E1.N1N2v induced complete protection by 3 and 28 DPI. All pigs survived infection and remained clinically normal, without significant changes in their hematological values (data not shown).

Viremia and virus shedding of vaccinated-exposed animals was examined at 4, 6, 8, 14 and 21 DPC (Table 4). As expected, in mock-vaccinated control animals, viremia was observed by 4 DPC, with virus titers remaining high by 8 DPC (approximately 10^7 $TCID_{50}$/ml of blood). Furthermore, virus was detected in nasal swabs and tonsil scrapings of these animals after 4 DPC. Conversely, no virus was detected in any of the samples obtained from pigs inoculated with E1.N3v and challenged with BICv at 3 DPI (Table 4). Whereas virus was detected in blood, nasal swabs, and tonsil scrapings at 4, 6, and 8 DPC in some animals infected with E1.N1N2v and challenged at 3 DPI (Table 4). Virus was undetectable in clinical samples obtained from any E1.N3v or E1.N1N2v infected pigs that were challenged at 28 DPI. Even though E1.N3v and E1.N1N2v showed a limited *in vivo* growth, a solid protection was induced shortly after vaccination.

Discussion

Virus glycoproteins are crucial in key steps of the virus cycle such as attachment to host cell receptors, entry, assembly of newly produced viral progeny, and exit. *In vivo*, viral glycoproteins have been shown to influence infectivity (Abe et al., 2004), virulence (Hulse et al., 2004; Panda et al., 2004), and host immune response (Panda et al., 2004). In the case of CSFV we have observed that modifications of glycosylation sites in envelope proteins E^{rns} (Fernandez Sainz et al., 2008), E2 (Risatti et al., 2007), and now E1 yielded viruses that *in vivo* induce either one of two well-defined outcomes: viruses as virulent as the parental CSFV Brescia, or viruses with a variable degree of attenuation.

Added oligosaccharides confer proper function to viral glycoproteins since alteration of those glycosylation sites in virus envelope proteins affect protein folding (Hebert et al., 1997; Kornfeld and Kornfeld, 1985; Shi and Elliott, 2004 and 2005; Slater-Handsby et al., 2004) and protein active conformation (Meunier et al., 1999). Modification of *N*-glycosylation sites seems to affect virus–host interactions. For instance, glycosylation patterns of the hemagglutinin gene of H5 avian influenza viruses (Deshpande et al., 1987; Horimoto and Kawaoka, 1994) and glycosylation patterns of the neuraminidase gene of highly pathogenic H5N1 avian flu viruses are important for virulence in birds (Hulse et al., 2004). Similarly, in the case of Pestiviruses, alteration of *N*-glycosylation patterns of two of the glycoproteins, E^{rns} and E2, affect virulence of CSFV strain Brescia (Risatti et al., 2007; Fernandez Sainz et al., 2008). CSFV strain Brescia E1 glycoprotein contains 3 *N*-linked putative glycosylation sites (http://www.cbs.dtu.dk/services/) (Moormann et al., 1990). Sequence analysis of CSFV E1 glycoproteins showed that 3 of the *N*-linked glycosylation sites are highly conserved in CSFV strains and two of them (N513 and N594) are also conserved among BVDV types I and II and BDV strains (data not shown), suggesting an important role for these *N*-glycosylation sites in Pestiviruses. Here we observed that single (E1.N3v) or multiple N to A substitutions (E1.N1N2v) within CSFV E1 rendered attenuated viruses with restricted *in vivo* replication capabilities (Table 2) while still highly immunogenic. Different from the acute fatal disease induced by virulent BICv, infections caused by these mutants were sub-clinical in swine and characterized by decreased viral loads in target organs and reduced virus shedding. Interestingly, mutants E1.N1v and E1.N2v retained the same capability of causing severe disease in swine as parental BICv, showing that *in vivo* E1 functions are retained and not influenced by the lack of glycans at positions N500 and N513. As with avian flu, the function altered as a result of modification of *N*-glycosylation target sites within CSFV glycoproteins has yet to be identified.

As shown in this study, single mutations of E1 putative glycosylation sites do not have a strong effect on *in vitro* replication or *in vivo* infectivity of CSFV, with the exception of residue N594 in the E1.N3v mutant. Furthermore, no viral progeny were obtained when the N594A substitution in E1.N3v was combined with N500A and/or N513A substitutions as in E1.N1N3, E1.N2N3 or E1.N1N2N3 (data not shown). Reactivity with mAbWH303 directed against CSFV E2 glycoprotein was always observed after transfection of swine cells with *in vitro* transcribed RNA from full-length cDNAs carrying E1.N1N3, E1.N2N3 or E1.N1N2N3 mutations. These observations suggest that lack of viral progeny may be linked to inefficient RNA replication,

Table 4
Detection of virus in nasal swabs, tonsil scrapings, and blood samples obtained from E1.N1N2v or E1N3v vaccinated pigs at times after challenge of with virulent BICv

Challenge group	Sample	Days post-challenge						
		0	4	6	8	12	14	21
E1.N1N2v 3 DPI	Nasal	0/4[a]	1/4 (1.9)	1/4 (2.5)	1/4 (3.1)	0/4	0/4	0/4
	Tonsil	0/4	1/4 (2.1)	1/4 (2.8)	2/4 (2.7)	0/4	0/4	0/4
	Blood	0/4	1/4 (2.9)[b]	3/4 (4)	1/4 (4.8)	0/4	0/4	0/4
E1.N1N2v 28 DPI	Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
E1.N3v 3 DPI	Nasal	0/4[a]	0/4	0/4	0/4	0/4	0/4	0/4
	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
E1.N3v 28 DPI	Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Control 3 DPI	Nasal	0/2	0/2	2/2 (2.1)	2/2 (5.1)	D[c]		
	Tonsil	0/2	1/2 (2.1)	2/2 (2.7)	2/2 (4.1)			
	Blood	0/2	1/2 (2.2)	2/2 (6.4)	2/2 (7.2)			
Control 28 DPI	Nasal	0/2	0/2	1/2 (2.1)	2/2 (3.6)	D[c]		
	Tonsil	0/2	1/2 (1.9)	2/2 (4.2)	2/2 (3.6)			
	Blood	0/2	1/2 (2.1)	2/2 (5.1)	2/2 (7.1)			

[a] Number of animals positive for virus isolation over total number of challenged animals.
[b] Number in parentheses indicate average virus titers of animals showing presence of virus (expressed as log_{10} $TCID_{50}$/ml).
[c] D, animals in this group were all dead by this time point.

improper proteolytic processing, and defects on protein trafficking or virus assembly.

Viable E1.N3v and E1.N1N2v showed a limited ability to establish a generalized infection in swine upon intranasal inoculation relative to the widespread distribution of parental BICv in control animals (Tables 3 and 4). Mutant viruses were detected in the spleen by day 2 post-inoculation (Table 3) but at significantly lower levels relative to BICv. Similarly, we have previously observed (Risatti et al., 2005a) that animals infected with live-attenuated CSFV vaccine CSICv or attenuated E2 chimera 319.1v showed CSFV antigen concentrated in the tonsil, with a significant relative reduction in immunoreactivity in the regional submandibular lymph node and spleen compared to the BICv-infected animals. Attenuation of these viruses and E1.N3v and E1.N1N2v in pigs could conceivably involve some aspect of virus attachment and/or efficiency of entry into critical target cells *in vivo*, or altered trafficking of the virus within infected host cells as been described for Human Respiratory Syncytial virus (Batonick et al., 2008), Sendai virus (Teng et al., 2001), and Measles virus (Moll et al., 2004).

In summary, our studies determined that individual *N*-linked glycosylation in glycoprotein E1 sites are not essential for viral particle formation or virus infectivity in cultured swine macrophages or the natural host, with one individual site, N594, involved in attenuation of the virulent parental virus. This study also showed that in the context of two or more putative glycosylation site modifications, residue N594 is critical for virus viability. The effective protective immunity elicited by E1.N3v and E1.N1N2v suggests that glycosylation of E1 could be modified for the development of live-attenuated vaccines. An improved understanding of the genetic basis of virus virulence and host range will permit future rational design of efficacious biological tools for controlling CSF.

Materials and methods

Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were cultured in Dulbecco's Minimal Essential Media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (IC) (Risatti et al., 2005a). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described by Zsak et al. (1996). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibodies WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was >1.8 $TCID_{50}$/ml. Plaque assays were performed using SK6 cells in 6-well plates (Costar). SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37 °C for 3 days. Plates were fixed with 50% (vol/vol) ethanol–acetone and stained by immunohistochemistry with mAb WH303.

Construction of CSFV glycosylation mutants

A full-length IC of the virulent CSFV Brescia strain (pBIC) (Risatti et al., 2005a) was used as a template in which *N*-linked glycosylation sites in the E1 glycoprotein were mutated. Glycosylation sites were predicted using analysis tools from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). N to A amino acid substitutions were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions and using the following primers (only forward primer sequences are shown); E1.N1v: TATGCCCTATCACCTTATTGT*GCT*G TGACAAGCAAAATAGGGTAC; E1.N2v: GGGTACATATGGTACACTAAC*GCC*TGTACC CCGGCTTGCCTCCCC; E1.N3v: GAAGGCTGTGACACAAACCAGCTG *GCT*TTAACAGT GGAACTCAGGACT.

In vitro rescue of CSFV Brescia and glycosylation mutants

Full-length genomic clones were linearized with SrfI and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates and incubated for 4 days at 37 °C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70 °C.

DNA sequencing and analysis

Full-length clones and *in vitro* rescued viruses were completely sequenced with CSFV specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977). Viruses recovered from infected animals were sequenced in the mutated region. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a ABI PRISM 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed using CAP3 (Huang and Madan, 1999). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Western blot analysis

Glycosylation status of the E1 glycoprotein of BICv and mutant viruses was analyzed in lysates of SK6 infected cells by Western immunoblots. CSFV E1 was detected with an anti-E1 antiserum produced in rabbits against CSFV Brescia E1 expressed in *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA). SK6 monolayers were infected (multiplicity of infection, MOI=1) with BICv or glycosylation mutants and harvested at 48 h post-inoculation (HPI) using the NuPAGE LDS sample buffer system (Invitrogen), and incubated at 80 °C for 20 min. Samples were run under reducing conditions in pre-cast NuPAGE 12% Bis–Tris acrylamide gels (Invitrogen). Western immunoblots were performed using the WesternBreeze Chemoluminescent Immunodetection System (Invitrogen).

Animal Infections

Each of the glycosylation mutants was initially screened for its virulence phenotype in swine relative to virulent Brescia strain. Swine used in all animal studies were 10 to 12 weeks old, forty-pound commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type virus. For screening, 10 pigs were randomly allocated into 5 groups of 2 animals each, and pigs in each group were inoculated with one of the single glycosylation mutants, E1.N1N2v or BICv. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment.

To assess the effect of the mutations in E1.N3v and E1.N1N2v on virus shedding and distribution in different organs during infection, pigs were randomly allocated into 3 groups of 9 animals each and intranasally inoculated (see above) with 10^5 $TCID_{50}$ of E1. N3v, E1.N1N2v or BICv. One pig per group was sacrificed at 2, 4, 6, 8 and

12 days post-infection (DPI). Blood, nasal swabs and tonsil scraping samples were obtained at times after infection. Tissue samples (tonsil, mandibular lymph node, spleen and kidney) collected at necropsy were snap-frozen in liquid nitrogen for subsequent virus titration. The remaining 4 pigs in each room were monitored daily for the appearance of clinical signs during a 21-day period.

For protection studies, pigs were randomly allocated into 6 groups. Pigs in groups 1 and 2 (n=4) were inoculated with 10^5 $TCID_{50}$ of E1. N1N2v, pigs in groups 3 and 4 (n=4) were inoculated with 10^5 $TCID_{50}$ of E1.N3v and pigs in groups 5 and 6 (n=2) were mock infected. At 3 DPI (groups 1 and 3) or 28 DPI (groups 2 and 4), animals were intranasally challenged with 10^5 $TCID_{50}$ of BICv along with animals in group 5 and 6. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood, serum, nasal swabs and tonsil scrapings were collected at times post-challenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer). Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman-Coulter, CA).

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References

Abe, Y., Takashita, E., Sugawara, K., Matsuzaki, Y., Muraki, Y., Hongo, S., 2004. Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. J. Virol. 78, 9605–9611.

Batonick, M., Oomens, A.G., Wertz, G.W., 2008. Human respiratory syncytial virus glycoproteins are not required for apical targeting and release from polarized epithelial cells. J. Virol. 82, 8664–8672.

Deshpande, K.L., Fried, V.A., Ando, M., Webster, R.G., 1987. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc. Natl. Acad. Sci. U. S. A. 84, 36–40.

Edwards, S., Moennig, V., Wensvoort, G., 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet. Microbiol. 29, 101–108.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), 2005. ICTV (2005). VIRUS TAXONOMY: VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, Hong Kong.

Fernandez Sainz, I., Holinka, L.G., Lu, Z., Risatti, G.R., Borca, M.V., 2008. Removal of a N-linked glycosylation site of classical swine fever virus strain Brescia E^{rns} glycoprotein affects virulence in swine. Virology 370, 122–129.

Hebert, D.N., Zhang, J.X., Chen, W., Foellmer, B., Helenius, A., 1997. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. J. Cell Biol. 139, 613–623.

Horimoto, T., Kawaoka, Y., 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68, 3120–3128.

Huang, X., Madan, A., 1999. CAP3: A DNA sequence assembly program. Genome Res. 9, 868–877.

Hulse, D.J., Webster, R.G., Russell, R.J., Perez, D.R., 2004. Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. J. Virol. 78, 9954–9964.

Hulst, M.M., Moormann, R.J., 1997. Inhibition of *Pestivirus* infection in cell culture by envelope proteins E(rns) and E2 of classical swine fever virus: E(rns) and E2 interact with different receptors. J. Gen. Virol. 78, 2779–2787.

Kornfeld, R., Kornfeld, S., 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54, 631–664.

Lindenbach, B.D., Thiel, H.J., Rice, C.M., 2007. Flaviviridae: the viruses and their replication. P. 1101, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A. (Eds.), Fundamental Virology, 5th ed. Lippincott Williams and Wilkins, Philadelphia.

Meunier, J.C., Fournillier, A., Choukhi, A., Cahour, A., Cocquerel, L., Dubuisson, J., Wychowski, C., 1999. Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. J. Gen. Virol. 80, 887–896.

Meyers, G., Saalmuller, A., Buttner, M., 1999. Mutations abrogating the RNase activity in glycoprotein E(rns) of the *Pestivirus* classical swine fever virus lead to virus attenuation. J. Virol. 73, 10224–10235.

Moll, M., Pfeuffer, J., Klenk, H.D., Niewiesk, S., Maisner, A., 2004. Polarized glycoprotein targeting affects the spread of measles virus in vitro and in vivo. J. Gen. Virol. 85, 1019–1027.

Moormann, R.J., Warmerdam, P.A., van der Meer, B., Hulst, M.M., 1990. Nucleotide sequence of hog cholera virus RNA: properties of the polyprotein encoded by the open reading frame spanning the viral genomic RNA. Vet. Microbiol. 23, 185–191.

Panda, A., Elankumaran, S., Krishnamurthy, S., Huang, Z., Samal, S.K., 2004. Loss of N-linked glycosylation from the hemagglutinin-neuraminidase protein alters virulence of Newcastle disease virus. J. Virol. 78, 4965–4975.

Reed, L.J., Muench, H.A., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Risatti, G.R., Borca, M.V., Kutish, G.F., Lu, Z., Holinka, L.G., French, R.A., Tulman, E.R., Rock, D.L., 2005a. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J. Virol. 79, 3787–3796.

Risatti, G.R., Holinka, L.G., Lu, Z., Kutish, G.F., Tulman, E.R., French, R.A., Sur, J.H., Rock, D.L., Borca, M.V., 2005b. Mutation of E1 glycoprotein of classical swine fever virus affects viral virulence in swine. Virology 343, 116–127.

Risatti, G.R., Holinka, L.G., Carrillo, C., Kutish, G.F., Lu, Z., Tulman, E.R., Fernandez Saiz, I., Borca, M.V., 2006. Identification of a novel virulence determinant within E2 structural glycoprotein of classical swine fever virus. Virology 355, 94–101.

Risatti, G.R., Holinka, L.G., Fernandez Sainz, I., Carrillo, C., Lu, Z., Borca, M.V., 2007. N-linked glycosylation status of Classical Swine Fever Virus strain Brescia E2 glycoprotein influences virulence in swine. J. Virol. 81, 924–933.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Shi, X., Elliott, R.M., 2004. Analysis of N-linked glycosylation of hantaan virus glycoproteins and the role of oligosaccharide side chains in protein folding and intracellular trafficking. J. Virol. 78, 5414–5422.

Shi, X., Brauburger, K., Elliott, R.M., 2005. Role of N-linked glycans on bunyamwera virus glycoproteins in intracellular trafficking, protein folding, and virus infectivity. J. Virol. 79, 13725–13734.

Slater-Handshy, T., Droll, D.A., Fan, X., Di Bisceglie, A.M., Chambers, T.J., 2004. HCV E2 glycoprotein: mutagenesis of N-linked glycosylation sites and its effects on E2 expression and processing. Virology 319, 36–48.

Teng, M.N., Whitehead, S.S., Collins, P.L., 2001. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo. Virology 289, 283–296.

Terpstra, C., Woortmeyer, R., Barteling, S.J., 1990. Development and properties of a cell culture produced vaccine for hog cholera based on the Chinese strain. Dtsch Tierarztl Wochenschr 97, 77–79.

Thiel, H.J., Stark, R., Weiland, E., Rumenapf, T., Meyers, G., 1991. Hog cholera virus: molecular composition of virions from a *Pestivirus*. J. Virol. 65, 4705–4712.

Van Gennip, H.G., Vlot, A.C., Hulst, M.M., De Smit, A.J., Moormann, R.J., 2004. Determinants of virulence of classical swine fever virus strain Brescia. J. Virol. 78, 8812–8823.

Wang, Z., Nie, Y., Wang, P., Ding, M., Deng, H., 2004. Characterization of classical swine fever virus entry by using pseudotyped viruses: E1 and E2 are sufficient to mediate viral entry. Virology 330, 332–341.

Weiland, E., Stark, R., Haas, B., Rumenapf, T., Meyers, G., Thiel, H.J., 1990. *Pestivirus* glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J. Virol. 64, 3563–3569.

Weiland, F., Weiland, E., Unger, G., Saalmuller, A., Thiel, H.J., 1999. Localization of pestiviral envelope proteins E(rns) and E2 at the cell surface and on isolated particles. J. Gen. Virol. 80, 1157–1165.

Zsak, L., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L., 1996. An African swine fever virus virulence-associated gene NL-S with similarity to the herpes simplex virus ICP34.5 gene. J. Virol. 70, 8865–8871.