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Substitution of Specific Cysteine Residues in the E1 Glycoprotein of Classical Swine Fever Virus Strain Brescia Affects Formation of E1-E2 Heterodimers and Alters Virulence in Swine[▽]

I. Fernández-Sainz,[1] L. G. Holinka,[1] D. Gladue,[1] V. O'Donnell,[1,3] Z. Lu,[2] B. K. Gavrilov,[3] G. R. Risatti,[3] and M. V. Borca[1]*

Plum Island Animal Disease Center, ARS, USDA, Greenport, New York 11944[1]; Plum Island Animal Disease Center, DHS, Greenport, New York 11944[2]; and Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut 06269[3]

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E1, along with E^{rns} and E2, is one of the three envelope glycoproteins of classical swine fever virus (CSFV). E1 and E2 are anchored to the virus envelope at their carboxyl termini, and E^{rns} loosely associates with the viral envelope. In infected cells, E2 forms homodimers and heterodimers with E1 mediated by disulfide bridges between cysteine residues. The E1 protein of CSFV strain Brescia contains six cysteine residues at positions 5, 20, 24, 94, 123, and 171. The role of these residues in the formation of E1-E2 heterodimers and their effect on CSFV viability *in vitro* and *in vivo* remain unclear. Here we observed that recombinant viruses harboring individual cysteine-to-serine substitutions within the E1 envelope protein still have formation of E1-E2 heterodimers which are functional in terms of allowing efficient virus progeny yields in infected primary swine cells. Additionally, these single cysteine mutant viruses were virulent in infected swine. However, a double mutant harboring Cys24Ser and Cys94Ser substitutions within the E1 protein altered formation of E1-E2 heterodimers in infected cells. This recombinant virus, E1ΔCys24/94v, showed delayed growth kinetics in primary swine macrophage cultures and was attenuated in swine. Furthermore, despite the observed diminished growth *in vitro*, infection with E1ΔCys24/94v protected swine from challenge with virulent CSFV strain Brescia at 3 and 28 days postinfection.

Classical swine fever (CSF) is a highly contagious disease of swine. The etiological agent, *Classical swine fever 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 *Flaviviridae* (2). The 12.3-kb CSFV genome contains a single open reading frame that encodes a 3,898-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 posttranslational processing of the polyprotein by cellular and viral proteases (5). 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 (22, 23). E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (19). E1 has been implicated (21), along with E^{rns} and E2 (4), in viral adsorption to host cells. Importantly, different modifications introduced into these glycoproteins appear to have an important effect on CSFV virulence (3, 6, 9, 10, 11, 12, 15, 20).

In lysates of CSFV-infected cells, E2 glycoprotein forms homodimers and heterodimers with E1 glycoprotein (14, 22) via disulfide bridges between cysteine residues. Formation of these disulfide-linked heterodimers also occurs in CSFV virions (19), suggesting that the interaction between envelope proteins plays an important role in virus assembly, virion maturity, and consequently in efficiency of viral infection. The E1 envelope protein of CSFV strain Brescia contains six cysteine residues at E1 positions 5, 20, 24, 94, 123, and 171. E1 residues that contribute to the formation of disulfide bridges with E2 glycoprotein are yet to be identified. Furthermore, the biological significance of E1-E2 heterodimer formation during infection and the role of E1 Cys residues during CSFV replication in target cells or in infection of swine are not known.

In this study, we have used oligonucleotide site-directed mutagenesis to construct a panel of single and double recombinant mutant viruses by substituting cysteine to serine residues within CSFV glycoprotein E1. These mutants were used to investigate whether substitutions of each of these Cys residues could affect formation of E1-E2 heterodimers in infected primary swine macrophages and virus virulence in swine. We observed that individual substitutions of these residues did not have an effect on the formation of E1-E2 heterodimers. Growth kinetics was not affected, nor was the ability to replicate or cause disease in swine. Interestingly, 11 out of 15 possible constructs containing double Cys-to-Ser substitutions were deleterious for the virus. A partial disruption of E1-E2 heterodimer formation was observed in cells infected with a virus harboring Cys24Ser and Cys94Ser substitutions in E1 protein. The virus, E1ΔCys24/94v, showed a decreased growth rate in primary swine macrophages and was attenuated in swine. The remaining constructs harboring double cysteine-to-

* Corresponding author. Mailing address: Plum Island Animal Disease Center, USDA/ARS/NAA, P.O. Box 848, Greenport, NY 11944-0848. Phone: (631) 323-3019. Fax: (631) 323-3006. E-mail: manuel.borca@ars.usda.gov.

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serine mutations retained the ability to produce virus progeny, formed heterodimers in infected cells, and were virulent in swine. Data presented here suggest that specific E1 cysteine residues are critical for virus viability and virus growth; most probably, these residues play a role in E1-E2 interactions, affecting assembly of mature viral particles.

MATERIALS AND METHODS

Viruses and cells. Swine kidney cells (SK6) (17), 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 strain Brescia was propagated in SK6 cells and used for the construction of an infectious cDNA clone (IC) (9). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described by Zsak et al. (24). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Presence of viral antigen was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody (MAb) WH303 (1) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (8) and expressed as 50% tissue culture infective doses ($TCID_{50}$)/ml. As performed, test sensitivity was $\geq$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 E1 cysteine mutant viruses (E1ΔCys). A full-length IC of the virulent CSFV strain Brescia (pBIC) (9) was used as a template in which Cys residues in the E1 glycoprotein were substituted with Ser residues. Cys-to-Ser amino acid substitutions (E1ΔCys) were introduced by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) performed per the manufacturer's instructions using the following primers (only forward primer sequences are shown; nucleotide changes compared with the native sequence are in boldface): C5, 5′ GCCTATGCCCT ATCACCTTAT**AGT**AATGTGACAAGCAAAATAGGG 3′; C20, 5′ TACATA TGGTACACTAACAAC**AGT**ACCCCGGCTTGCCTCCCCAAA 3′; C24, 5′ ACTAACAACTGTACCCCGGCT**AGC**CTCCCCAAAAATACAAAGATA 3′; C94, 5′ TCCCATGAAGAACCTGAAGGC**AGT**GACACAAACCAGCTGAA TTTA 3′; C123, 5′ TGGAATGTTGGCAAATATGTG**AGT**GTTAGACCAGA CTGGTGGCCA 3′; C171, 5′ TCAACCACGGCATTCCTCATC**AGC**TTGAT AAAAGTATTAAGAGGA 3′.

***In vitro* rescue of CSFV Brescia and E1ΔCys recombinant mutant viruses.** Full-length genomic clones were linearized with SrfI and *in vitro* transcribed using the MEGAscript T7 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 were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (16). *In vitro* rescued viruses and viruses recovered from infected animals were sequenced in the mutated region, whereas the E1ΔCys24/94v genome was sequenced completely. Sequencing reactions were prepared with the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on an ABI Prism 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). The final DNA consensus sequence represented an average 5-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Comparison of predicted alpha-helices and beta strands between parental BICv E1, double mutant E1ΔCys24/94 E1, and quadruple mutant E1ΔCys24/94/123/171 E1 proteins were done with NetSurfP ver. 1.1 (7).

Western blot analysis. Formation of E1-E2 heterodimers by BICv and the mutant viruses was analyzed in lysates of SK6-infected cells by Western immunoblot assays. CSFV E1 was detected with an anti-E1 serum produced in rabbits against CSFV strain Brescia E1 glycoprotein expressed in *Escherichia coli* (3). E2 detection was performed using anti-E2 monoclonal antibody WH303 (1). SK6 monolayers were infected (multiplicity of infection [MOI] = 1) with BICv or E1ΔCysv mutants, harvested at 48 h postinoculation (hpi) using the NuPAGE LDS sample buffer system (Invitrogen), and incubated at 70°C for 10 min. Samples were run under either reducing or nonreducing conditions in precast NuPAGE 12% Bis-Tris acrylamide gels (Invitrogen). Western immunoblot assays were performed using the WesternBreeze chemiluminescent immunodetection system (Invitrogen).

Animal infections. Each of the E1ΔCys mutants was initially screened for its virulence phenotype relative to the virulent Brescia strain using 40-pound commercial-breed swine that were 10 to 12 weeks old. Pigs were inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type virus. For screening, 22 pigs were randomly allocated into 11 groups of 2 animals each, and pigs in each group were inoculated with either BICv or one of the E1ΔCysv mutants. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough) and changes in body temperature were recorded daily throughout the experiment.

For protection studies, pigs were randomly allocated into 3 groups. Pigs in groups 1 and 2 ($n = 4$) were inoculated with E1ΔCys24/94v, while pigs in group 3 ($n = 4$) were mock infected. At 3 days postinfection (dpi) (group 1) or 28 dpi (groups 2 and 3), animals were intranasally challenged with BICv. 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 postchallenge, 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).

RESULTS

Viability of CSFV E1ΔCys recombinant mutant viruses. Highly conserved Cys residues within E1 protein of CSFV strain Brescia (Fig. 1) were targeted for mutagenesis in the context of a full-length cDNA clone. Single Cys-to-Ser substitutions in E1 protein yielded viruses E1ΔCys5v, E1ΔCys20v, E1ΔCys24v, E1ΔCys94v, E1ΔCys123v, and E1ΔCys171v, while double Cys-to-Ser substitutions yielded only viruses E1ΔCys5/171v, E1ΔCys20/171v, E1ΔCys94/171v, and E1ΔCys24/94v (Table 1). Partial nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming the fidelity of the RNA sequence in rescued viruses. Double substitutions within E1 protein that included combinations of residues 5/20, 5/24, 5/94, 5/123, 20/24, 20/94, 20/123, 24/123, 24/171, 94/123, and 123/171 did not yield infectious viral progeny after three independent transfection experiments using SK6 cells (Table 1). These data suggest that more than one Cys residue in E1 is necessary for proper assembly of infectious virus particles, particularly Cys20 and Cys123, since 8 of 11 combined substitutions that yielded nonviable viruses involved these two residues.

Replication of CSFV E1ΔCys mutants in primary swine macrophages. *In vitro* growth characteristics of rescued single and double mutant viruses were evaluated in primary swine macrophage cell cultures, these being primary CSFV target cells during infection of swine, and compared to parental BICv in a multistep growth curve (Fig. 2A and B). All recombinant viruses, with the exception of E1ΔCys24/94v, displayed growth kinetics indistinguishable from parental BICv (Fig. 2A and B). Growth of the double mutant E1ΔCys24/94v was significantly slower than that of BICv, exhibiting titers 10- to 15-fold lower than those of parental virus. Additionally, E1ΔCys24/94v showed reduced-size foci of infection on SK6 cells relative to those of parental BICv (Fig. 2C). Although these substitutions in E1 were not lethal like mutations observed with the majority of double mutants (Table 1), substitutions of E1 Cys24 and Cys94 significantly affected virus growth in macrophages and virus spreading in SK6 cells. The complete genome sequence of E1ΔCys24/94v confirmed that the introduced Cys-to-Ser substitutions are the only deviations from the original parental BICv genomic sequence. Double mutants that did not affect

	CSFV E1 protein residues		
	1-30	91-130	161-180
BICv	LSPY**C**NVTSK IGYIWYTNN**C** TPA**C**LPKNTK	PEG**C**DTNQLN LTVELRTEDV IPSSVWNVGK YV**C**VRPDWWP	NSASTTAFLI **C**LIKVLRGQV
ALD	....	....	I
Eystrup	....	....	I
Alfort/187	....	....	I
Alfort A19	....	....	I
Alfort/Tuebingen	.....R.	....K..... V.....I..	....
GPE-	....	....	.....M.... I
Chinese strain	....	....	.....I.... I
Thiverval	....	L.....	I
Riems	....	....	.....I.... I
CS	....V.....	.K..... ....R	I
Paderborn	.....R.	....G..... V.....I..	....I.....
Borken	.....R.	....K..... V.....I..	....
Sp01	.....R.	....K..... V.....I..	....
India	....	....	....V.....I
BVDV NADL	A....D.DR. .....K..	VMD..KT... .....T.AE. ..G....L.. ...I..N...	.A.T.....V ..V.IV...M
BVDV VEDEVAC	A....E.ERR L.....K..	IHD..K.... .....T.AE. ..G....L.. ...I.....	.A.T.....V ..V..A....
BVDV CP7	A....E.ER. L.....K.. .....R...	IQD..K.... .....T.AE. ..G....L..	TA.T.....V ..V..V....
BVDV Osloss	A....E.ERR L.....K..	IHD..K.... ...G.T.AE. ..G....L.. ...I.....	TA.T.....V ..V..V....
BVDV Nose	A....D.DR. L....F.K..	IMD..KT... .....T.A.. ..G....L.. ...I.....	.A.T.....V ..V.MV...M
BVDV NY'93	A....G.ER. ...V...K.. .....R..R	VDT..K.... ...ATTVAE. ..GT...L.. ...I.....	.A.T..... F.V.A....L
BVDV Oregon C24v	A....D.DR. L....F.K..	ITD..KT... .....T.A.. ..G....L.. ...I.....	.A.T.....V ..V.MV...M
BVDV 890	A....G.ER. ...V...K.. .....R...	VDT..K.... ...ATTVAE. ..GT...L.. ...I.....	.A.T..... F.V.A....L
BVDV SD1	A....D.DR. .....F.K..	ITD..KT... ..I..T.A.. ..G....L.. ...I.....	.A.T.....V ....MV....
BDV C413	A....G.EK. ...V...K.. .....R...	VDT..K.... ...ATTVAE. ..G....L.. ...I.....	YA.T..... F.VIA....L
BD78	A....G.EK. ...V...K.. .....R...	VDT..K.... ...ATTVAE. ..R....L.. ...I.....	.P.T..... F.V.A....L
R2727	A....D.DR. L....F.K..	IMD..KTK.. .....T.A.. ..G....L.. ...I.....	.A.T.....V ..V.MI...M
H2121 Chamois-1	Q.....K. ...V...S..	.DD..... ...S.KV... .....LE. ...I..Y...	....V.L....I
Gifhor	Q.....R. ....G...	.DQ..M.... ...D.KV... .....L.. ...I.....	S..... ..V.....
X818	Q.....R. ...V.....	.SV..... ...S..VD.. .....L..	....V.....
BD31	Q.....R.	.SE..... ...G..VD.. .....L..	....A.....
L83/84	.....R.	.SE..... ...G..VD.. .....L..	....V.....
BM01	Q.....R. ....R...	.....L.... ...G..... V..... ...I.....	....V.....

FIG. 1. A multiple alignment of CSFV core proteins revealed the presence of highly conserved putative Cys residues (bold and underlined) among pestiviruses (CSFV, classical swine fever virus; BVDV, bovine viral diarrhea virus; BDV, border disease virus).

TABLE 1. CSFV E1 Cys-to-Ser mutant viruses rescued from SK6 cells and their virulence phenotype in infected swine

Rescued mutant virus	Substituted Cys residue(s)	Phenotype
E1ΔCys5v	5	Virulent
E1ΔCys20v	20	Virulent
E1ΔCys24v	24	Virulent
E1ΔCys94v	94	Virulent
E1ΔCys123v	123	Virulent
E1ΔCys171v	171	Virulent
–[a]	5/20	–
–	5/24	–
–	5/94	–
–	5/123	–
E1ΔCys5/171v	5/171	Virulent
–	20/24	–
–	20/94	–
–	20/123	–
E1ΔCys20/171v	20/171	Virulent
E1ΔCys24/94v	24/94	Attenuated
–	24/123	–
–	24/171	–
–	94/123	–
E1ΔCys94/171v	94/171	Virulent
–	123/171	–

[a] –, no virus rescued.

virus growth in swine macrophages included Cys171, suggesting that this residue plays a minor role in the process of formation of infectious viral particles.

Detection of E1-E2 heterodimers in infected SK6 cells. Since formation of E1-E2 heterodimers mediated by disulfide bridges is observed in lysates from cells infected with CSFV (22) and in mature CSFV virions (19), we tested for the presence of these dimers in SK6 cells infected with parental or recombinant viruses. Under nonreducing conditions, Western immunoblot assays using anti-E2 MAb WH303 showed that single or double Cys-to-Ser substitutions in E1 generally did not affect formation of E2 homodimers or E1-E2 heterodimers in infected SK6 cells, since the complexes are still formed and appear partially functional (Fig. 3A). Only lysates from cells infected with recombinant E1ΔCys24/94v showed changes in the proportions of homo-/heterodimers expressed. A similar pattern was observed when formation of homo- and heterodimers was detected with a polyclonal anti-E1 antibody (Fig. 3B). Infection with E1ΔCys24/94v leads to a shift in the ratio of homo-/heterodimers relative to parental virus infections, with an increased accumulation of E2 homodimers over E1-E2 heterodimers while proportions of E2 monomers remained unchanged. Additionally, it was observed that electrophoretic mobility of E1ΔCys24/94v E1-E2 heterodimers is slower than that of any of the other mutants or parental BICv.

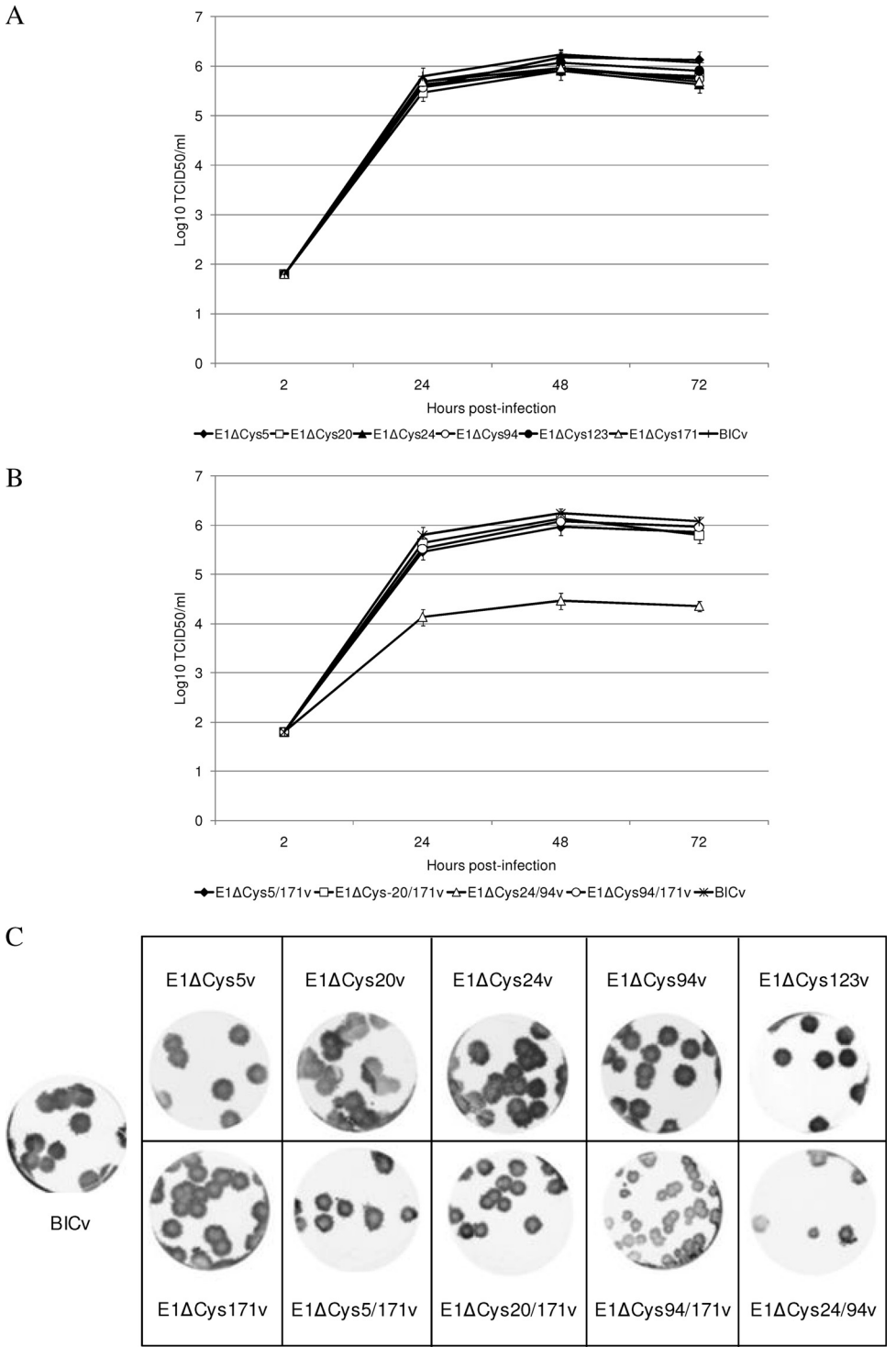

FIG. 2. *In vitro* growth characteristics of E1ΔCys mutant viruses relative to parental BICv. (A and B) Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the single (A) and double (B) E1ΔCys mutant viruses or BICv. Virus yields were calculated by titration at times after infection of SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection, $\geq \log_{10} 1.8$ $TCID_{50}$/ml. (C) Foci of infection of single and double E1ΔCys mutant viruses and parental BICv were detected on SK6 cell monolayers by immunoperoxidase staining using anti-E2 MAb WH303.

This may happen with the E1-E2 heterodimer in the absence of critical disulfide bonds, causing its configuration to become less compact. We observed minor changes in the levels of expression of E1 and E2 proteins in SK6 cells infected with E1ΔCys24/94v relative to levels of expression observed in cells infected with BICv (Fig. 3C and D). Data confirm that Cys residues 24 and 94 are required for the proper formation of E1-E2 heterodimers.

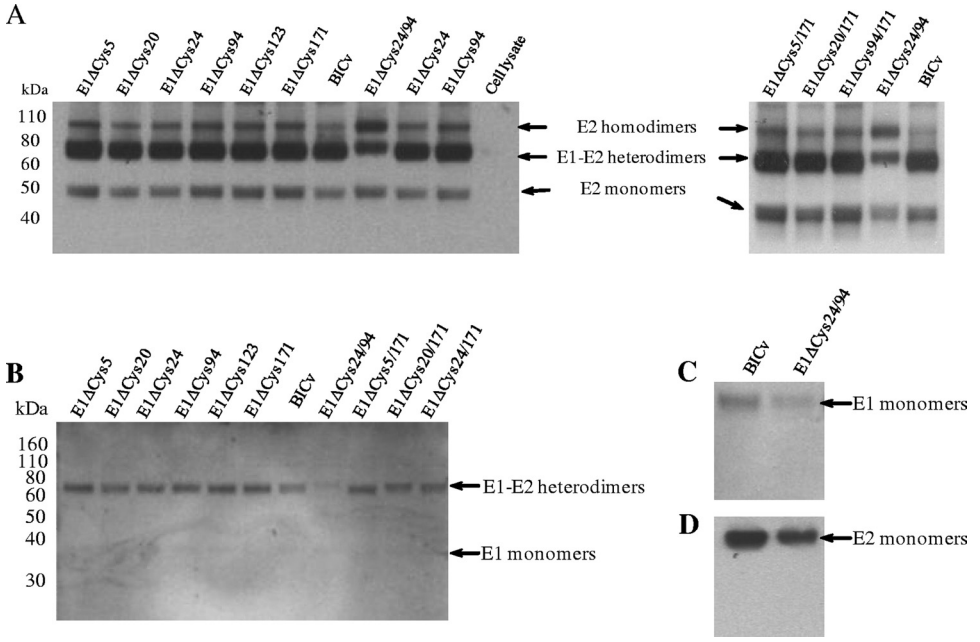

FIG. 3. Western blot analysis of SK6 cell lysates infected with E1ΔCys single and double mutant viruses. Detection of E1-E2 heterodimers with anti-E2 MAb WH303 (A) or with an anti-E1 rabbit anti serum (B) and monomeric forms of E1 (C) and E2 (D). SDS-PAGE was performed under nonreducing (A and B) or reducing (C and D) conditions.

Substitutions of Cys24 and Cys94 residues in E1 lead to virus attenuation. *In vivo* infections using viable single and double mutant E1 recombinant viruses (Table 1) were assessed in swine. Naïve pigs were intranasally inoculated and monitored for clinical disease using either BICv or a mutant virus. Single and double mutant viruses, with the exception of E1ΔCys24/94v, induced a clinical disease indistinguishable from disease induced by parental BICv (Table 2). Pigs infected with these viruses presented clinical signs of CSF starting at 3 to 5 dpi, with clinical presentation and severity similar to those observed in animals inoculated with BICv. Viremia, viral loads in nasal swabs and tonsils, and white blood cell counts were similar in animals inoculated with parental or recombinant viruses containing single (data not shown) or double (Fig. 4) Cys-to-Ser substitutions. Interestingly, pigs ($n = 8$) infected with E1ΔCys24/94v survived the infection and remained normal throughout the observation period (21 days) (Table 2 and Fig. 4). 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). Viral loads in these animals were significantly lower than those observed in pigs inoculated with parental virus, indicating a rather limited replication of this recombinant virus harboring substitutions in E1 at Cys24 and Cys94, resulting in an attenuated phenotype. Additionally, it appears that Cys171 plays a considerable role in pathogenesis since animals infected with double mutant viruses E1ΔCys5/171v and E1ΔCys20/171v survived longer (3 and 7 days, respectively) than did animals infected with parental BICv (Table 2).

E1ΔCys24/94v induces protection against lethal CSFV challenge. The limited *in vivo* replication kinetics shown by

TABLE 2. Swine survival and fever response following infection with CSFV E1ΔCys mutants and parental BICv

Virus	No. of survivors/total no.	Mean time to death, days (SD)	Fever		
			No. of days to onset (SD)	Duration, days (SD)	Maximum daily temp, °C (SD)
BICv	0/6[a]	9.5 (1.3)	3.5 (0.57)	5.75 (0.95)	41.3 (0.18)
E1ΔC5v	0/2	9.5 (2.1)	3.5 (0.7)	6 (1.4)	41.3 (0.08)
E1ΔC20v	0/2	7.5 (0.7)	3.5 (0.7)	4 (0)	41.2 (0.54)
E1C24v	0/2	13.5 (0.7)	3.5 (0.7)	10 (1.4)	41.2 (0.07)
E1ΔC94v	0/2	9.5 (3.5)	3.5 (0.7)	6 (2.8)	41.2 (0.15)
E1ΔC123v	0/2	9.5 (0.7)	3.5 (0.7)	6 (0)	41.1 (0.07)
E1ΔC171v	0/2	9.5 (4.9)	5 (1.4)	4.5 (3.5)	41.1 (0.16)
E1ΔC5/171v	0/3	12.5 (0.7)	4 (0)	8.5 (0.7)	41.7 (0.71)
E1ΔC20/171v	0/3	16.5 (0.7)	5 (1.4)	11.5 (0.7)	41.1 (0.08)
E1ΔC94/171v	0/3	11 (0)	4 (0)	7 (0)	41.4 (0.22)
E1ΔC24/94v	8/8[a]				39.8 (0.26)

[a] Values represent two different but identical experiments.

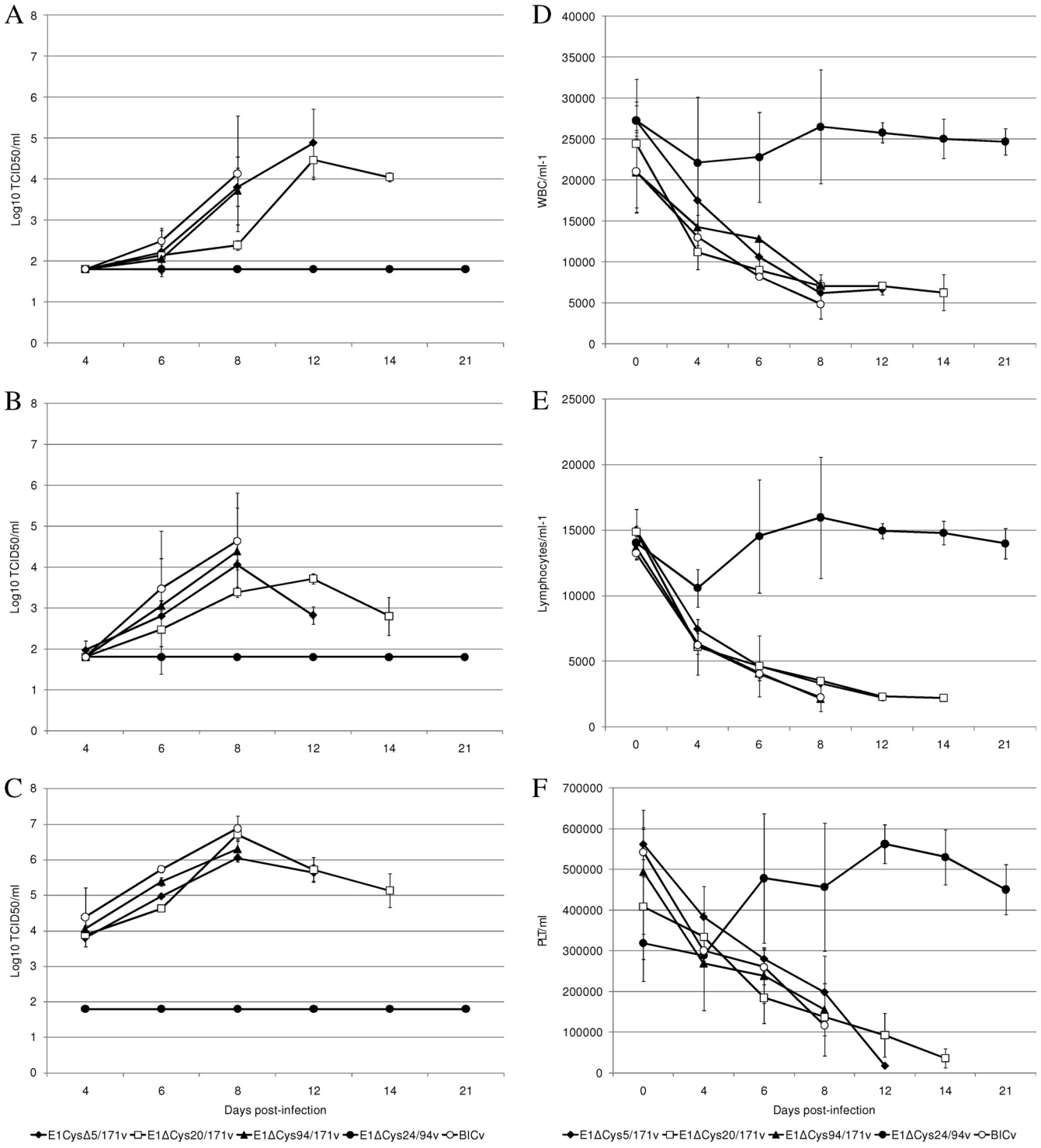

FIG. 4. Viral loads and hematologic changes detected in pigs inoculated with double E1ΔCys mutant and parental viruses. (A to C) Virus titers detected in nasal swabs (A), tonsil scrapings (B), and blood (C) after infection with E1ΔCys5/171v, E1ΔCys20/171v, E1ΔCys94/171v, E1ΔCys24/94v, or parental BICv. Each point represents the mean $\log_{10}$ $TCID_{50}$/ml and standard deviations from at least two animals. Sensitivity of virus detection, $\geq\log_{10}$ 1.8 $TCID_{50}$/ml. (D to F) White blood cell (WBC) (D), lymphocyte (E), and platelet (PLT) (F) counts after infection.

E1ΔCys24/94v is similar to that observed with CSICv (9), a live-attenuated vaccine virus that induces protection against BICv. In order to assess the ability of this double mutant to induce protection against CSFV, pigs were inoculated with E1ΔCys24/94v and challenged at 3 dpi (early) and at 28 dpi (late) with virulent BICv. Pigs were protected against an early and late challenge with BICv (Table 3 and Fig. 5). Mock-vaccinated control pigs developed anorexia, depres-

TABLE 3. Swine survival and fever response in E1ΔCys24/94v-infected animals after the challenge with parental virulent BICv

Challenge group	No. of survivors/total no.	Mean time to death, days (SD)	Fever		
			No. of days to onset (SD)	Duration, days (SD)	Maximum daily temp, °C (SD)
Mock	0/4	10.5 (1.5)	4.5 (0.57)	5.75 (0.95)	41.2 (0.19)
E1ΔCys24/94v, 3 dpi	3/4	10[a]	4[a]	6[a]	40.3 (0.57)
E1ΔCys24/94v, 28 dpi	7/7	0	0	0	39.7 (0.51)

[a] Data correspond to the only animal out of the four inoculated that presented CSFV-related symptoms.

sion, and fever by 4 days postchallenge (dpc) and a marked reduction in circulating leukocytes and platelets by 4 dpc (Fig. 5). Pigs died or were euthanized *in extremis* by 9 dpc (Table 2). Pigs inoculated with E1ΔCys24/94v exhibited partial protection by 3 dpi. Three out of four pigs survived infection and remained clinically normal, without significant changes in their hematological values (Fig. 5). All animals challenged at 28 days post-E1ΔCys24/94v infection were also protected, remaining clinically normal, without alterations of hematological profiles (Fig. 5). Virus was detected in low titers from blood, nasal swabs, and tonsil scrapings at 4, 6, and 8 dpc in some animals infected with E1ΔCys24/94v and challenged at 3 dpi (Fig. 5). BICv was undetectable in clinical samples obtained from any E1ΔCys24/94v-infected pigs that were challenged at 28 dpi.

Altogether, these results suggest that Cys24 and Cys94 are not critical for E1-E2 heterodimer formation in infected cells but that they have an effect on the overall rate of formation of these dimers that ultimately affects virus growth *in vitro* and *in vivo*, leading to a complete attenuation of the parental virus.

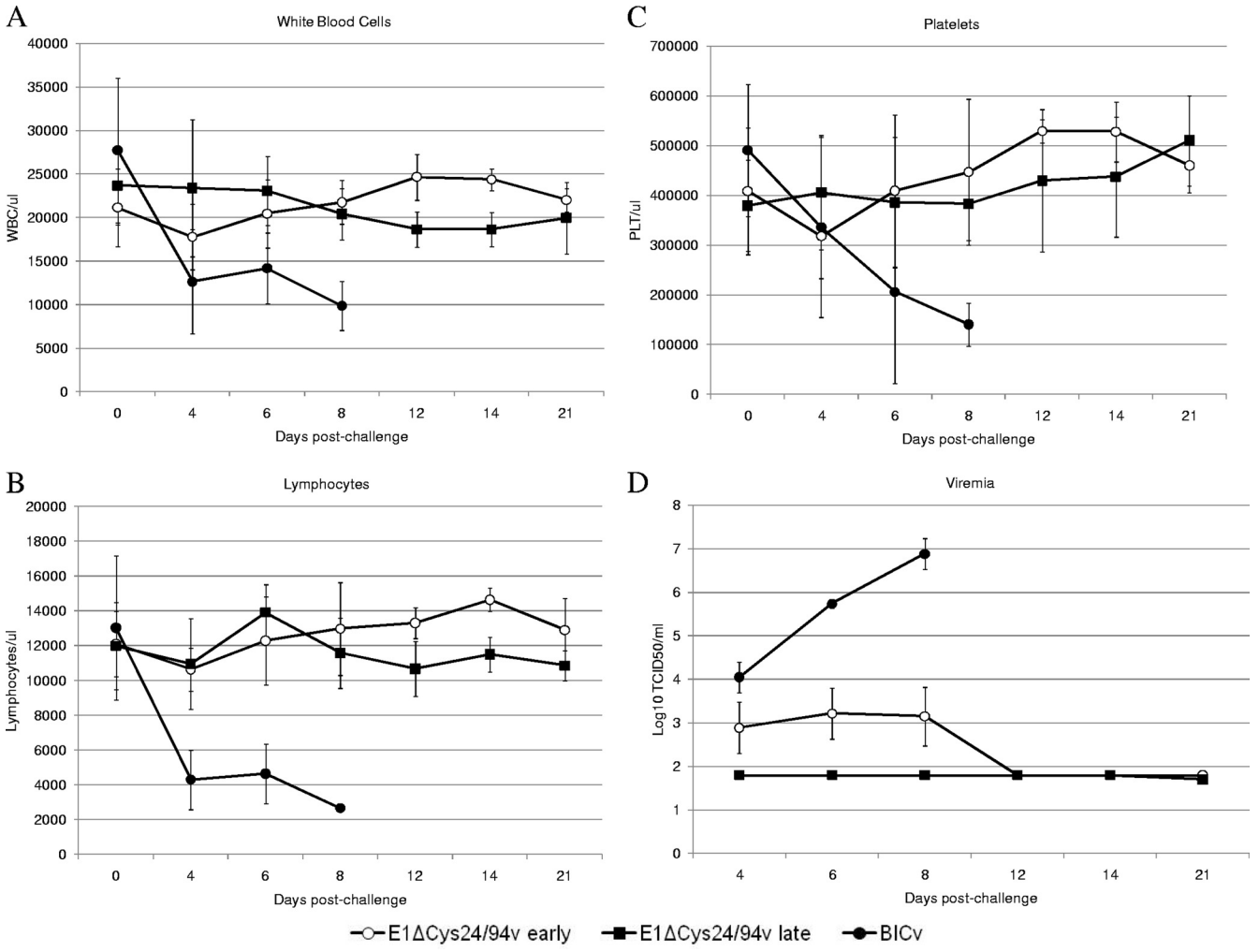

FIG. 5. Hematologic changes (A, B, and C) and viremia (D) detected in pigs inoculated with attenuated E1ΔCys24/94v and challenged with virulent BICv at 3 (early) or 28 (late) days postinfection. Values from four animals at each time point.

DISCUSSION

Cysteine bridges seem to play an important role in the formation of E1-E2 heterodimers (19, 22) and consequently impact the functioning of these glycoproteins during infection. We therefore performed a mutational analysis of the six cysteine residues in the E1 envelope protein within the context of a full-length infectious CSFV cDNA clone and analyzed infectivity of rescued recombinant viruses in primary swine macrophage cultures and in swine.

Substitution of each cysteine residue at positions 5, 20, 24, 94, 123, and 171 to serine in BICv E1 yielded viable mutant viruses. Introduced mutations did not affect formation of E1-E2 heterodimers in infected cells or virus infectivity either *in vitro* or *in vivo*, suggesting that more than one disulfide link may contribute to the formation of E1 and E2 heterodimers and to the functions of these proteins. A similar observation has been made with other viruses; a single substitution of conserved Cys171 to Ser (Fig. 1), located in the putative transmembrane domain of E1 protein, in the context of a BVDV E1- and E2-pseudotyped vesicular stomatitis virus, did not preclude formation of heterodimers or virus infectivity (13). It is possible that in these single-site mutants other interactions may contribute to formation of heterodimers and overall efficiency of the viral infection. Ronecker et al. (13) observed that substitutions of conserved Lys174Ala and Arg177Ala in BVDV E1 (Fig. 1) significantly reduced the formation of heterodimers in cells infected with pseudotyped viruses, indicating that these charged amino acids in the transmembrane domains of BVDV E1 also contribute to the interaction with E2.

The precise mapping of E1 Cys residues involved with active CSFV infection could not be determined in this study since 11 out of 15 possible combinations of double substitutions to Ser (Table 3) were deleterious, indicating that all these changes most probably affected morphogenesis of the virion. However, we also observed that three out of four double Cys mutant viruses rescued (E1ΔCys24/94, E1ΔCys5/171, E1ΔCys20/171, and E1ΔCys94/171) involve substitutions at Cys171, suggesting that this residue is not critical for virus infection. When inoculated into swine, these mutants retained the same capability of causing severe disease in swine as did parental BICv, showing that *in vivo* E1 functions are retained and not influenced by the lack of Cys residues at position 171. Conversely, no viral progeny was obtained when combined substitution involved Cys123, suggesting a critical role of this residue in the formation of mature virions.

The combined substitutions of Cys24 and Cys94 were not deleterious but significantly reduced BICv infectivity of swine macrophages and the ability of the virus to spread in SK6 cells. The decreased efficiency of viral infection by E1ΔCys24/94v corresponds with an impaired ability of mutated E1 to form heterodimers with E2 in infected cells, where a shift toward the accumulation of homodimers over heterodimers was observed. This finding suggests a contribution of these residues to form disulfide bonds with E2 that when modified may exert subtle conformational changes within E1 that impact active viral infection. Significant secondary structure changes in E1 due to Cys substitutions were not predicted (data not shown).

Our results suggest that Cys24 and Cys94 mutations affect BICv infectivity in swine. Different from the acute fatal disease induced by virulent BICv, infections caused by E1ΔCys24/94v were subclinical in swine and characterized by decreased viral loads in target organs and reduced virus shedding. No reversions to wild-type genotype were observed in viruses isolated from these animals. Attenuation of E1Cys24/94v 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. The effective protective immunity elicited by E1Cys24/94v suggests that modified E1 could be used for the development of live-attenuated vaccines. Similar studies have demonstrated that mutation or deletion of Cys171, one out of nine cysteine residues found in CSFV E^{rns} protein that is involved in the formation of homodimers via disulfide bonds, yielded infectious viruses lacking the capability to form homodimers in infected SK6 cells (18). These CSFV E^{rns} mutants were attenuated in pigs and induced a significant neutralizing antibody response in these animals.

In summary, our studies determined that substitutions of individual Cys residues in glycoprotein E1 are not essential for *in vivo* infectivity, exhibiting virulence characteristics similar to those of parental virus in the natural host. Data suggest that Cys24, Cys94, and Cys123 play major roles in E1 function, likely affecting virion morphogenesis by interacting with E2 protein. An improved understanding of the genetic basis and mechanisms of virus infection and virus virulence will permit rational design of efficacious biological tools for controlling CSF. Additionally, double Cys-to-Ser substitutions at positions 24 and 94 produce complete attenuation of the virulent Brescia isolate.

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