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Identification of a novel virulence determinant within the E2 structural glycoprotein of classical swine fever virus

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Abstract

Classical swine fever virus (CSFV) E2 glycoprotein contains a discrete epitope (TAVSPTTLR, residues 829–837 of CSFV polyprotein) recognized by monoclonal antibody (mAb) WH303, used to differentiate CSFV from related ruminant pestiviruses, *Bovine Viral Diarrhea Virus* (BVDV) and *Border Disease Virus* (BDV), that infect swine without causing disease. Progressive mutations were introduced into mAb WH303 epitope in CSFV virulent strain Brescia (BICv) to obtain the homologous amino acid sequence of BVDV strain NADL E2 (T*SFNMD*TL*A*). *In vitro* growth of mutants T1v (T*SF*SPTTLR), T2v (T*SFN*PTTLR), T3v (T*SFNM*TTLR) was similar to parental BICv, while mutants T4v (T*SFNMD*TLR) and T5v (T*SFNMD*TL*A*) exhibited a 10-fold decrease in virus yield and reduced plaque size. *In vivo*, T1v, T2v or T3v induced lethal disease, T4v induced mild and transient disease and T5v induced mild clinical signs. Protection against BICv challenge was observed at 3 and 21 days post-T5v infection. These results indicate that E2 residues TAVSPTTLR play a significant role in CSFV virulence.

Keywords: Virulence; Pathogenesis; Attenuation; Vaccine; Protection; Glycoproteins; Classical swine fever virus

Introduction

Classical swine fever (CSF) is a highly contagious disease of swine that can be either acute or chronic in nature (Van Oirschot, 1986). 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* (Francki et al., 1991). The 12.5 kb CSFV genome contains a single open reading frame which encodes a 4000-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 (Rice, 1996). 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 by their carboxyl termini and E^{rns} loosely associated with the viral envelope (Thiel et al., 1991; Weiland et al., 1990, 1999).

Virulence and host range phenotypes vary among CSFV isolates and between pestiviruses. Infection with highly virulent CSFV strains leads to death in infected animals, whereas isolates of moderate to low virulence induce a prolonged chronic disease (Van Oirschot, 1986). In addition, BVDV and BDV, while etiologic agents of diseases in bovine and ovine species, respectively, can also infect swine without inducing clinical disease (Van Oirschot, 1986). Despite availability of genomic sequences from CSFV of differing virulence

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phenotypes, BVDV and BDV, the genetic basis of CSFV virulence in the natural host remains poorly understood (Van Oirschot, 1986). The use of reverse genetics has enabled the identification of viral determinants of virulence, facilitating development of candidate live attenuated CSF vaccines (Mayer et al., 2004, 1999; Moormann et al., 1996; Moser et al., 2001; Risatti et al., 2005a, 2005b; Ruggli et al., 1996; Tratschin et al., 1998; van Gennip et al., 2000). Notably, all three viral glycoproteins have been associated with CSFV virulence (Meyers et al., 1999; Risatti et al., 2005a, 2005b; van Gennip et al., 2004). E^{rns} mutants of strain Alfort/187 exhibited different degrees of attenuation when inoculated in swine (Meyers et al., 1999), and a 19 nucleotide insertion in the carboxyl-end of the E1 gene caused complete attenuation of the highly virulent strain Brescia (Risatti et al., 2005b). Recently, the E2 gene from vaccine strain CS was shown to attenuate chimeric CSFV containing a Brescia strain genetic background (Risatti et al., 2005a). Further analysis of that chimeric virus mapped the residues responsible for the attenuation at the carboxyl-terminal half of E2 (Risatti et al., unpublished data).

E2 glycoprotein is considered essential for CSFV replication, as virus mutants containing partial or complete deletions of the E2 gene have proven non-viable (van Gennip et al., 2002). E2 has been implicated, along with E^{rns} (Hulst and Moormann, 1997) and E1 (Wang et al., 2004), in viral adsorption to host cells; indeed, chimeric pestiviruses exhibit infectivity and cell tropism phenotypes consistent with those of the E2 gene donor (Liang et al., 2003; van Gennip et al., 2000). E2 is the most immunogenic of the CSFV glycoproteins (Konig et al., 1995; Weiland et al., 1990), inducing neutralizing antibodies and protection against lethal challenge (Hulst et al., 1993). CSFV E2 also contains between amino acid residues 829 and 837 an epitope recognized by monoclonal antibody (mAb) WH303 (Lin et al., 2000), a reagent which fails to react with BVDV or BDV E2 and is routinely used for CSF diagnostics. The amino acid sequence comprising this epitope (TAVSPTTLR) is invariant among known CSFV strains and differs substantially from homologous sequences encoded by other pestiviruses, suggesting that the WH303 epitope may play a significant role during CSFV infection. Here we report the effects of mutations within the WH303 epitope of CSFV E2 made progressively toward the homologous amino acid sequence of BVDV strain NADL, demonstrating an additive effect for viral virulence in swine and complete attenuation after six amino-acid changes. Interestingly, animals infected with virus mutants were protected when challenged with virulent Brescia virus at 3 and 21 days post-vaccination.

Results

Construction of CSFV T1–T5 mutant viruses

Infectious RNA was *in vitro* transcribed from full-length infectious clones (ICs) of CSFV strain Brescia or mutants T1 to T5 and used to transfect SK6 cells (Fig. 1). Virus was rescued from transfected cells by day 4 post-transfection. Nucleotide sequences of the rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at the locus encoding the WH303 epitope were reflected in T1v–T5v.

	CSFV residues								
	829	830	831	832	833	834	835	836	837
Brescia	T	A	V	S	P	T	T	L	R
NADL	T	*S*	*F*	*N*	*M*	*D*	T	L	*A*
T1v	T	*S*	*F*	S	P	T	T	L	R
T2v	T	*S*	*F*	*N*	P	T	T	L	R
T3v	T	*S*	*F*	*N*	*M*	T	T	L	R
T4v	T	*S*	*F*	*N*	*M*	*D*	T	L	R
T5v	T	*S*	*F*	*N*	*M*	*D*	T	L	*A*

Fig. 1. Comparison of CSFV strain Brescia, BVDV strain NADL and CSFV T1–5 mutant viruses in the mAb WH303 epitope region of E2 glycoprotein. Amino acid residue positions in the CSFV polyprotein are indicated. Italics indicate residues in BVDV strain NADL and CSFV T1v to T5v that are different from those in Brescia.

Replication of T1v–T5v in vitro

In vitro growth characteristics of T1v–T5v relative to parental BICv were evaluated in a multistep growth curve (Fig. 2A). SK6 cell cultures were infected at a multiplicity of infection (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. While mutants T1v, T2v, and T3v exhibited growth characteristics practically indistinguishable from BICv, T4v and T5v exhibited a 10-fold decrease in the final virus yield. T4v and T5v also exhibited a noticeable reduction in plaque size relative to BICv, T1v, T2v and T3v (Fig. 2B). Finally, while immunocytochemical reactivity with the mAb WH303 was equivalent for T1v, T2v and BICv, reactivity was partially lost in T3v and completely abolished in T4v and T5v-infected cells (Fig. 2B). These results indicate that mutations of the WH303 epitope affecting the ability of CSFV to replicate *in vitro* have a similar effect on WH303 reactivity.

Mutants T4v and T5v lack determinants necessary for CSFV virulence in swine

To examine the effect progressive WH303 epitope mutation on CSFV virulence in swine, virulence phenotypes of T1v–T5v mutant and BICv wild type viruses were compared in 6 groups of pigs intranasally inoculated with 10^5 $TCID_{50}$ of virus and monitored for clinical disease. Results from this experiment are shown in Table 1 and Figs. 3–5. Identity and stability of WH303 epitope mutations were confirmed by nucleotide sequence analysis of virus recovered from tonsils of T1v–T5v-infected animals at 6 DPI (data not shown). While BICv, as expected, was highly pathogenic, effectively inducing fever, clinical signs and death in swine, T1v–T5v mutants appeared to have virulence phenotypes that were increasingly attenuated (Table 1, Fig. 3). T1v and T2v were also highly pathogenic, inducing fever and death in swine in a manner similar to that of

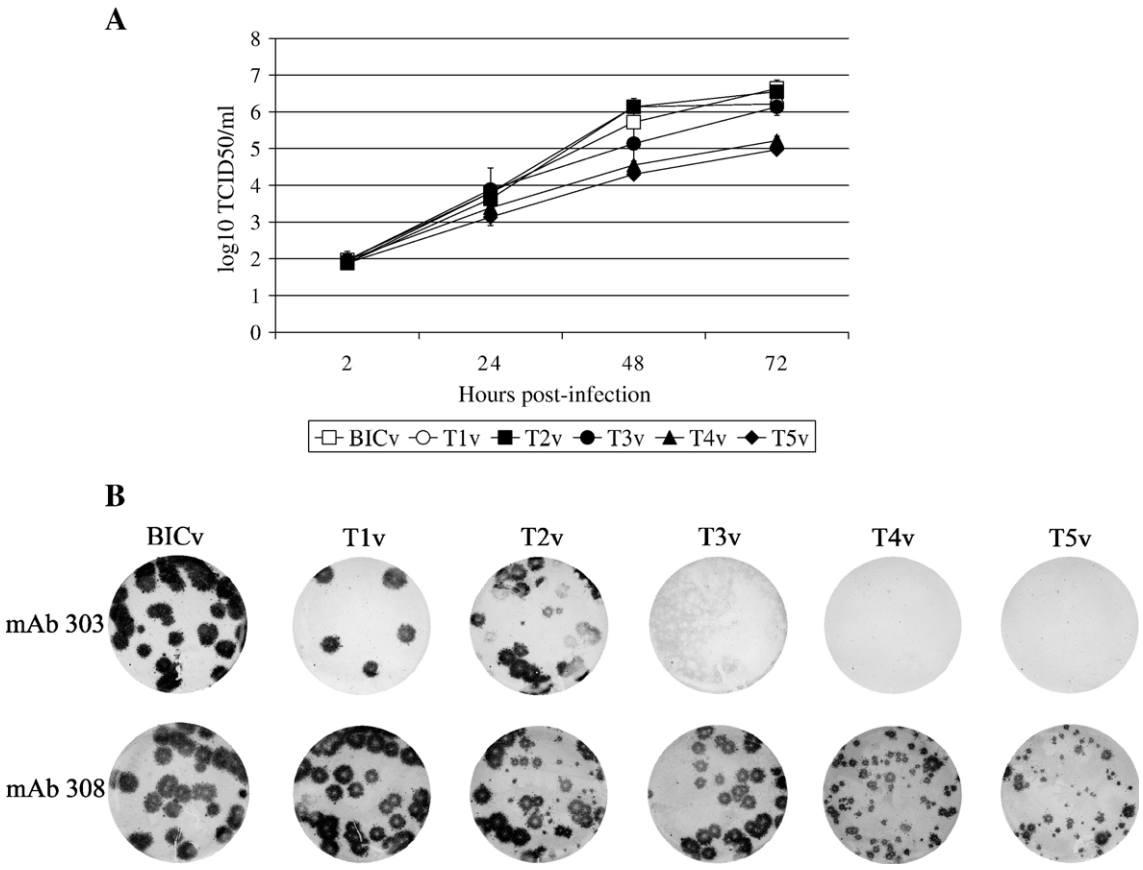

Fig. 2. (A) In vitro growth characteristics of T1v–T5v mutants and BICv. SK6 monolayers were infected (MOI=0.01) with T1v, T2v, T3v, T4v, T5v or BICv and virus yield titrated at times post-infection. Data represent means and standard deviations from two independent experiments. (B) Plaque formation and mAb reactivity of mutants T1v–T5v and BICV. SK6 monolayers were infected with 50 to 100 $TCID_{50}$, overlaid with 0.5% agarose and incubated at 37 °C for 3 days. Plates were fixed with 50% (vol/vol) ethanol–acetone and differentially stained by immunohistochemistry with mAb WH303 and mAb WH308.

BICv (Table 1); however, T2v demonstrated a slight delay in clinical scores relative to BICv (Fig. 3). T3v induced lethal disease but with delayed kinetics relative to BICv, as death occurred 4–8 days later (Table 1). Remarkably, T4v and T5v failed to induce lethal disease, with T4v inducing only a mild and transient clinical disease and T5v inducing mild clinical signs (Table 1, Fig. 3). Similarly, BICv, T1v, T2v and T3v infection by 6 DPI resulted in a drastic reduction in white blood cell (WBC) and platelet counts which remained low until death, while T4v and T5v-infected induced a transient and much less dramatic effect (Fig. 4).

Attenuation of T1v–T5v was also reflected in viremia and virus shedding. While T1v and T2v induced viremic titers comparable to those induced by BICv, T3v titers were reduced by 10^1 to 10^2 $\log_{10}$, and T4v and T5v induced titers 10^3 to 10^4 $\log_{10}$ lower than BICv titers at similar times post-infection (Fig.

Table 1
Swine survival and fever response following infection with T1v–T5v or BICv

Virus	Number of survivors/ total	Mean time to death days (SD)	Mean time of fever onset days (SD)	Mean time duration of fever days (SD)	Max daily temperature average (SD)
T1v	0/2	8.5 (2.1)	3.0 (1.4)	4.5 (2.1)	107.2 (0.0)
T2v	0/2	14.5 (2.1)	4.5 (0.7)	9.5 (0.7)	106.6 (1.7)
T3v	0/2	19.0 (0.0)	5.0 (0.0)	6.0 (1.4)	105.7 (1.6)
T4v	4/4[a]	–	5.0 (0.0)	2.5 (2.1)[b]	106.2 (0.6)
T5v	6/6[c]	–	–	–	104.8 (0.6)
BICv	0/6[c]	12.5 (2.1)	4.5 (0.7)	4.5 (0.7)	105.8 (0.3)

SD: standard deviation.
[a] Two independent experiments with 2 pigs in each experiment.
[b] Two out of 4 animals presented fever.
[c] Includes animals used in protection studies.

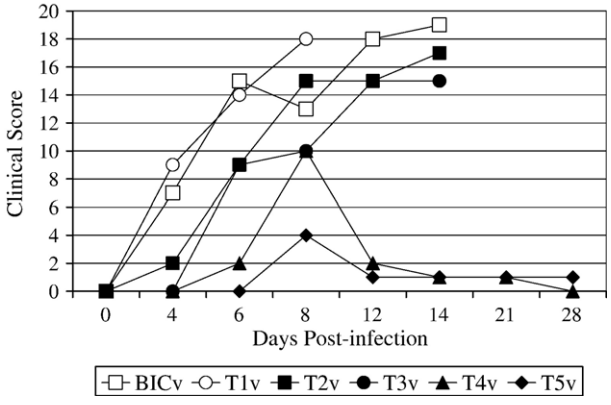

Fig. 3. Clinical scores recorded from pigs infected with recombinant viruses T1v–T5v and BICv. Clinical Scores were calculated as previously described with modifications (see Materials and methods) and were based on observations of two (T1v, T2v, T3v and T4v) or six (T5v and BICv) animals.

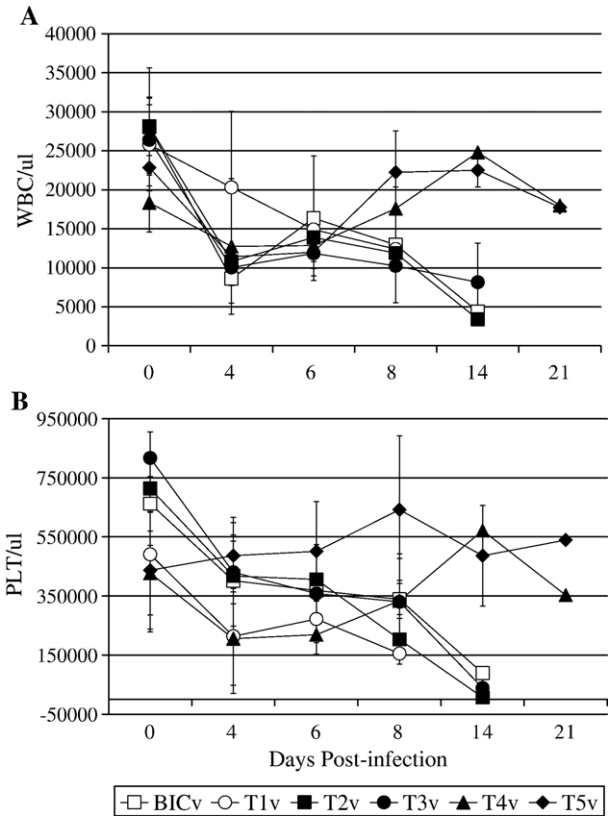

Fig. 4. (A) Peripheral white blood cell and (B) platelet counts in pigs infected with recombinant viruses T1v–T5v and BICv. Counts are expressed as numbers/μl and each point represents the mean and standard errors for two (T1v, T2v, T3v and T4v) or six (T5v and BICv) animals.

5A). A similar pattern was observed for virus titers from nasal swabs and tonsil-scrapings (Figs. 5B and C, respectively), with T3v, T4v and T5v titers falling below detectible levels at later DPI.

For a more detailed study of T5v pathogenesis, T5v and BICv-infected animals were euthanized at 2, 4, 6, 8 and 14 DPI (one animal/time point/group) and virus titers determined for tonsil, submandibular lymph node, spleen, blood and kidney and for nasal swab and tonsil scraping samples (Table 2). T5v exhibited significantly lower levels of virus replication in tonsils (approximately 10^2 to 10^4 $\log_{10}$) relative to BICv. Similar differences between T5v and BICv were observed in virus titers from mandibular lymph node, spleen and kidney (Table 2).

These results indicate that an increasing number of mutations within the WH303 epitope of CSFV E2 had an additive effect in attenuating the virus for swine, with mutations present in T4v and T5v resulting in significant decrease in viral virulence. Furthermore, T5v infection is characterized by a decreased viral replication in tonsils and target tissues, and dramatically reduced virus shedding.

T5v infection protects swine against challenge with pathogenic BICv

The ability of T5v to induce protection against BICv challenge was evaluated. Swine vaccinated with T5v was challenged at 3 or 21 DPI with 10^5 $TCID_{50}$ of pathogenic BICv. Mock vaccinated control pigs receiving no T5v and challenged with BICv developed anorexia, depression and fever by 4 days post-challenge (DPC), developed a marked reduction of circulating leukocytes and platelets by day 7 DPC and had died or were moribund and euthanized by 12 DPC (Table 3).

Notably, T5v induced by 3 DPI complete protection against BICv-induced clinical disease. All pigs survived infection and remained clinically normal, with only two animals presenting with a transient fever at 4 DPC (Table 3) and without significant changes in their hematological values (Fig. 6). Similarly, pigs challenged at 21 days post-T5v infection remained clinically normal (Table 3).

Viremia and virus shedding of BICv challenge virus as specifically detected with mAb WH303 was also examined at 4, 6, 8, 14 and 21 DPC (data not shown). As expected in mock-vaccinated control animals, BICv viremia was observed by

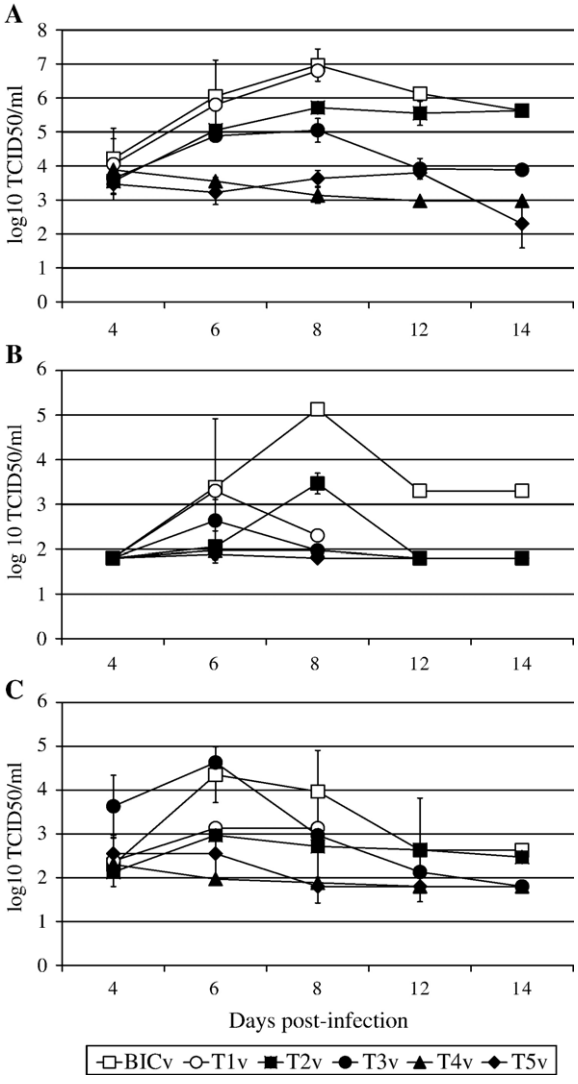

Fig. 5. Virus titers in (A) blood, (B) nasal swabs and (C) tonsil scrapings from pigs infected with T1v–T5v mutants or BICv. Each point represents the mean log10 TCID50/ml and standard deviation for two (T1v, T2v, T3v and T4v) or six (T5v and BICv) animals.

Table 2
Virus titers[a] in clinical samples and tissues obtained from pigs following infection with T5v or BICv

Virus	T5v					BICv				
DPI[b]	2	4	6	8	14	2	4	6	8	14
Nasal swabs	Neg[c]	1.97	Neg	Neg	Neg	Neg	Neg	1.97	4.63	3.97
Tonsil scrapings	Neg	Neg	2.63	Neg	Neg	Neg	Neg	1.97	4.97	2.97
Blood	Neg	ND[d]	2.97	3.97	Neg	Neg	3.13	6.47	6.3	6.80
Tonsil	2.13	2.97	3.30	1.97	2.80	1.97	4.63	5.13	5.47	6.13
Mandibular lymph node	2.80	3.30	3.63	2.47	3.30	1.8	2.80	4.80	5.80	5.97
Spleen	Neg	2.97	2.63	3.47	2.80	Neg	2.13	2.80	5.63	6.30
Kidney	Neg	2.47	2.977	3.13	2.13	Neg	Neg	2.80	4.80	5.97

[a] Titers expressed as log 10 TCID50/ml.
[b] DPI, days post-infection.
[c] Neg: ≤1.80 log 10 TICD50/ml.
[d] ND: not determined.

5 DPC, with virus titers remaining high (10^6 TCID50/ml by 8 DPC) until death, and BICv was titrated from nasal swabs and tonsil scrapings by 4 DPC, reaching titers of 10^4–10^5 TCID50/ml) by 8 DPC. In contrast, BICv was absent in all clinical samples (blood, nasal swabs or tonsil scrapings) from T5v-vaccinated swine following challenge. These results indicate that T5v is able to rapidly induce complete protection against lethal CSFV challenge, that T5v-immune swine demonstrate no detectible viremia or shedding from challenge virus and that T5v can be differentiated from wild-type virus via absence of the WH303 epitope.

Discussion

Here, we have shown that mutations introduced into the CSFV E2 glycoprotein at the CSFV-specific, mAb WH303 epitope resulted in attenuation of highly pathogenic Brescia strain. Amino acids of the CSFV WH303 epitope (TAVSPTTLR) were progressively changed to resemble homologous residues (TSFNMDTLA) of the BVDV strain NADL E2 glycoprotein. Interestingly, mutant viruses encoding T***SFNMD***TLR (T4v) or T***SFNMD***TL***A*** (T5v) sequences lack reactivity to mAb WH303, show small plaque morphology and are significantly attenuated *in vivo*. Unlike the acute fatal disease induced by BICv, T4v and T5v infections were subclinical in swine and characterized by decreased viral replication in target organs and reduced virus shedding.

Table 3
Swine survival and fever response of T5v-infected animals following challenge with BICv

Challenge DPI[a]	Number of survivors/ total	Mean time to death days (SD)	Mean time of fever onset days (SD)	Mean time of fever duration days (SD)	Average max daily temperature (SD)
3	4/4	–	4 (0.8)[b]	1 (0.0)	103.1 (0.6)
21	4/4	–	–	–	102.9 (0.3)
Control[c]	0/4	12 (0.0)	4 (0.0)	5 (0.7)	105.9 (0.7)

SD: standard deviation.
[a] Days post-infection with T5v.
[b] Two out of 4 animals presented fever.
[c] Control: animals were mock vaccinated.

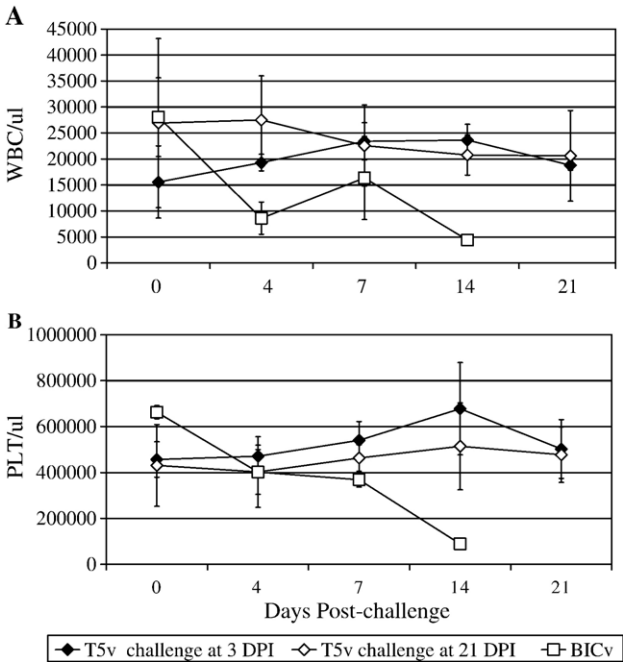

Fig. 6. (A) Peripheral white blood cell and (B) platelet counts in pigs mock vaccinated or vaccinated with T5v and challenged at 3 or 21 DPI with BICv. Values for control, mock vaccinated and challenged animals, are represented with empty squares. Counts are expressed as numbers/μl and represent the mean of four individuals with error bars indicating standard error.

In general, the genetic bases and the molecular mechanisms underlying CSFV virulence remain obscure, with few studies associating specific viral proteins or genomic region with virulence. Single or double codon mutation of E^{rns} that abrogated its RNase activity resulted in attenuation of CSFV strain Alfort in swine and, similarly, mutation of the E^{rns} RNase domain of BVDV attenuated the virus in cattle (Meyers et al., 1999, 2002). Complete deletion of N^{pro} from virulent CSFV strains Alfort/187 and Eystrup attenuated these viruses in swine (Mayer et al., 2004), as did an in frame insertion of 19 amino acids into the E1 gene of CSFV strain Brescia (Risatti et al., 2005b). Replacement of the E2 gene in CSFV strain Brescia with that of vaccine strain CS resulted in a chimeric virus that has a significant *in vivo* attenuation, including significantly decreased virus replication in tonsils, a transient viremia, limited generalization of infection and decreased virus shedding (Risatti et al., 2005a).

Findings presented here are the first to identify a discrete amino acid sequence within E2 as a specific determinant of CSFV virulence. A L710H substitution in E2 was previously shown to affect CSFV virulence in swine; however, the attenuated phenotype also required concomitant mutations in E^{rns} (van Gennip et al., 2004). Comparative sequence analysis previously indicated that Brescia and CS strain E2 proteins differed by 22 amino acids (Risatti et al., 2005a). Notably, the mAb WH303 epitope identified here as a CSFV strain Brescia virulence determinant was identical in strain CS, consistent with strong reactivity of these viruses with mAb WH303 and

suggestive of a second, WH303-independent genetic determinant associated with attenuation of Brescia/CS E2 chimeras (Risatti et al., 2005a). Indeed, such a determinant is currently being mapped in the carboxyl terminal half of E2, downstream of mAb WH303 epitope (Risatti et al., unpublished data).

Notable is the association of the novel CSFV virulence determinant found here with the mAb WH303 epitope. Recent studies support the target epitope of mAb WH303 as an immunodominant feature of CSFV. Neutralizing monoclonal antibodies which bind the SPTxL motif within the WH303 epitope (SPTTL) were independently generated using a phage-displayed random peptide library of CSFV E2 and E^{rns} (Zhang et al., 2006). The mAb WH303 is also highly specific for CSFV, strongly reactive with all known strains and failing to react with E2 encoded by other pestiviruses (Edwards et al., 1991). Consistent with this reactivity, amino acid sequences mapped to the mAb WH303 epitope are invariant among sequenced strains of CSFV and divergent from homologous sequences encoded by BVDV and BDV (data not shown). Such conservation in epitope sequence and reactivity bolsters rationale for WH303 use in diagnostics, but conversely suggests that engineering the epitope to serve as a negative marker could aid in rational design of a CSF vaccine. Epitope conservation also suggests that the CSFV-specific sequences in the WH303 epitope of E2, identified here as a CSFV virulence determinant, involve a mechanism specific and critical for CSFV infection.

Although the mechanism by which the WH303 epitope affects virulence of CSFV is unknown, attenuating mutations in the E2 glycoprotein conceivably involve aspects of virus attachment and/or efficient entry into critical target cells *in vivo*. Anchored to the virion envelope, pestivirus E2 appears as both homo and heterodimers linked by disulfide bridges (Thiel et al., 1991; Wang et al., 2004; Weiland et al., 1990) and, along with E^{rns} (Hulst and Moormann, 1997) and E1 (Wang et al., 2004), has been shown to be important for virus-receptor binding and host-cell specificity. The bovine pathogen BVDV engineered to express the complete E2 gene of BDV, a sheep pestivirus, retained its ability to form plaques in sheep cell cultures but lost its ability to form plaques in bovine kidney (MDBK) cells, a phenotype associated with 100-fold decrease in virus yield and not complete loss of replication by the BVDV/BDV E2 chimera in MDBK (Liang et al., 2003). Similarly, BVDV strain CP7 engineered to express the complete E2 gene of CSFV strain Alfort 187 acquired the ability to propagate efficiently in porcine cells but not in bovine cells (Reimann et al., 2004). On the other hand, CSFV strain C engineered to express homologous BVDV sequences in the amino terminus of E2 demonstrated a 10-fold decrease in virus progeny yield in swine kidney (SK6) cells. While this impaired growth in SK6 cells resembled wild-type BVDV, the amino-terminal region of BVDV E2 failed to impart on the CSFV/BVDV E2 chimera the BVDV-like ability to infect fetal bovine epithelial cells (van Gennip et al., 2000). This observation is consistent with results presented here: CSFV engineered to express BVDV-like sequences at the WH303 epitope in T4v and T5v mutants demonstrated a 10-fold reduction in viral progeny yield in SK6 cells relative to BICv, T1v, T2v and T3v (Fig. 2A) but lacked the ability to replicate efficiently in bovine kidney cells (MDBK) (data not shown). Thus, while BVDV-like residues contained in T4v and T5v are sufficient to affect replication of CSFV in SK6 cells and attenuate the virus *in vivo*, they are insufficient in imparting a wild-type BVDV host-range phenotype *in vitro*.

Also suggestive of a role for E2 mutations in virus attachment and/or entry was the small plaque phenotype exhibited by T4v and T5v, in which mAb WH303 epitope mutations imparted a plaque size reduction relative to parental BICv in SK6 cells (Fig. 2B). Although an association between *in vitro* plaque size reduction and *in vivo* attenuation of CSFV has yet to be firmly established, certain observations suggest a relationship. While serially passed, heparan sulfate binding-dependent variants of CSFV strain Brescia found to produce reduced plaque size and contain a single amino acid mutation in the E^{rns} protein remained virulent in pigs (Hulst et al., 2000, 2001), other Brescia-derived viruses producing reduced plaque size and containing recombinant mutations in E1 (Risatti et al., 2005b) and E2 (Risatti et al., 2005a) were attenuated in swine. Finally, BVDV strain NADL demonstrated a small plaque phenotype in SK6 cells similar to that of T4v and T5v (data not shown), again suggesting that BVDV-like mutations in the WH303 epitope impart to CSFV similar capabilities for attachment and/or spreading *in vitro* and inability to produce disease *in vivo*.

In summary, a novel CSFV genetic virulence determinant associated with the WH303 mAb epitope within the E2 glycoprotein has been identified. Interestingly, progressively added mutations within the epitope correlated with incremental loss of mAb WH303 reactivity and virulence *in vivo*, indicating that normally invariant sequence within the mAb WH303 epitope is critical for CSFV virulence in swine. Although the mechanism of attenuation remains unknown, the complete attenuation of CSFV mutants containing BVDV-like sequence within the epitope also suggested a link between the lack of the CSFV epitope sequence and the inability of other pestiviruses (BVDV and BDV) to induce disease in swine. Improving the understanding of the genetic basis of CSFV virulence will permit rational design of live attenuated CSF vaccines of enhanced safety, efficacy and utility. Specifically, engineering of the WH303 epitope as both a means of viral attenuation and means to remove WH303 reactivity may ultimately prove useful in rational design of attenuated marker CSF vaccines.

Materials and methods

Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were cultured in Dulbecco's minimal essential medium (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 (Risatti et al., 2005a). 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 immunoperoxidase assay using the CSFV monoclonal antibodies WH303 or WH308 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) (Risatti et al., 2003). Titers were calculated using the method of Reed and Muench (1938) and expressed as TCID50/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 TCID50/ml.

Construction of CSFV infectious clones (ICs) T1–5

A full-length IC of the virulent Brescia isolate (pBIC) (Risatti et al., 2005a) was used as a template in which six residues of the WH303 epitope (TAVSPTTLR) between residues 829 to 837 of E2 were mutated to reflect those of homologous residues present in the BVDV isolate NADL (T*SFNMD*TL*A*) (Lin et al., 2000). Mutations were added progressively, yielding five IC for rescue of the following viral mutants: T1v (T*SF*SPTTLR), T2v (T*SFN*PTTLR), T3v (T*SFNM*TTLR), T4v (T*SFNMD*TLR) and T5v (T*SFNMD*TL*A*) (Fig. 1). Mutations 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 primers described in Table 4.

In vitro rescue of CSFV brescia and T1v–T5v mutant viruses

Full-length genomic clones were linearized with *Srf*I 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 ohms and 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were plated in 12 well plates and 25 cm^2 flasks and incubated for 4 days at 37 °C and 5% CO_2. Virus was detected by immunoperoxidase staining 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 area. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730×l automated DNA Sequencer (Applied Biosystems). 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).

Animal infections

Each of the T1v–T5v mutants was initially screened for its virulence phenotype in swine relative to virulent Brescia virus. Swine used in all animal studies here were 10 to 12 weeks old, 40-pound commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type virus. For screening, 12 pigs were randomly allocated into 6 groups of 2 animals each, and pigs in each group were inoculated with one of the T1v–T5v mutants or BICv. Pigs kept in isolation rooms were observed throughout the experiment and clinical scores were recorded based on a scoring system described by Mittelholzer et al. (2000) with modifications. Briefly, observed clinical parameters include liveliness, body tension, body shape, gait, skin condition, eyes condition, appetite, defecation and food leftovers. Scores varied from normal=0 to severe CSF symptoms=3. Scores for all clinical parameters were added and plotted. Body temperature variations were assessed daily, with values above 104 °F considered as fever.

To assess the effect of T5v mutations on virus shedding and distribution in different organs during infection, 10 pigs were randomly allocated into 2 groups of 5 animals each and inoculated with T5v or BICv. One pig per group was sacrificed at 2, 4, 6, 8 and 14 DPI. Blood, nasal swabs and tonsil scraping samples were obtained from pigs at necropsy. Tissue samples (tonsil, mandibular lymph node, spleen and kidney) were snap-frozen in liquid nitrogen for virus titration.

For protection studies, 12 pigs were randomly allocated into 3 groups of 4 animals each. Pigs in groups 1 and 2 were inoculated with T5v, animals in group 3 were mock-infected. At 3 DPI (group 1) or 21 DPI (group 2), animals were challenged with BICv along with animals in group 3. 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 after challenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer) for total and differential white blood cell counts. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

Table 4
Primers used to construct T1–T5 viruses

Primer[a]	Sequence
T1 F	5′ GGGTGTTATAGAGTGCACGTCATTTAGCCCGACAACTCTGAGAAC 3′
T2 F	5′ GGGTGTTATAGAGTGCACGTCATTTAATCCGACAACTCTGAGAAC 3′
T3 F	5′ GGGTGTTATAGAGTGCACGTCATTTAATATGACAACTCTGAGAAC 3′
T4 F	5′ GAGTGCACGTCATTTAATATGGACACTCTGAGAACAGAAGTGGTA 3′
T5 F	5′ TCATTTAATATGGACACTCTGGCAACAGAAGTGGTAAAGACCTTC 3′

[a] Only forward primer sequences are provided. Reverse primers correspond to the complementary sequence.

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