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Mutations in the carboxyl terminal region of E2 glycoprotein of classical swine fever virus are responsible for viral attenuation in swine

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Abstract

We have previously reported [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] that chimeric virus 319.1v containing the *E2* glycoprotein gene from Classical Swine Fever Virus (CSFV) vaccine strain CS with the genetic background of highly virulent CSFV strain Brescia (BICv) was markedly attenuated in pigs. To identify the amino acids mediating 319.1v attenuation a series of chimeric viruses containing CS E2 residues in the context of the Brescia strain were constructed. Chimera 357v, containing CS E2 residues 691 to 881 of CSFV polyprotein was virulent, while chimera 358v, containing CS E2 residues 882 to 1064, differing in thirteen amino acids from BICv, was attenuated in swine. Single or double substitutions of those amino acids in BICv E2 to CS E2 residues did not affect virulence. Groups of amino acids were then substituted in BICv E2 to CS E2 residues. Mutant 32v, with six substitutions between residues 975 and 1059, and mutant 33v, with six substitutions between 955 and 994, induced disease indistinguishable from BICv. Mutant 31v, with seven substitutions between residues 882 and 958, induced a delayed onset of lethal disease. Amino acids abrogating BICv virulence were then determined by progressively introducing six CS residues into 31v. Mutant 39v, containing nine residue substitutions, was virulent. Mutant 40v, containing ten residue substitutions, induced mild disease. Mutant 42v, containing twelve substitutions, and mutant 43v, with an amino acid composition identical to 358v, were attenuated in swine indicating that all substitutions were necessary for attenuation of the highly virulent strain Brescia. Importantly, 358v protected swine from challenge with virulent BICv at 3 and 28 days post-infection.
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Introduction

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine, presenting as either an acute or chronic infection characterized by fever and hemorrhage (van Oirschot, 1986). Disease outbreaks occur intermittently in Europe and other parts of the world, resulting in significant economic losses.

Classical swine fever virus (CSFV), the causative agent of the disease, is a member of the *Pestivirus* genus of the *Flaviviridae* family (Becher et al., 2003). CSFV is a small, enveloped virus with a single-stranded 12.3 kb RNA genome of positive polarity that contains a long open reading frame encoding a 3898-amino-acid polyprotein. Cellular and viral proteases are used for co- and post-translational processing of eleven to twelve final cleavage products (Rice, 1996). Virus particles contain four structural proteins: the core protein C and the envelope glycoproteins E^{rns}, E1 and E2 (Thiel et al., 1991). E1 and E2 are anchored to the envelope by their carboxy termini, with E^{rns} loosely associated with the envelope. E^{rns} and E2 are present as homodimers linked by disulfide bridges on the surface of CSFV virions, whereas E2

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is found dimerized with E1 (Thiel et al., 1991; Weiland et al., 1990, 1999).

CSFV can cause severe disease in pigs, characterized by high morbidity and mortality (van Oirschot, 1986). Infection with highly virulent CSFV strains generally leads to death of infected animals, whereas isolates of moderate to low virulence induce a prolonged chronic disease (van Oirschot, 1986). Development of infectious CSFV cDNA clones has permitted genetic approaches for exploration of mechanisms involved with viral replication and pathogenesis. Infectious clones (IC) of attenuated and pathogenic strains have been constructed and used to identify viral proteins or protein domains functioning in viral replication and virulence, and to engineer live-attenuated CSF marker vaccines (Mayer et al., 2004; Meyers et al., 1999; Moormann et al., 1996; Moser et al., 2001; Risatti et al., 2005a, 2005b, 2006, 2007; Ruggli et al., 1996; Tratschin et al., 1998; van Gennip et al., 2000, 2002; Widjojoatmodjo et al., 2000).

E^{rns} has been shown to function in viral virulence. Depending on the extent of the engineered mutation, specific Alfort/187 E^{rns} mutants exhibited different degrees of attenuation when inoculated in swine, indicating a role for E^{rns} in viral pathogenesis (Meyers et al., 1999). N^{pro} also has been shown to be involved in virulence (Mayer et al., 2004). The deletion of N^{pro} in the Alfort/187 and Eystrup strains abrogates their virulence for swine. Recently, it has been demonstrated that E1 plays a role in CSFV virulence. A 19-nucleotide insertion in the carboxyl-end of the *E1* gene resulted in complete attenuation of the highly virulent strain Brescia (Risatti et al., 2005b).

E2, the most immunogenic of the CSFV glycoproteins, induces a protective immune response in swine (Hulst et al., 1993; Konig et al., 1995; van Zijl et al., 1991). E2 is an essential protein for CSFV since virus mutants containing partial or complete deletions of the *E2* gene were nonviable (van Gennip et al., 2002). E2 has been implicated, along with E^{rns} and E1, in viral adsorption to the cell (Hulst and Moormann, 1997; Wang et al., 2004). Additionally, experiments using chimeric viruses containing partial or complete replacement of the *E2* gene by homologous genomic regions of other pestiviruses exhibited changes in their infectivity efficacy and/or cell tropism consistent with that of the donor virus (Liang et al., 2003; van Gennip et al., 2000).

Previously, we have shown that chimeric virus 319.1v (Risatti et al., 2005a) containing the E2 glycoprotein of vaccine strain CS in the genetic background of highly virulent strain Brescia (BICv) was completely attenuated in pigs. Here we have mapped amino acid residues responsible for mediating that attenuation. A series of mutant viruses were constructed based on the genetic background of BICv and tested for their virulence in swine. Individual or groups of amino acids were substituted in BICv E2 glycoprotein, between amino acid positions 691 and 1064 of CSFV polyprotein, to residues located at similar positions in vaccine strain CS. Results indicate that attenuation of chimeric virus 319.1v requires the presence of at least 12-amino-acid substitutions located near the carboxyl terminus of E2 glycoprotein, between residues 882 and 1032 of the CSFV polyprotein. Additionally, attenuated viruses induced solid protection against challenge with virulent CSFV at 3 and 28 days post-infection.

Results

The carboxyl terminal region of CS E2 glycoprotein in chimera 319.1v is responsible for viral attenuation

Chimeric virus 319.1v, containing the E2 glycoprotein of vaccine strain CS within the genetic background of the highly virulent strain Brescia (BICv), was markedly attenuated in pigs, exhibiting significantly decreased virus replication during infection and decreased virus shedding (Risatti et al., 2005a). To further define the amino acid residues mediating attenuation, two chimeric viruses, 357v and 358v, were constructed. Chimera 357v contains CS residues 691 to 881 of the CSFV polyprotein, while chimera 358v contains CS residues 882 to 1064; within the genetic background of virulent BICv (Fig. 1A).

Growth kinetics of 357v, 358v and BICv were compared in a multistep growth curve. Primary porcine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time 0) and samples were collected at different time points after infection. Replication kinetics of 357v and 358v were comparable to parental virus BICv (Fig. 2A). However, 358v showed an impaired capability to spread in SK6 cells, similar to the CSICv plaque phenotype. 358v plaque size was significantly reduced when compared to chimera 357v and parental BICv plaque phenotypes (Fig. 6A). Accordingly, parental virus 319 also presented with a reduced plaque size (Risatti et al., 2005a).

Virulence phenotype of 357v and 358v was assessed in swine. Groups of 4 pigs were intranasally inoculated with 10^5 $TCID_{50}$ of each virus. Notably, the 358v-infected group survived infection, remaining clinically normal throughout the observation period, whereas the 357v-infected group showed clinical signs of disease compatible to those observed for BICv (Risatti et al., 2005a)-infected animals (Table 1). White blood cell (WBC), lymphocyte and platelet counts dropped drastically by 6 DPI in 357v-infected animals, and remained low until death, while similar counts dropped transiently in 358v-infected animals (Fig. 3). Viremia in 358v-infected animals was transient (6 DPI) and significantly reduced by 10^4 to 10^5 $\log_{10}$ from titers observed for 357v-infected animals (Fig. 3D). A similar pattern was observed for nasal swab and tonsil scraping samples (Fig. 3E and F). Thus, the carboxyl terminal region of CS E2 glycoprotein (358v) encompassing 182 amino acids and differing from BICv in 13 residues (Fig. 1B) is responsible for complete attenuation of CSFV strain Brescia.

Multiple E2 amino acid residues are associated with 358v-attenuated phenotype

Comparative sequence analysis of Brescia and CS E2 between CSFV polyprotein residues 882 and 1064 reveals no deletions or insertions and 7% amino acids divergence including 7 non-conserved substitutions (T886M, P889L, S927L, D958G, A975E, R979S, and L1059P), 3 semi-conserved substitutions (T928A, H955R, and A988T) and 3 conserved substitutions (Q892R, R994K, and I1032V). The role of these non-conserved and semi-conserved amino acid substitutions in BICv attenua-

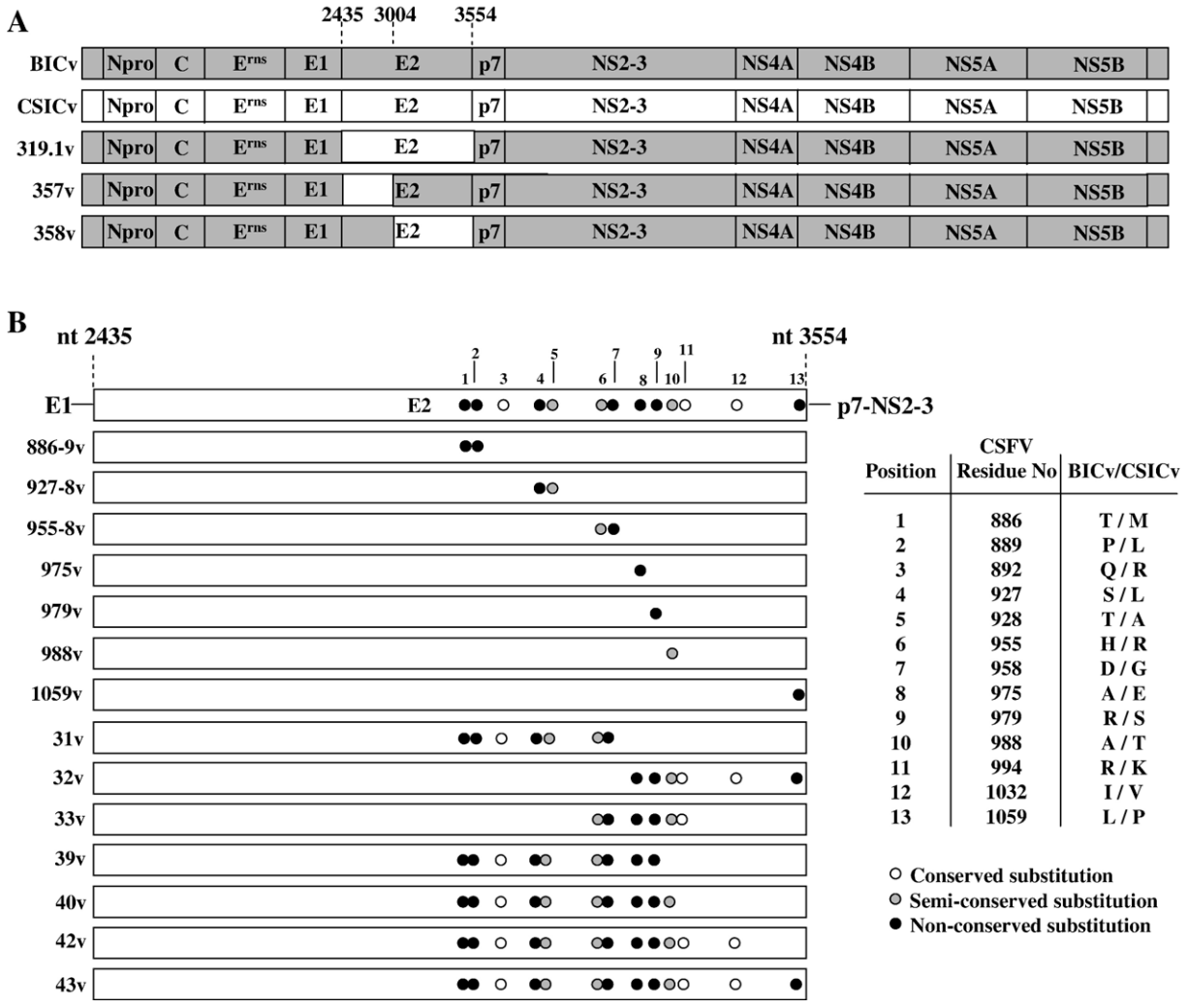

Fig. 1. Construction of CSFV chimeric viruses. (A) cDNA for viruses 357 and 358. (B) cDNA for chimeric viruses 886/9, 927/8, 955/8, 975, 979, 988, 1059, 31, 32, 33, 39, 40, 42 and 43. Corresponding nucleotide numbers are depicted. Substituted amino acid residues in the carboxyl terminus of E2 between Brescia and CS strains are depicted.

tion was assessed. Replication kinetics and virus yields of seven viruses containing single or double non-conserved or semi-conserved substitutions (Fig. 1B), were comparable to chimera 358v in multistep growth curves in primary porcine macrophage cell cultures (Fig. 2B). *In vivo*, groups of pigs ($n=2$) were intranasally inoculated with 10^5 $TCID_{50}$ of each of these seven mutant viruses (Fig. 1B). All seven viruses induced disease in swine indistinguishable from that induced by Brescia virus, with animals dying by 11 DPI (Table 1). WBC, lymphocyte and platelets counts dropped drastically by 6 DPI in all infected animals, and remained low until death (Fig. 4). Virus titers in blood, nasal swabs and tonsil scraping samples were high (Fig. 5), resembling those observed with BICv- or 357v-infected animals.

Mapping amino acid residues inducing attenuation in 358v

To further define relevant E2 residues inducing attenuation of 358v. Three additional chimeric viruses (31v, 32v, 33v) were constructed by introducing all 13 CS E2 substitutions grouped in three partially overlapping regions: 31v (T886M, P889L, Q892R, S927L, T928A, H955R, and D958G), 32v (A975E, R979S, A988T, R994K, I1032V, and L1059P) and 33v (H955R, D958G, A975E, R979S, A988T, and R994K) (Fig. 1B) into BICv. Replication kinetics and virus yields for 31v, 32v, 33v, and 358v in primary swine macrophage cultures were similar (Fig. 2C). Interestingly, 31v-infected swine presented a delayed onset of lethal disease (Table 1), unlike swine infected with 32v and 33v that manifested a disease pattern similar to 357v. This indicates that 7-amino-acid substitutions in 31v (T886M, P889L, Q892R, S927L, T928A, H955R, and D958G), located between CSFV polyprotein residues 886 and 958, while not sufficient to completely attenuate BICv significantly affected disease course. Despite the observed delayed phenotype, a drastic drop in blood cells counts was observed for all groups from days 4 to 6 DPI, remaining low until death (Fig. 4), with high virus titers in blood, nasal, and tonsil scraping samples in all infected animals until death (Fig. 5).

Determination of CS amino acid residues required to attenuate BICv was accomplished by constructing 4 additional

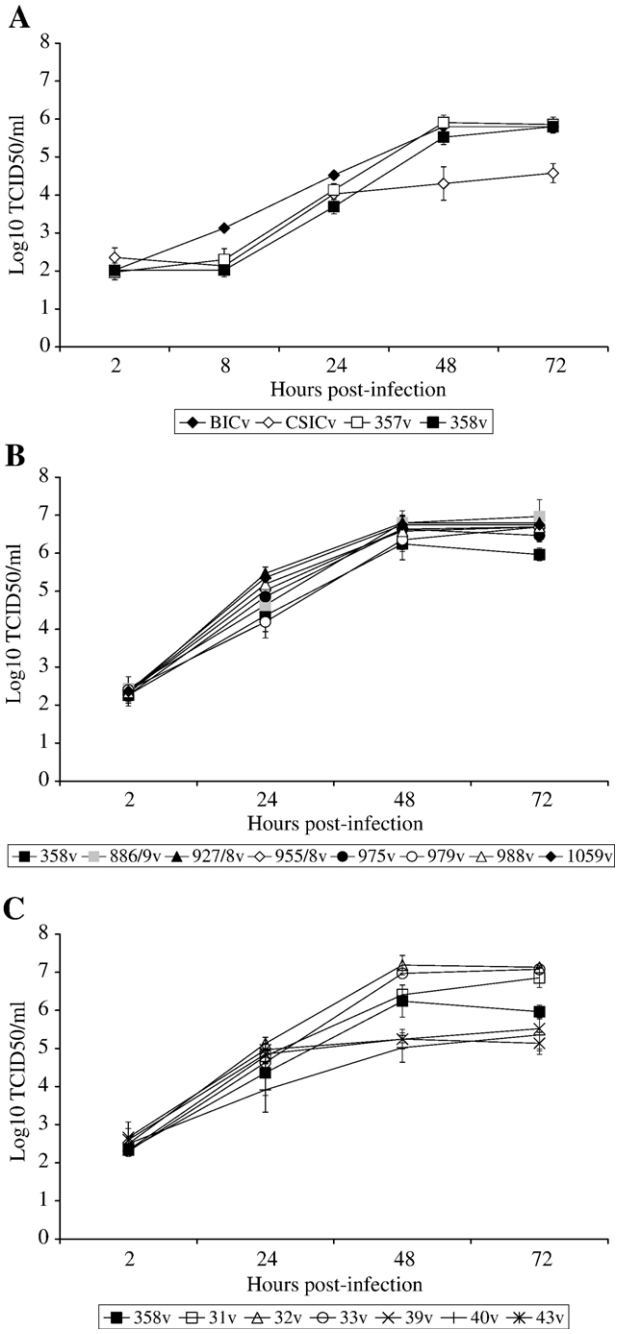

Fig. 2. Growth characteristics of chimeric viruses on swine macrophage cell cultures. (A) CSFV BICv, CSICv and chimeras 357v and 358v. (B) Chimeric viruses 886/9, 927/8, 955/8, 975, 979, 988 and 1059. (C) Chimeric viruses 31, 32, 33, 39, 40, 42 and 43. Primary swine macrophage cell cultures were infected (MOI of 0.1). At times post infection, samples were collected and titrated on SK6 cells for virus yield. Data are means and standard deviation of three independent experiments.

chimeric viruses (39v, 40v, 42v, and 43v). Six-amino-acid substitutions (A975E, R979S, A988T, R994K, I1032V, and L1059P) were progressively introduced into 31v (Fig. 1B): 39v (T886M, P889L, Q892R, S927L, T928A, H955R, D958G, A975E, R979S), 40v (T886M, P889L, Q892R, S927L, T928A, H955R, D958G, A975E, R979S, A988T), 42v (T886M, P889L, Q892R, S927L, T928A, H955R, D958G, A975E, R979S, A988T, R994K, I1032V), and 43v (T886M, P889L, Q892R, S927L, T928A, H955R, D958G, A975E, R979S, A988T, R994K, I1032V, L1059P). Replication kinetics and final virus yields of 39v, 40v, 42v, and 43v, in primary swine macrophage cell cultures, were similar to attenuated chimera 358v (Fig. 2C). *In vivo*, a pattern of progressive attenuation of BICv was observed with these mutants, extending from delayed onset of lethal disease in 39v-infected pigs, mild disease induced by 40v, with animals surviving infection, to complete attenuation observed in animals infected with 42v and 43v. Blood cell counts dropped by 6 DPI in 39v and 40v-infected animals, remaining low until death (39v) or the end of the experimental period (40v), while in swine infected with 42v, and 43v counts dropped by 6 DPI, but recovered to normal levels toward the end of the observation period (Fig. 4). Viremia and virus shedding in 39v- and 40v-infected swine resembled that observed for the virulent chimera 357v. Virus titers in blood and the oronasal cavity of 42v- and 43v-infected swine resembled those observed for the attenuated chimera 358v (Fig. 5).

Our data indicate that virulence attenuation observed with chimera 358v is mediated by 12 substitutions (T886M, P889L, Q892R, S927L, T928A, H955R, D958G, A975E, R979S, A988T, R994K, and I1032V), including 6 non-conserved substitutions and a conserved one (I1032V) near the E2 carboxyl-terminal transmembrane domain.

Comparative analysis in the secondary structure of BICv and 358.1v

The prediction shows that the secondary structure of BICv E2 glycoprotein is composed of a random coil (~51%), an extended strand (~32%), and an alpha helix (~15%). The rest of the

Table 1
Swine survival, and fever response following infection with CSF chimeric viruses

Virus	Number of survivors/total [a]	Mean time to death, days (±SD)	Fever		
			Days to onset (±SD)	Duration, no. of days (±SD)	Max temperature, °F (±SD)
357v	0/4	11.2 (1.5)	6.25 (0.5)	6.5 (1.5)	105.2 (1.4)
358v	4/4	–	–	–	103.1 (0.7)
886/9v	0/2	11 (0)	3.5 (0.7)	6.5 (0.7)	106.4 (0.1)
927/8v	0/2	11.5 (0.7)	3 (0)	6 (1.4)	105.3 (0.4)
955/8v	0/2	11.5 (0.7)	3.5 (0.7)	7.5 (0.7)	105.8 (0.2)
975v	0/2	11 (4.2)	3 (0)	7 (2.8)	107.4 (0)
979v	0/2	11.5 (0.7)	3 (0)	7 (1.4)	106.4 (0.4)
988v	0/2	9 (1.4)	4 (0)	4 (1.4)	106.6 (0)
1059v	0/2	11 (0)	4 (0)	5.5 (1.2)	105.7 (0.9)
31v	0/2	16 (1.4)	5 (0)	8.5 (3.5)	106.9 (0.7)
32v	0/2	8.5 (0.7)	4.5 (0.7)	5.5 (0.7)	105.6 (0.2)
33v	0/2	9 (0)	3 (0)	7 (0)	107.7 (0.1)
39v	0/2	14 (1.4)	5.5 (0.7)	5.5 (0.7)	106.5 (0.2)
40v	2/2	–	8.5 (4.9)	10 (7)	105.9 (1.5)
42v	2/2	–	7 [b] (0)	2 (0)	103.6 (2.2)
43v	2/2	–	5 [b] (0)	2 (0)	103.7 (0.4)

[a] Animals were infected intranasally with 10^5 $TCID_{50}$ of each virus.
[b] Only one animal presented fever.

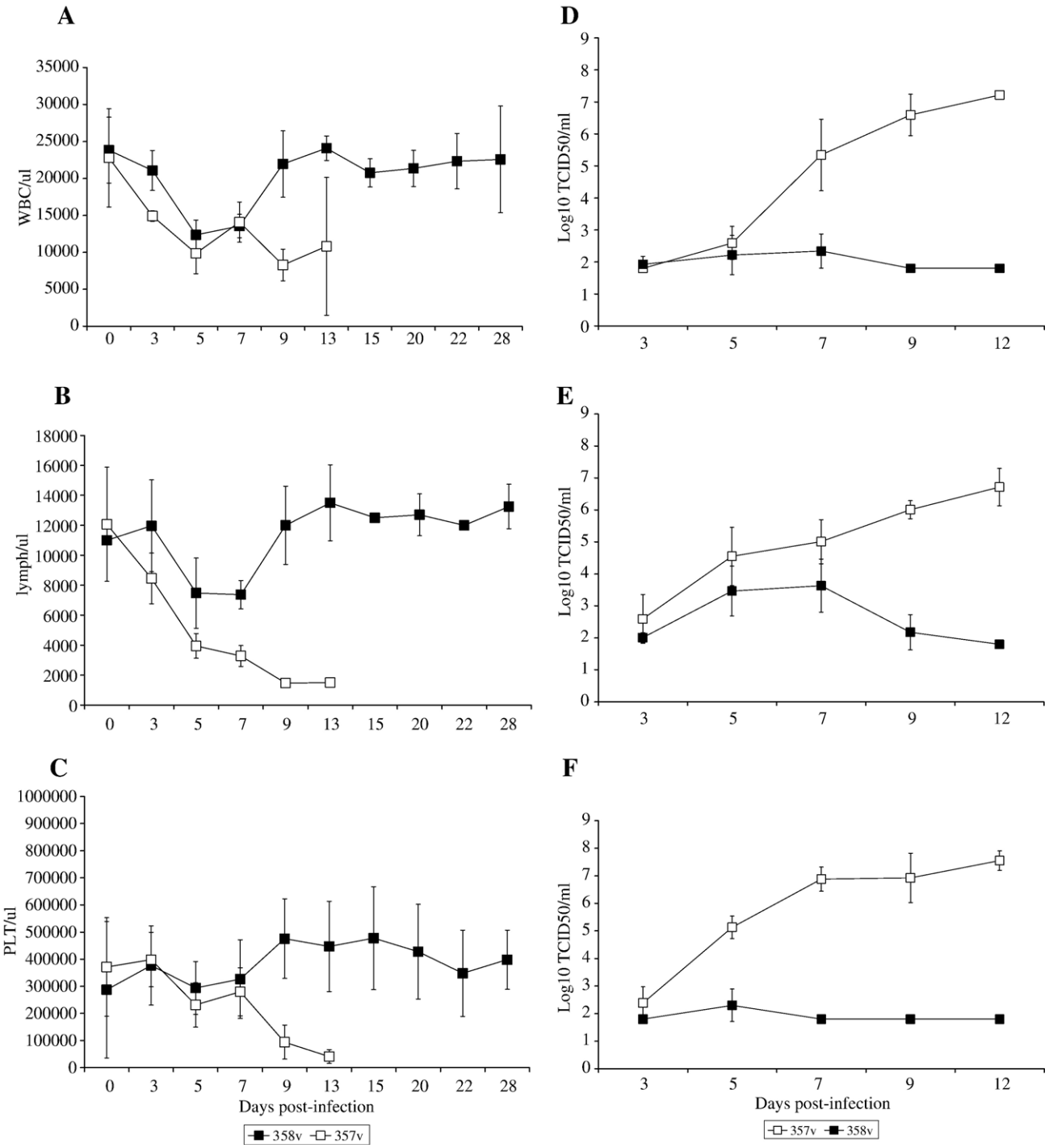

Fig. 3. Peripheral blood cell counts (A–C) and virus titers (D–F) in pigs infected with chimeric viruses 357 and 358: (A) peripheral white blood cells (WBC), (B) lymphocytes (Lymph) and (C) platelets (PLT). Counts were determined as described in Materials and methods. Virus titers of (D) nasal swabs, (E) tonsil scrapings and (F) blood, from pigs infected with chimeric viruses 357 and 358. Each point represents the mean and standard deviation of 4 animals.

structure (~3%) remains ambiguous based on the consensus sequence. The thirteen-amino-acid substitutions in chimera 358v between CSFV residues 882 and 1064, reduced 14 amino acid residues in the alpha helix and increased 13 amino acids in the extended strand, compared to BICv (Fig. 7). The differences in the distribution of the E2 alpha helix and extended strand between the BICv and 358v viruses are mainly located in the region after CSFV amino acid positions 889 to 895 when adding the extended strand, and positions 986 to 991 when disrupting the alpha helix region (Fig. 7). Comparing the less virulent 39v and partially attenuated 40v, it could be hypothesized that viral attenuation would require addition and disruption, ultimately leading to alteration of the secondary structures within this particular area of E2. Based on that prediction, amino acid

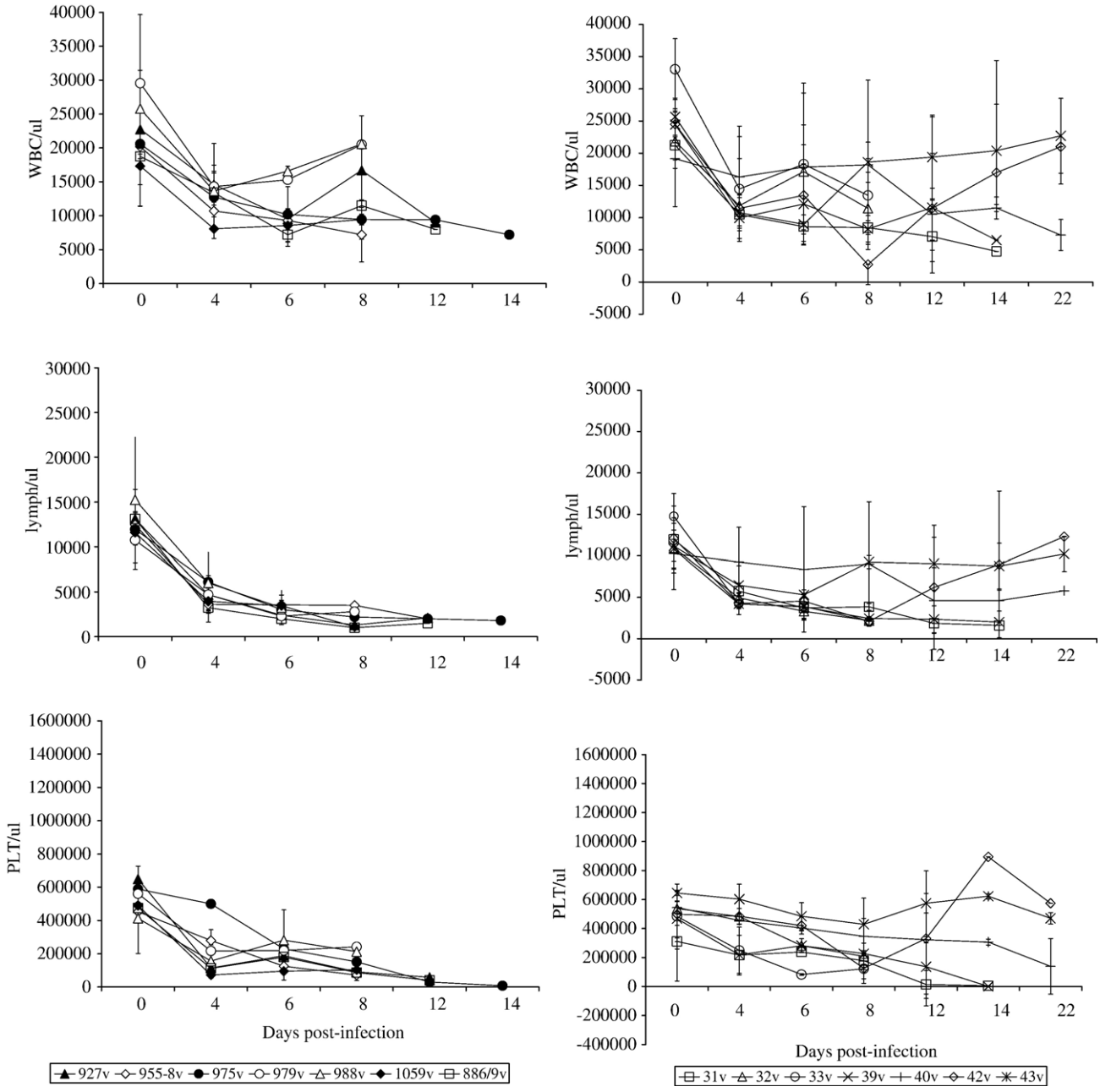

Fig. 4. Cell counts of peripheral white blood cells (WBC), lymphocytes (Lymph) and platelets (PLT) in pigs infected with chimeric viruses 886/9, 927/8, 955/8, 975, 979, 988 and 1059 (panels on the left) or 31, 32, 33, 39, 40, 42 and 43 (panels on the right). Counts were determined as described in Materials and methods. Each point represents the mean and standard deviation of at least two animals.

substitutions at positions P889L and A988T would generate a secondary structure similar to viruses CS and 358, which contain the 13-amino-acid substitutions in the area between CSFV residues 882 and 1064, compared with BICv. To test this hypothesis, a recombinant BICv containing substitutions P889L and A988T (called 45v) was generated and its virulence tested in swine. Results demonstrated that animals infected with 45v presented a clinical disease and virus replication profiles indistinguishable from that produced by infection with BICv (data not shown). These results indicate that attenuation of 358v could be explained by those substitutions causing alterations of the secondary structure of the BICv E2 protein in the area between CSFV residues 882 and 1064.

Attenuated 358v induces protection against challenge with virulent CSFV Brescia strain

The attenuate chimera 358v showed a limited ability to spread during the infection in swine, making itself a candidate experimental vaccine strain. Therefore, the ability of 358v to induce a protective immune response against challenge with virulent BICv was evaluated in swine at 3 and 28 DPI. Mock-

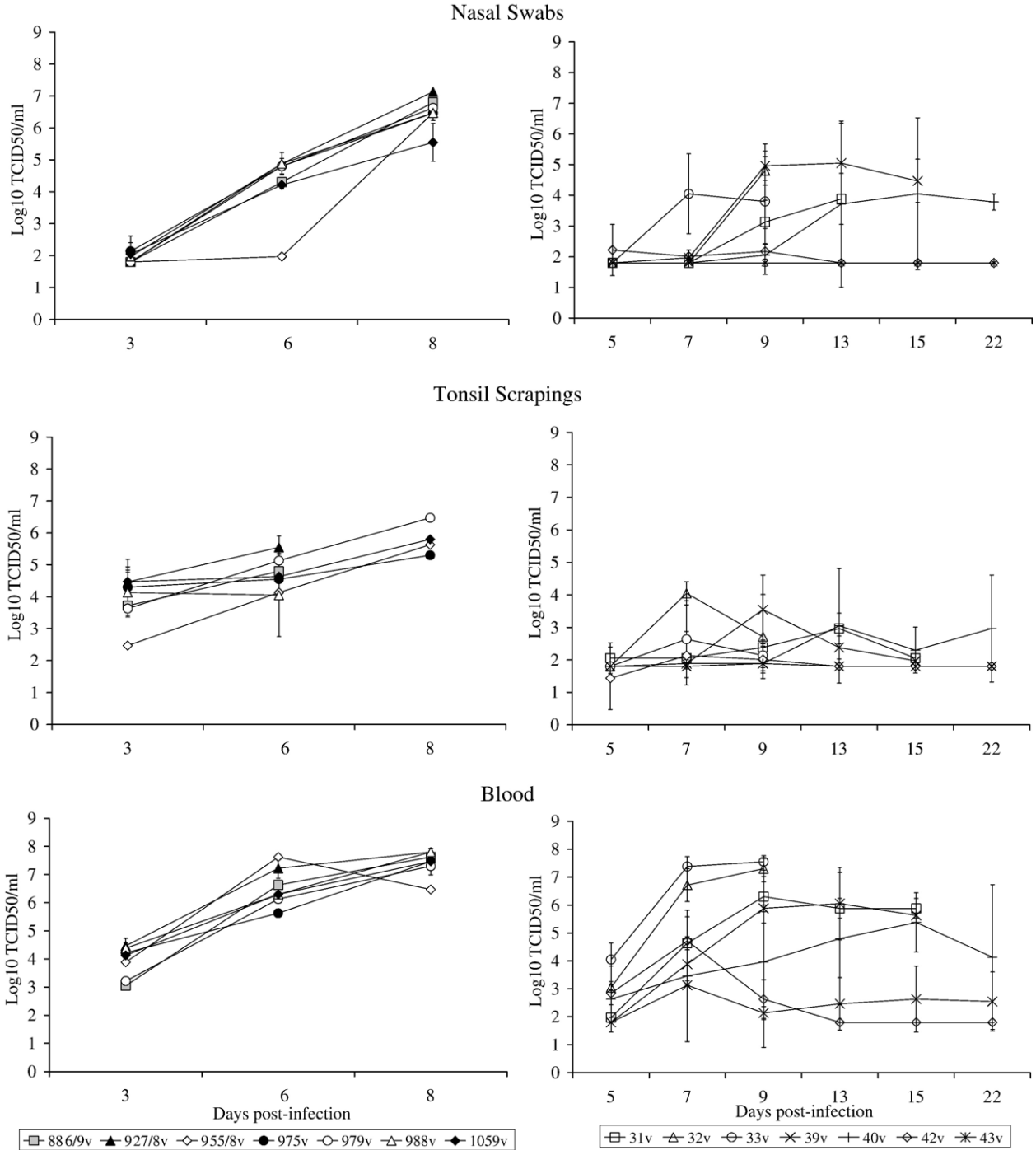

Fig. 5. Virus titers of nasal swabs, tonsil scrapings and blood, from pigs infected with chimeric viruses 886/9, 927/8, 955/8, 975, 979, 988 and 1059 (panels on the left) or 31, 32, 33, 39, 40, 42 and 43 (panels on the right). Each point represents the mean and standard deviation of at least two animals.

vaccinated pigs challenged with BICv developed anorexia, depression, and fever by 4 days post-challenge (DPC), with a marked reduction of circulating leukocytes and platelets by 7 DPC (data not shown), and died or were euthanized *in extremis* by 12 DPC (Table 2). Notably, 358v-vaccinated pigs challenged at 3 DPI showed complete protection against BICv-induced clinical disease. All pigs survived infection and remained clinically normal, with only two animals presenting a transient fever at 4 DPC (Table 2) and without significant changes in their hematological values (data not shown). Similarly, pigs challenged at 28 days post 358v vaccination remained clinically normal (Table 2). Viremia and virus shedding (nasal swabs, and tonsil scrapes) of BICv challenge virus was examined at 4, 7, 14 and 20 DPC. Mock-vaccinated animals showed viremia by 5 DPC, with virus titers remaining high (10^6 $TCID_{50}$/ml by 8 DPC) until death (data not shown). Virus shedding was detected by 4 DPC reaching titers of 10^4–10^5 $TCID_{50}$/ml by 8 DPC (data not shown). In contrast, virus

Table 2
Swine survival and fever response in 358v-infected animals following challenge with BICv

Time of challenge	Number of survivors/total	Mean time to death, days (±SD)	Fever		
			Days to onset (±SD)	Duration, no. of days (±SD)	Max temperature, °F (±SD)
3	3/3	–	4.5 [a] (0.7)	2.2 (3.2)	103.8 (0.4)
28	3/3	–	–	–	102.5 (0.9)
Mock	0/3	12 (2)	4 (0)	7 (0)	107.5 (0.1)

[a] Two of three animals presented with fever.

titers were below the level of detection in blood, nasal, and tonsil samples obtained from all pigs vaccinated with attenuated chimera 358v (data not shown).

Unlike most of the virulent CSFV strains, CS vaccine strain does not react with E2-specific mAb WH308 (Edwards et al., 1991) (Data not shown). Therefore, reactivity with mAb WH308 can potentially be used as a viral antigenic marker. Chimeras 357v and 358v were analyzed for the ability to react with mAb WH308. SK6 cells infected with chimera 357v strongly react with mAb WH308, while cells infected with chimera 358v lack reactivity with the monoclonal antibody (Fig. 6B). This indicates that the mAb WH308 epitope resides in an area between residues 882 and 1064 of the CSFV polyprotein (Fig. 1B). Further mapping of the epitope was assessed by analyzing the reactivity of 31v, 32v, and 33v against mAb WH308 in SK6-infected cells. 31v lack of reactivity indicates that the epitope is located between residues 882 and 958 of the CSFV polyprotein (Fig. 6C). Among the three double mutants within this area, 886/9v, 927/8v and 955/8v used in these studies, only mutant 886/9v did not react with mAb WH308. This lack of reactivity indicates that T886 and P889 in BICv are directly involved with mAb WH308 binding.

Discussion

The genetic bases and the molecular mechanisms underlying CSFV virulence remain unclear, with few studies associating specific viral proteins or genomic regions with virulence. Single or double codon mutation of E^{rns} that inhibited its RNase activity resulted in attenuation of the CSFV Alfort/Tübingen strain in swine (Meyers et al., 1999) and, similarly, mutation of the E^{rns} RNase domain of BVDV attenuated the virus in cattle (Meyer et al., 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). Recently, E2 of the CSFV Brescia strain has been shown to harbor determinants associated with virulence. Mutations, either abrogating glycosylation of the amino acid at position 805 or replacing amino acid residues 829 to 837 with the homologous

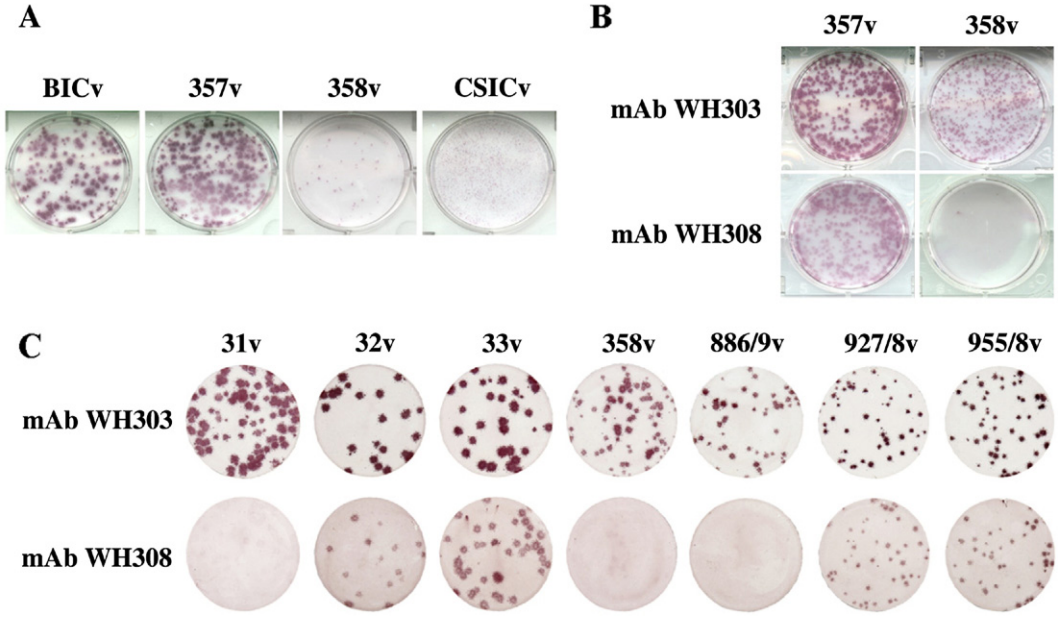

Fig. 6. Plaque formation of CSFV chimeric viruses on SK6 cell cultures. (A) BICv, CSICv, 357v and 358v. (B) Reactivity with mAb WH303 and WH308 of chimeric viruses 357v and 358v; and (C) 31v, 32v, 33v, 358v, 886/9v, 927/8v and 955/8v. Cell cultures were infected with 50–100 $TCID_{50}$, overlaid with 0.5% agarose, and incubated at 37 °C. Plates were fixed with 50% (v/v) ethanol/acetone and stained by immunohistochemistry with the corresponding mAb as described in Materials and methods.

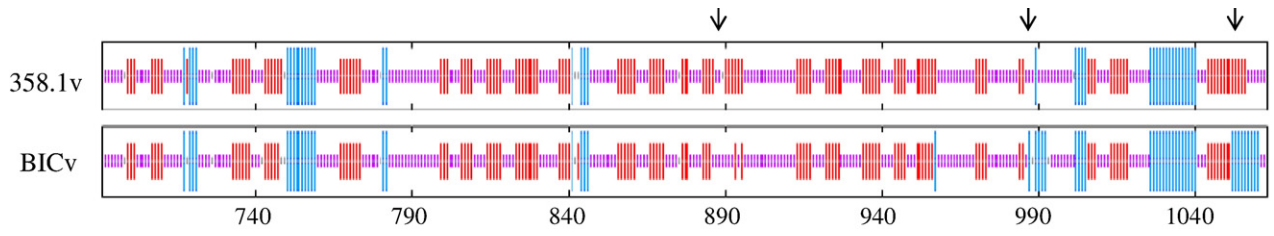

Fig. 7. The predicted secondary structures of 358.1v and BICv E2 proteins. Numbers represent amino acid residues in the CSFV polyprotein. The purple, red, and blue vertical lines indicate amino acid residues with random coil, extended strand, and alpha helix structures, respectively. The arrows indicate the mutations at amino acid positions 889 (P→L), 988 (A→T), and 1059 (L→P) associated with three most significant structural differences between the two strains. The dash indicates the transmembrane domain.

sequence of the BVDV NADL strain produced a significant attenuation of the highly virulent parental strain (Risatti et al., 2006, 2007). Additionally, it has been reported that changes at residue 710 in E2, in combination with point mutations in E^{rns}, induced a reduced virulence of CSFV strain Brescia (Van Gennip et al., 2004).

In a previous report, we have shown that replacement of the *E2* gene in CSFV strain Brescia with that of vaccine strain CS resulted in a chimeric virus, 319.1v, that was significantly attenuated *in vivo*, demonstrating considerably decreased virus replication in tonsils, a transient viremia, a limited generalization of infection, and decreased virus shedding (Risatti et al., 2005a). Here we have demonstrated that attenuation of chimeric virus 319.1v is due to the presence of a stretch of 12-amino-acid substitutions lying within the carboxyl terminus of the E2 glycoprotein, between residues 882 and 1032 of the CSFV polyprotein. This represents a third area within the *E2* gene associated with virulence; two other independent determinants are located in a glycosylation site at position 805 and the other in a region between amino acid residues 805 and 837 of the CSFV Brescia polyprotein (Risatti et al., 2006, 2007).

Chimeric virus 358v, which contains the genetic background of virulent BICv and 182 residues encompassing the carboxyl terminal half of CS E2 (Fig. 1A) was attenuated in swine. The amino acid sequence in this portion of the *E2* gene differs in 13 amino acids between attenuated CS and virulent BICv. Only when 12 of the 13 residues were substituted from BICv to CS sequence (42v) attenuation was observed.

Interestingly, none of the 12 substitutions, tested as a single- or double-amino-acid mutation, affected BICv virulent phenotype. Even double mutant P889L/A988T (45v) containing the only two substitutions predicted to significantly alter the secondary structure of the area between amino acid residues 882 and 1032 was unable to revert the virulent phenotype. These observations suggested that a larger number of residues were necessary to impart an attenuated phenotype. Moreover, viruses 31v, 32v and 33v, where stretches of up to six residues were substituted from BICv to CS amino acids (Fig. 1B), were still unable to induce a completely attenuated phenotype in pigs. Thus, viruses 39v, 40v, 42v, and 43v (Fig. 1B) were constructed by progressively introducing 2, 3, 5, and 6 BICv to CS amino acid substitutions, respectively, into the genetic background of 31v. This approach resulted in the production of viruses that showed progressive degrees of attenuation from a virulent phenotype, with no surviving animals, to 39v, with a transient fever in animals inoculated with 42v and 43v (Table 1).

The mechanisms mediating this attenuation remain unknown. It could be hypothesized that attenuating substitutions in BICv E2 glycoprotein conceivably involve aspects of virus attachment and/or efficient entry into critical target cells *in vivo*. Also suggestive of a role for E2 mutations in virus attachment and/or entry was the small plaque phenotype exhibited by 358v, in accordance with the same phenotype showed by the vaccine strain CSICv. 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 (Risatti et al., 2005a, 2005b, 2006, 2007).

Finally, we observed that chimera 358v, despite having a limited replication *in vivo*, characterized by reduced viremia, generalization of infection, and virus shedding, induced a rapid and effective protective response against virulent BICv. Similar protection patterns have been described previously (Bironi et al., 1987; Terpstra and Wensvoort, 1988) for live-attenuated vaccine (LAV) strains of CSFV. This rapid, antibody-independent protection likely involves mechanisms of innate immunity (e.g. macrophages/monocytes, dendritic cells, and NK cells, and their products). Additionally, the lack of reactivity of 358v to mAb WH308, which recognizes a highly specific CSFV epitope, makes this virus a potential live-attenuated marker vaccine candidate.

In summary, a novel CSFV genetic virulence determinant associated with the E2 glycoprotein has been identified. Although the mechanism underlying the attenuation remains unknown, improving the understanding of the genetic basis of CSFV virulence will permit future rational design of live-attenuated CSF vaccines of enhanced safety, efficacy and utility.

Materials and methods

Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of Bovine Viral Diarrhea Virus (BVDV) were used throughout this study. SK6 cells 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 (obtained from the Animal and Plant Health Inspection Service, Plum Island Animal Disease Center) was propagated in SK6 cells and used for the construction of an

infectious cDNA clone (Risatti et al., 2005a). Titration of CSFV in clinical samples was performed in 96 well plates (Costar, Cambridge, MA) using SK6 cells. After 4 days in culture, viral infectivity was detected by immunoperoxidase assay using the CSFV monoclonal antibody WH303 (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 $TCID_{50}$/ml. As performed, test sensitivity was >1.97 $\log_{10}$ $TCID_{50}$/ml. Primary swine macrophage cell cultures were prepared as described by Zsak et al. (1996).

Construction of CSFV Brescia/CS chimeric cDNA infectious clones (IC)

Two chimeric viruses, designated 357v and 358v, were constructed containing the amino (CSFV residues 691–881) and the carboxyl (CSFV residues 882–1064) regions of the CS E2 glycoprotein in the context of the Brescia (BICv) strain, respectively (Fig. 1). Construction of these chimeric viruses was accomplished through the generation of several intermediate plasmids. First, the CSFV genomic region containing the *E2*, *p7*, and *NS23* genes from strains Brescia and CS was PCR amplified using the CSFV-specific primers F2379 and R6636 (Table 3) to include the native restriction endonuclease sites *Nae*I (position 2438) and *Bam*HI (position 6435). The amplified products were then cloned into a pCR4Topo vector, using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) to create the plasmids pBreN/B and pCSN/B. Next, a unique *Sac*II restriction enzyme site (Table 3) was introduced into both plasmids at the 3′ end of the *E2* gene (position 3540) by site-directed mutagenesis using the QuikChange® XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) to generate the plasmids pBreN/B*Sac*II and pCSN/B*Sac*II. These plasmids were then digested with *Sac*II/*Bam*HI and *Nae*I/*Sac*II, independently. The *Sac*II/*Bam*HI fragment and *Nae*I/*Sac*II fragment from pCSN/B*Sac*II were then introduced into the *Sac*II/*Bam*HI-digested pBreN/B*Sac*II and the *Nae*I/*Sac*II-digested pBreN/B*Sac*II to yield the plasmids pN(Bre)S/B(CS) and pN(CS)S/B(Bre), respectively. A second unique restriction enzyme site, *Age*I, was then introduced by site-directed mutagenesis into both of these plasmids at position 3004, located approximately in the middle of the *E2* gene, to generate the plasmids pN(Bre)S/B(CS)*Age*I and pN(CS)S/B(Bre)*Age*I (Table 3). These plasmids were independently digested with restriction enzymes *Nae*I/*Age*I and *Age*I/*Sac*II. The *Nae*I/*Age*I fragment and *Age*I/*Sac*II fragment from pN(Bre)S/B(CS)*Age*I were cloned into pN(CS)S/B(Bre) to obtain the plasmids pN/BBre5′E2CS and pN/BBre3′E2CS, respectively. In a final step, the *Nae*I/*Bam*HI fragments from pN/BBre5′E2CS and pN/BBre3′E2CS were independently cloned into pBIC to obtain the full-length plasmids pB(5′E2)CS (357) and pB(3′E2)CS (358), respectively (Fig. 1A).

Seven mutant full-length clones containing double- or single-amino-acid substitutions in the *E2* gene area between CSFV polyprotein residues 886 and 1059 were generated by PCR-based site-directed mutagenesis of full-length cDNA clone pBIC as described above. Full-length mutant clones were named p886/9, p927/8, p955/8, p975, p979, p988 and p1059 (Fig. 1B). The mutagenic oligonucleotide primer sets (Table 3) were designed following manufacturer's instructions (Stratagene).

Chimeric full-length clones, p31, p32 and p33, contain all CS amino acids in the region between residues 882 and 958 (p31), between 955 and 994 (p33) and between 975 and 1064 (p32) of the CSFV BICv polyprotein (Fig. 1B). Chimeras were generated by progressively introducing mutations by PCR-based site-directed mutagenesis of full-length cDNA clone pBIC, as described above. Full-length cDNA chimera p31 was obtained using p886/9 as a DNA template and subsequently substituting residues 892, 927/8 and 955/8 for CS residues (Fig. 1B). Full-length cDNA chimera p32 was obtained using p975 as a DNA template and subsequently substituting residues 979, 988, 994, 1032 and 1059 for CS residues. Full-length cDNA chimera p33 was obtained using p955/8 as a DNA template and subsequently substituting residues 975, 979, 988 and 994 for CS residues (Fig. 1B).

Chimeric full-length cDNA clones p39, p40, p42 and p43 are derived from p31 and they were obtained by progressively substituting BICv residues with CS E2 residues between amino acid positions 882 and 979 (p39), between 882 and 988 (p40), between 882 and 1032 (p42), and between 882 and 1064 (p43) of the CSFV polyprotein (Fig. 1B). Full-length chimera p39 was obtained using plasmid p31 as DNA template and subsequently

Table 3
Oligonucleotides used for construction of the chimeric infectious clones

Primer	Sequence	Function
F2379	5′ CCTCATCTGCTTGATAAAAG 3′	PCR pBreN/B and pCSN/B
R6636	5′ CCTTCTCTGGGCTTGTTC 3′	
F*Sac*II	5′ GAACAACTCG<u>CCG**C**GG</u>GTCTACAGTTAGG 3′	Insert *Sac*II site
F*Age*I	5′ ATGTGTGAAAGGTGA<u>ACCG**G**T</u>GACCTACACGGGGGG 3′	Insert *Age*I site
F886/9	5′ CCAGTGACCTACA**T**GGGGGGGC**T**AGTAAAACAATGC 3′	Mutate residues 886/9
F927/8	5′ GGTTACAGAATAGTGGATT**TA**G**C**GGACTGTAACAGAGATGGC 3′	Mutate residues 927/8
F955/8	5′ CACAACTGTCAAGGTGC**G**TGCATTAG**GC**GAAAGACTAGGCCCT 3′	Mutate residues 955/8
F975	5′ GATCGCTTCTAGTG**A**GGGACCTGTAAGG3′	Mutate residue 975
F979	5′ TAGTGCGGGACCTGTAAG**T**AAAACTTCCTGTACATTC 3′	Mutate residue 979
F988	5′ CTTCCTGTACATTCAACTAC**A**CAAAAACTCTGAGGAACAGG 3′	Mutate residue 988
F1059	5′ GTTCTAACAGAAC**C**CGCCGCGGGTCTACAG 3′	Mutate residue 1059

Bold characters indicated mutations. When not showed, reverse primers are complementary to the forward primers. Enzymatic restriction sites are <u>underlined</u>.

substituting residues 975 and 979 for CS residues. p40 full-length cDNA was obtained after introducing CS residue 988 into plasmid p39. p42 full-length cDNA was obtained after introducing CS residues 994 and 1032 into plasmid p40, and p43 full-length cDNA was generated by introducing CS residue 1059 into plasmid p42.

Rescue of CSFV chimeric 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. The RNA was then transfected into SK6 cells by electroporation at 500 V, 720 Ω, 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 using a CSFV E2-specific monoclonal antibody, WH303 (Edwards et al., 1991). Stocks of rescued viruses were stored at −70 °C.

DNA sequencing

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). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Boston, MA). Reaction products were sequenced on an ABI-PRISM 3700 automated DNA Sequencer (PE Biosystems, Foster City, CA). Sequence data was assembled with the Phrap software program, with confirmatory assemblies performed using CAP3 (Huang and Madan, 1999). The final DNA consensus sequence represented, on average, fivefold redundancy at each base position.

Animal infections

To assess virulence of all chimeric viruses, 40-lb pigs aged 10 to 12 weeks old were randomly allocated into groups of two animals each. Pigs were intranasally inoculated with 10^5 $TCID_{50}$ of BICv, or one of the chimeric viruses. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) were observed daily throughout the experiment.

For protection studies, 9 pigs were randomly allocated into 3 groups of 3 animals each. Pigs in groups 1 and 2 were inoculated with 358v, and animals in group 3 were mock-infected. At 3 (group 1) or 28 DPI (group 2), animals were intranasally challenged with 10^5 $TCID_{50}$ of BICv, along with animals in group 3. Blood, serum, nasal swabs and tonsil scrapings were collected at designated times after challenge. Clinical signs and body temperature were recorded daily throughout the experiment as described above.

Total and differential white blood cells counts

Blood was obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer). Total white blood cell, lymphocyte and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

Protein sequence analysis

Protein secondary structure of E2 wild-type and mutants was predicted using a consensus method described by Combet et al. (2000). Functional domains of the protein were also predicted with SMART (Letunic et al., 2006), PSI-BLAST (Altschul et al., 1997), and Interpro (Mulder et al., 2005).

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