Virology 526 (2019) 38–44

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Identification of structural glycoprotein E2 domain critical to mediate replication of Classical Swine Fever Virus in SK6 cells

M.V. Borca[a,*], L.G. Holinka[a], E. Ramirez-Medina[b], G.R. Risatti[b], E.A. Vuono[a,c], K.A. Berggren[a,c], D.P. Gladue[a,*]

[a] ARS, USDA, Plum Island Animal Disease Center, Greenport, NY 11944, USA
[b] Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269, USA
[c] Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN, USA

ARTICLE INFO

Keywords:
CSFV
BVDV
Classical Swine Fever Virus
Bovine viral diarrhea virus
E2
Glycoprotein
Pestivirus

ABSTRACT

Envelope glycoprotein E2 of Classical Swine Fever Virus (CSFV) is involved in several critical virus functions. To analyze the role of E2 in virus replication, a series of recombinant CSFVs harboring chimeric forms of E2 CSFV and Bovine viral diarrhea virus (BVDV) were created and tested for their ability to infect swine or bovine cell lines. Substitution of native CSFV E2 by BVDV E2 abrogates virus replication in both cell lines. Substitution of individual domains in CSFV Brescia E2 by the homologous from BVDV produces chimeras that efficiently replicate in SK6 cells with the exception of a chimera harboring BVDV E2 residues 93–168. Further mapping revealed a critical area in E2 required for CSFV replication in SK6 cells between protein residues 136–156. This is the first report categorically defining a discrete portion of E2 as essential to pestivirus infection in susceptible cells.

1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine caused by CSF virus (CSFV), a small enveloped virus with a positive-sense, single-strand RNA genome (Becher et al., 2003). The approximately 12.5-kb CSFV genome contains a single open reading frame that encodes a polyprotein composed of 3898 amino acids that ultimately yields up to 12 final cleavage products (NH2-Npro-C-Erns-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 virion include the capsid (C) protein and glycoproteins: E^{rns}, E1 and E2. E^{rns}, a secreted protein, is loosely associated with the viral envelope (Weiland et al., 1990, 1999; Thiel et al., 1991) and does not have a hydrophobic transmembrane anchor domain. E^{rns} does, however, possess a C-terminal charged amphipathic segment that can mediate translocation of E^{rns} across bilayer membranes (Langedijk, 2002). E1 and E2 are transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor (Thiel et al., 1991). E2 is considered essential for CSFV replication, as virus mutants containing partial or complete deletions of the E2 gene are nonviable (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 (van Gennip et al., 2000; Liang et al., 2003) and also, modifications introduced into this glycoprotein appear to have an important effect on CSFV virulence (Risatti et al., 2005, 2006, 2007a, 2007b; Van Gennip et al., 2004).

The role of E2 in host range specificity has been analyzed obtaining dissimilar results among different pestiviruses. For instance, a C-strain virus with a chimeric E2 having the amino terminus 275 residues of BVDV did not have a drastic alteration in cell tropism compared to the parental virus, just showing a slower growth rate in SK6 cells without gaining the ability to replicate in bovine cells, as BVDV does (van Gennip et al., 2000). Conversely, a chimera having E2 of CSFV Alfort in the BVDV backbone, in contrast to the parental BVDV strain, only efficiently grows on porcine cells and very inefficiently infected bovine cells (Reimann et al., 2016). Additionally, a BVDV chimera harboring E2 from Border disease virus (BDV) replicated in sheep cells but showed a remarkable reduction in its ability to propagate in MDBK (Madin-Darby bovine kydney) cells, a phenotype that is characteristic of the BDV E2 donor (Liang et al., 2003). A similar model, but using an E2 from a different BDV strain, showed a change in its tropism from bovine to ovine cells (Rasmussen et al., 2007). Finally, the substitution of all structural proteins in a BVDV backbone by the homologous proteins of

* Corresponding authors.
E-mail addresses: Manuel.Borca@usda.gov (M.V. Borca), Douglas.Gladue@usda.gov (D.P. Gladue).

https://doi.org/10.1016/j.virol.2018.10.004
Received 16 August 2018; Received in revised form 12 September 2018; Accepted 2 October 2018
Available online 16 October 2018
0042-6822/ Published by Elsevier Inc.

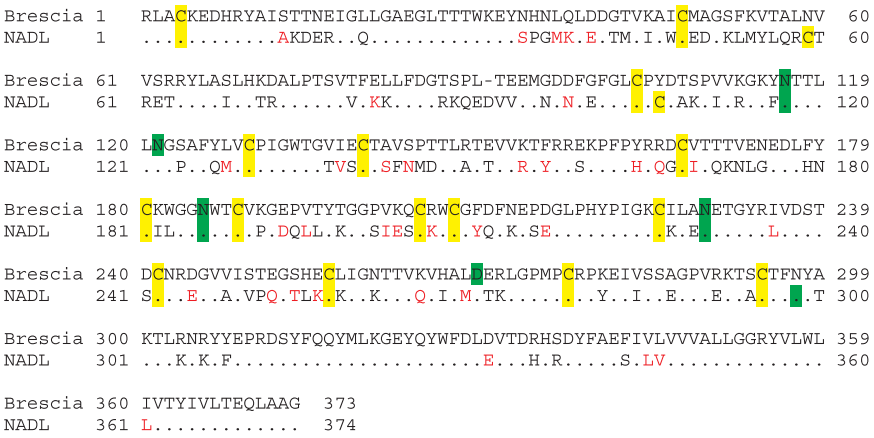

Fig. 1. Comparison of E2 amino acid sequence between CSFV Brescia and BVDV NADL. Alignment of amino acid sequences from CSFV and BVDV (gene bank # M31768.1 and AJ133738.1, respectively) showed identical (.), conserved (red) and non-conserved residues substitutions. Presence of cysteine residues are highlighted in yellow and putative glycosylation sites are highlighted in green.

the Bungowannah virus were not sufficient to completely transfer the Bungowannah cell tropism. Chimera virus RNA replication was either markedly reduced or absent in a number of different cell lines susceptible to Bungowannah infection but not to BVDV (Richter et al., 2014). Therefore, the precise role of E2 in the process of host cell interaction and tropism in pestiviruses is far from being completely understood.

Interestingly, our results demonstrated that substitution of CSFV E2 by BVDV E2 instead leading to a change in cell tropism from a swine (SK6) to a bovine (MDBK) cell line produced a chimeric virus unable to replicate. Moreover, we present here the identification of the CSFV E2 domain that is critical for replication in SK6 cells. A large series of recombinant CSF viruses harboring chimeric forms of E2 derived from the CSFV Brescia strain and the BVDV NADL strain were developed and tested for their ability to infect SK6 cells. We identified a specific stretch of amino acid residues between positions 132–157 of E2 as the critical area allowing CSFV Brescia strain to replicate in SK6 cells.

2. Materials and methods

2.1. Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990) and Madin-Darby bovine kidney (MDBK) cells, free of BVDV, were cultured in Dulbecco's minimal essential media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV Brescia strain was propagated in SK6 cells and was used for the construction of an infectious cDNA clone (Risatti et al., 2005). Titration of CSFV was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). After 4 days in culture, viral infectivity was assessed using an immunoperoxidase assay utilizing the CSFV monoclonal antibody WH303 (mAb WH303) (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titration of BVDV NADL was performed in MDBK cells. Titers were calculated according to the method that has been previously described (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml.

2.2. Construction of CSFV mutants

A full-length infectious clone (IC) of the virulent Brescia strain (pBIC) (Risatti et al., 2005) was used as a template to obtain all cDNA IC constructs described in this report. Chimeric constructs used in this report were obtained by DNA synthesis (Epoch Life Sciences, Sugar Land, TX, USA). Each of the IC constructs were completely sequenced to verify that only site-directed mutagenesis-induced changes were present.

2.3. In Vitro Rescue of CSFV Brescia and derivative mutants

Full-length genomic clones were linearized with *Srf*I and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX) (Risatti et al., 2005). 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 6-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.

2.4. 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 transfected cells were sequenced in the region of the genome that contained the desired mutations. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data was assembled using Sequencher 4.7™ software (Genes Codes Corporation, Ann Arbor, MI). The final DNA consensus sequence represented, on average, a three- or four-fold redundancy at each base position.

3. Results

3.1. Amino acid analysis between CSFV Brescia E2and BVDV NADL E2

Comparison of the amino acid sequences of CSFV Brescia E2 and BVDV NADL E2 using BLAST (Altschul et al., 1990) results in 65% identity and 76% similarity between the two proteins. Brescia E2 is 373 amino acids long while NADL E2 is 374, having an additional aspartic acid residue inserted at position 91 (Fig. 1). Brescia possesses 5 predicted N-glycosylation sites (Gupta and Brunak, 2002) at positions 116, 121,185, 229 and 269, while NADL has 4 predicted N-glycosylation sites at positions 117, 186, 230, and 298. It was previously shown (Branza-Nichita et al., 2002) that cysteine bonds occur between E2 homodimers and E2-E1 heterodimers. All fifteen cysteine residues present in Brescia E2 are also present in NADL E2 with the addition of two cysteine residues at positions 59 and 106 in NADL E2. Therefore, in general, both proteins present a high degree of similarity at the structural level.

3.2. BVDV E2does not transfer BVDV host range specificity to CSFV Brescia infectious clone

CSFV derived from the Brescia infectious clone (BICv) efficiently

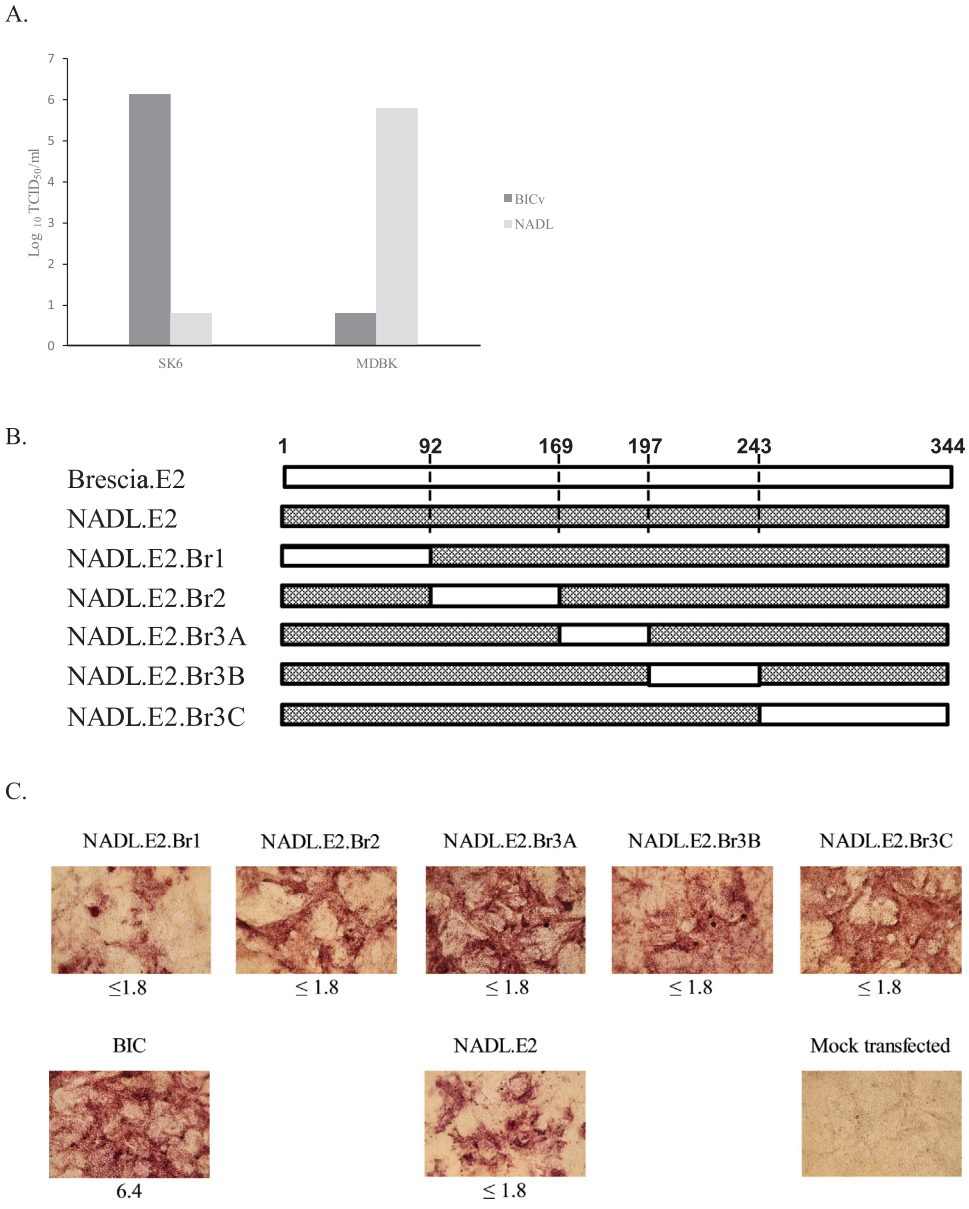

Fig. 2. (A) Replication of CSFV Brescia and BVDV NADL in SK6 and MDBK cells. Either cell line was infected (MOI = 1) with BICv and BVDV NADL and presence of virus progeny detected at 96 (BICv) or 48 hs (BVDV NADL). Data show a representative experiment of three repetitions. (B) Schematic representation of NADL.E2, NADL.E2.Br1, NADL.E2.Br2, NADL.E2.Br3A, NADL.E2.Br3B, and NADL.E2.Br3C constructs. (C) SK6 cells transfected with constructs NADL.E2, NADL.E2.Br1, NADL.E2.Br2, NADL.E2.Br3A, NADL.E2.Br3B, and NADL.E2.Br3C were stained at the fourth day post transfection (dpt) with anti-E^{rns} monoclonal antibody WH174. Virus yield (shown in the bottom of each transfection picture) were detected in cell extracts also at the fourth dpt. Titrations were performed in SK6 cells (additionally in MDBK for NADL.E2 construct) and titers expressed as TCID50/ml. Sensitivity of titration log10 > 1.8 TCID50/ml.

replicates in a porcine cell line, SK6, but not in MDBK, a bovine-derived cell line. Conversely, BVDV strain NADL efficiently replicates in MDBK but not in SK6 cells (Fig. 2A). To analyze the role of E2 in defining species host range a chimeric CSFV infectious clone (IC) construct, based in a BICv genetic backbone where CSFV E2 was replaced with that of BVDV NADL E2 (NADL.E2), was developed (Fig. 2B). The first 10 native CSFV amino acid residues on the N-terminus and C-terminus of the protein were conserved to ensure that E2 would be cleaved in the CSFV background. NADL.E2 construct was *in vitro* transcribed and the RNA used to transfect SK6 cells. Transfection was followed by protein expression, detected by immunocytochemistry reactivity against E^{rns} specific monoclonal antibody (mAb) WH174 (Fig. 2C). Titration of extracts of the transfected cells in both SK6 (Fig. 2C) and MDBK cells (data not shown) demonstrated that NADL.E2 is completely unable to replicate in both cell lines. This indicates that, in this model, E2 is not fully responsible for host cell species tropism. Attempts to reinstate the ability of the NADL.E2 construct to replicate in SK6 cells by substituting its individual E2 domains by the homologous domains from CSFV Brescia were largely unsuccessful. Five chimeric CSFV IC constructs (NADL.E2.Br1, NADL.E2Br.2, NADL.E2.Br3A, NADLE.2Br.3B, and NADL.E2Br.3C) were developed where each of the constructs has a single Brescia E2 domain. Domains of E2 were defined based on the crystal structure analysis identifying three distinct domains (Br1, Br2, Br3), with the third domain consisting of three distinct small B-sheet modules (Br.3A, Br.3B, Br.3C) (Li et al., 2013), in the context of the NADL E2. RNA transcripts were transfected in SK6 cells followed by evident levels of protein expression (Fig. 2C) as evidenced by immunocytochemistry using mAb WH174. However, titration in SK6 cells of transfected cell extracts were negative (Fig. 2C).

3.3. Determination of CSFV Brescia E2domains critical for virus replication

Since complete replacement of CSFV Brescia E2 using BVDV E2 (NADL.E2 construct) entirely abrogates virus replication in SK6 cells we developed 5 chimeric constructs of CSFV IC (Br.E2.NADL1, Br.E2.NADL2, Br.E2.NADL3A, Br.E2.NADL3B, and Br.E2.NADL3C) where each of the constructs has a single NADL E2 domain in the context of the Brescia E2 glycoprotein. These constructs were used to identify areas of Brescia E2 that are critical for CSFV replication in SK6 cells. (Fig. 3A). Each of the cDNA constructs were *in vitro* transcribed and the corresponding RNA was transfected into SK6 cells. Presence of protein expression (assessed by immunocytochemistry staining using

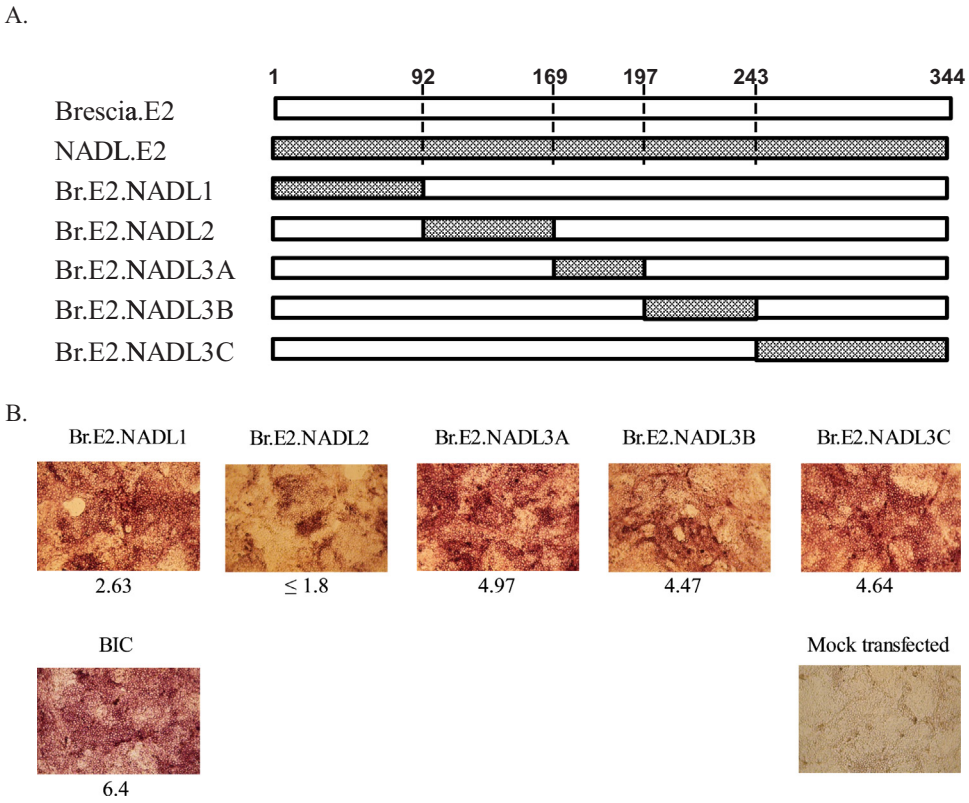

Fig. 3. (A) Schematic representation of Br.E2.NADL1, Br.E2.NADL2, Br.E2.NADL3A, Br.E2.NADL3B, and Br.E2.NADL3C constructs. (B) SK6 cells transfected with constructs indicated in (A) were stained and titrated as it was described in Fig. 2C.

E^{rns} specific mAb WH174) were clearly detected in cells transfected with all the constructs (Fig. 3B). Interestingly, cell extracts of transfections from all constructs produced different levels of virus progeny with the exception of construct Br.E2.NADL2 presenting no detectable level of virus (Fig. 3C). Therefore, although replacement of homologous domains of Brescia E2 by the corresponding domain in NADL mostly supports virus replication in SK6 cells, the replacement of domain 2 eliminated the ability of the chimeric virus to replicate in SK6 cells, suggesting that domain 2 of E2 must play a critical role for CSFV replication in SK6 cells.

Domain 2 of Brescia E2, as design in this report, consists of a 78 amino acid stretch that spans the area of amino acids 92–168 in the glycoprotein (Fig. 3B). To identify the critical amino acids responsible for virus replication in SK6 cells, we developed three chimeric constructs (Br.E2.NADL2A, Br.E2.NADL2B and Br.E2.NADL2C) where each of these constructs has a portion of domain 2 of NADL E2 in the context of Brescia E2. Therefore, the chimeras harbor NADL E2 amino acid areas between amino acids 92–135, 136–156 and 157–168, respectively. Alignment of E2 amino acid sequences between Brescia and NADL showed 12, 12 and 10 amino acid substitutions in regions 2A, 2B and 2C, respectively (Fig. 4A). SK6 cells were transfected with *in vitro* transcribed viral RNAs, and presence of protein expression in the transfected cells was clearly detected four days later by immunocytochemistry staining using E^{rns} specific mAb WH174 (Fig. 4B). Cell extracts of these transfections were titrated in SK6 cells to assess the presence of virus progeny. Interestingly, constructs Br. E2.NADL2A and Br. E2.NADL2C produced evident virus yield while cells transfected with construct Br.E2.NADL2B failed to produce any detectable virus (Fig. 4C). In order to confirm that swapping areas 2A and 2C with NADL E2 in Brescia E2 had no deleterious effect on CSFV replication in SK6 cells, we developed a new chimera construct (Br.E2.NADL2AC) containing both areas 2A and 2C of E2 NADL in the context of Brescia E2 (Fig. 4C). SK6 cells transfected with Br.E2.NADL2AC *in vitro* transcribed RNA showed evident protein expression in immunocytochemistry staining using E^{rns} specific mAb WH174 as well as presence of infectious virus (Fig. 4C), confirming that areas 2A and 2C in Brescia E2 can be substituted with the corresponding homologous areas of NADL E2, retaining the ability of CSFV replication in SK6 cells. This result further suggests that area 2B or residues 137–156 in E2 are required for CSFV replication in SK6 cells.

3.4. Identification of critical amino E2acid residues mediating CSFV replication in SK6 cells

To determine the critical area between residues 136 and 156 of CSFV Brescia E2 required to allow virus infectivity, a new set of chimeric constructs was constructed where native Brescia E2 amino acid residues were progressively substituted from E2 Brescia to E2 NADL amino acids in the backbone of Br.E2.NADL2B. Thus, a series of seven chimeric mutants (Br.E2.NADL.T1–7) were generated, progressively incorporating NADL residues to replace divergent homologous ones in Brescia E2 between amino acid resides 136 and 160 (Fig. 5). All the BrE2NADL.T1–7 cDNA constructs were *in vitro* transcribed and the obtained RNA transfected into SK6 cells. Presence of protein expression in the transfected cells was clearly detected four days later by immunocytochemistry staining using E^{rns} specific mAb WH174. Titers of rescued chimeric viruses showed a progressive decreased along the accumulation of native CSFV residues being substituted by the corresponding ones in BVDV. Complete absence of virus progeny were observed only when all native CSFV divergent amino acid residues in domain 2B were replaced with the homologous NADL residues. These results indicate that the 2B domain or residues 136–157, in E2 is required for CSFV replication in SK6 cells.

To rule out the possibility that expanding 2B with additional amino acids from BVDV would result in viral replication in SK6 cells a series of additional mutants were made, T8–15. All the Br.E2.NADL.T8–15 cDNA

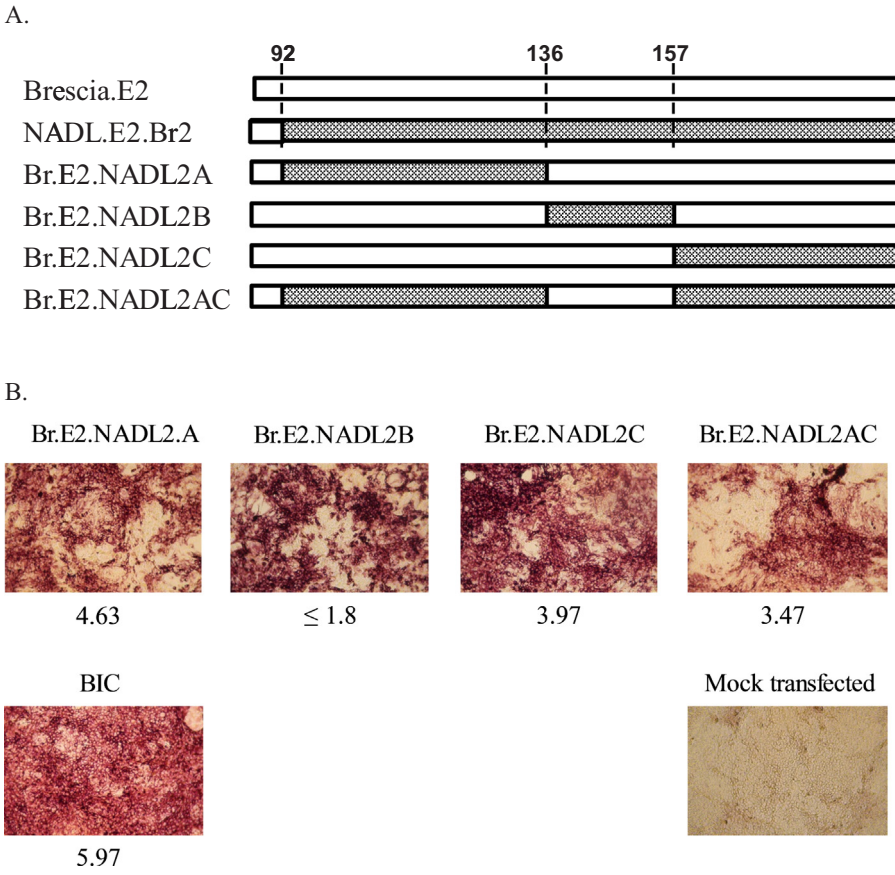

Fig. 4. (A) Schematic representation of Br.E2.NADL2A, Br.E2.NADL2B, Br.E2.NADL2C, and Br.E2.NADL2AC constructs. (B) SK6 cells transfected with constructs indicated in (A) were stained and titrated as it was described in Fig. 2C.

constructs were *in vitro* transcribed and the obtained RNA transfected into SK6 cells and presence of protein expression in the transfected cells was clearly detected four days later by immunocytochemistry staining using E^{rns} specific mAb WH174. However, none of the additional mutants produced virus progeny, further suggesting that the area within 2B is the minimal area required for CSFV to replicate in SK6 cells (Fig. 5).

4. Discussion

CSFV structural glycoprotein E2 is essential for CSFV replication (van Gennip et al., 2002) and has been critically implicated in viral adsorption to host cells (van Gennip et al., 2000; Liang et al., 2003). Accordingly, many efforts have been made in linking pestivirus E2 in the determination of species host range. Results obtained using different approaches were not conclusive, in fact they are quite contradictory. In this report, swapping E2 from BVDV into the CSFV Brescia background does not increase the ability of BrE2NADL to replicate in bovine MDBK cells although it does abolish replication in swine SK6 cells.

Similarly, a C-strain virus harboring a chimeric BVDV E2 showed no gain in its ability to grow in bovine cells and slowed its growth rate in

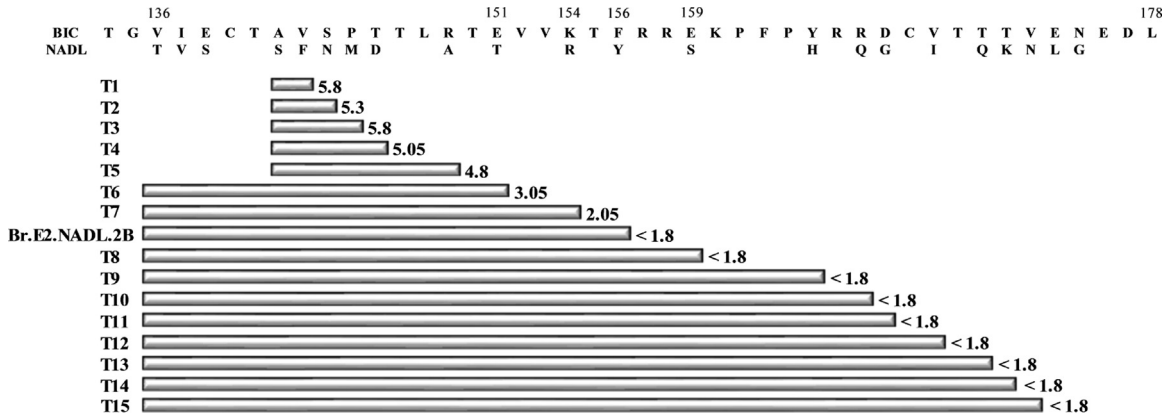

Fig. 5. (A) Schematic representation of constructs Br.E2.NADL.T1 to Br.E2.NADLT.15 and Br.E2.NADL.2B. Alignment of CSFV Brescia E2 and BVDV NADL amino acid sequence between residues at positions 136–179 are shown in the top. Red horizontal bars depict area where native CSFV residues were substituted by their homologous in BVDV in each of the sixteen constructs. Virus yield (shown in the right end of each bar) were detected in cell extracts also by the fourth dpt. Titrations were performed in SK6 cells and titers expressed as TCID50/ml. Sensitivity of titration log10 $\geq$ 1.8 TCID50/ml.

SK6 cells (van Gennip et al., 2000). In addition, substitution of structural proteins in a BVDV backbone by the homologous proteins of the Bungowannah virus did not transfer its cell tropism to the receptor chimera (Richter et al., 2014). Conversely, other models produced different results when swapping cell tropisms between the donor E2 virus and the receptor virus backbone. Chimeric BVDV harboring E2 of CSFV Alfort or from Border disease virus (BDV) gained donor E2 cell tropism, acquiring the ability to efficiently grow in porcine and ovine cells, respectively, while losing the capability to replicate in bovine cells (Liang et al., 2003; Reimann et al., 2004). Therefore, the role of E2 in host cell tropism remains unclear in these pestivirus models. Perhaps in some of the models, as in the one reported here, cell tropism is not only restricted by the presence of E2 but also by additional cellular mechanisms required to completely mediate virus replication (Richter et al., 2014). Further research is needed to fully understand the molecular virus/host requirements defining species tropism among different pestiviruses.

Regardless of these contradictory results determining the role of E2 in defining host cell tropism, there is no debate about the involvement of the protein in the process of virus replication (van Gennip et al., 2002). Particularly, E2 has been implicated, along with E^{rns} (Hulst and Moormann, 1997) and E1 (Wang et al., 2004), in viral adsorption to host cells (van Gennip et al., 2000; Liang et al., 2003). In this regard, there is little direct information on the regions of E2 functionally critical in the process of virus infection. We took advantage of the fact that BVDV NADL E2 completely abrogates the ability of CSFV Brescia to identify those critical regions using a large set of CSFV ICs harboring chimeric Brescia/NADL E2 glycoproteins. Our results identified E2 amino acid residues between positions 138–151 of E2 as playing a critical role in CSFV Brescia replication in SK6 cells.

This particular area of E2 has been the focus of diverse research activities. It harbors a unique amino acid sequence conserved among CSFV isolates but absent in other pestiviruses (Edwards et al., 1991). Consequently, the area is recognized by a CSFV-specific monoclonal antibody, WH303 (Edwards et al., 1991; Lin et al., 2000) that has been used for the development of a DIVA antigenic marker in CSFV experimental vaccines (Risatti et al., 2006). Additionally, this area harbors linear epitopes that induce virus neutralizing antibodies and synthetic peptides representing this E2 fragment have been shown to induce protection in pigs against challenge with virulent isolates (Liu et al., 2006). Also, previous results from our laboratory already demonstrated that specific substitutions in the CSFV Brescia E2 area between amino acid residues 140–148 with the homologous residues from BVDV (TAVSPTTLR) reduced virus replication in SK6 cells up to 10-fold, suggesting that this area is involved in the process of virus attachment/replication (Risatti et al., 2006). Interestingly, indirect evidence has been reported indicating the role of this area of E2 in the process of cell attachment (Li et al., 2011). A synthetic peptide resembling the area between residues 141 and 170 of glycoprotein CSFV E2 specifically attaches to target cell surfaces and inhibits virus replication.

Using the crystal structure of BVDV E2 as a guide (Li et al., 2013) (Fig. 6), the area identified within E2 in this study (residues 137–156) that is required for replication in SK6 cell cultures is located in an region that is mostly projecting outwards from the rest of E2 in a small loop-like arrangement of amino acids with an additional adjacent domain of accessible residues. Interestingly, residue substitutions in mutant BrE2NADL.T5, harboring only mutations in the outward projecting loop, do not appear to induce important alterations in the process of CSFV replication. However, additional mutational changes in BrE2-NADL.T6 and T7, comprising both the outward projecting loop and the adjacent molecular structure, causes a progressive decrease in virus growth ability in SK6 cells. In the case of mutant Br.E2.NADL2B, growth in SK6 cells was completely abolished. This suggests the possibility that these mutations, when fully changed to NADL residues, may suffer conformational changes affecting potential binding domains in E2 that prevents the ability of growth in SK6 cultures. Further work will need to be performed to determine what structural changes actually do occur.

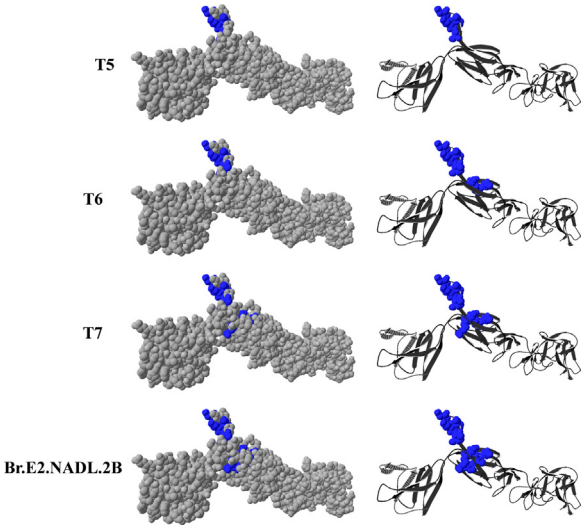

Fig. 6. Three-dimensional location of E2 amino acid residues identified as critical for CSFV replication in SK6 cells. The crystal structure of BVDV E2 (PDB# 4JNT) (Gladue et al., 2014) was used as a template to visualize the locations of the mutated amino acids in the indicated E2 mutants. Amino acids that were mutated from CSFV to BVDV residues are colored in blue in each of the indicated mutants. On the left is a space filling model while on the right is a ribbon model of BVDV E2.

We have previously shown that CSFV protein E2 specifically interacts with several swine host proteins (Gladue et al., 2014). Although no mapping of E2 residues mediating these interactions have been reported, it appears that most of these E2 interacting sites are conformational dependent (Gladue et al., 2014). Therefore, it is possible that small structural differences between CSFV and BVDV E2 might affect the ability of the chimeric protein to interact with swine host partner proteins and that this binding could be necessary to allow for virus particle formation or virus replication. Again, further work needs to be performed to determine if these disruptions of interaction among proteins do actually occur.

In summary, we present here the identification of CSFV E2 domain (s) critical in the process of replication in SK6 cells. A series of recombinant CSF viruses harboring chimeric forms of E2 between CSFV Brescia isolate and the BVDV strain NADL were developed and tested for their ability to infect a swine derived cell line, SK6. Results reported here clearly identified a specific stretch of amino acid residues between positions 136–157 of E2 as the critical area allowing CSFV Brescia strain replication in SK6 cells. We believe data presented here is the first report categorically defining in a direct manner a discrete portion of E2 as essential to the progress of pestivirus infection in susceptible cells.

Acknowledgements

We specially thank Melanie Prarat for editing the manuscript. We thank Dr. Trevor Drew and Dr. Helen Crooke from Animal and Plant Health Agency (APHA) and Dr. Edward Joseph Dubovi at Cornell University for supplying antibodies that were used in this study. This research was supported in part by an appointment to the Plum Island Animal Disease Center (PIADC) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). ORISE is managed by ORAU under DOE contract number DE-SC0014664. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, APHIS, DHS, DOE, or ORAU/ORISE.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., Konig, M., Schweizer, M., Stalder, H., Schirrmeier, H., Thiel, H.J., 2003. Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. Virology 311, 96–104.

Branza-Nichita, N., Lazar, C., Durantel, D., Dwek, R.A., Zitzmann, N., 2002. Role of disulfide bond formation in the folding and assembly of the envelope glycoproteins of a pestivirus. Biochem. Biophys. Res. Commun. 296, 470–476.

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

Gladue, D.P., Baker-Bransetter, R., Holinka, L.G., Fernandez-Sainz, I.J., O'Donnell, V., Fletcher, P., Lu, Z., Borca, M.V., 2014. Interaction of CSFV E2 protein with swine host factors as detected by yeast two-hybrid system. PLoS One 9, e85324.

Gupta, R., Brunak, S., 2002. Prediction of glycosylation across the human proteome and the correlation to protein function. Pac. Symp. Biocomput. 310–322.

Hulst, M.M., Moormann, R.J., 1997. Inhibition of pestivirus infection in cell culture by envelope proteins E(rns) and E2 of classical swine fever virus: E(rns) and E2 interact with different receptors. J. Gen. Virol. 78 (Pt 11), 2779–2787.

Langedijk, J.P., 2002. Translocation activity of C-terminal domain of pestivirus Erns and ribotoxin L3 loop. J. Biol. Chem. 277, 5308–5314.

Li, X., Wang, L., Zhao, D., Zhang, G., Luo, J., Deng, R., Yang, Y., 2011. Identification of host cell binding peptide from an overlapping peptide library for inhibition of classical swine fever virus infection. Virus Genes 43, 33–40.

Li, Y., Wang, J., Kanai, R., Modis, Y., 2013. Crystal structure of glycoprotein E2 from bovine viral diarrhea virus. Proc. Natl. Acad. Sci. USA 110, 6805–6810.

Liang, D., Sainz, I.F., Ansari, I.H., Gil, L.H., Vassilev, V., Donis, R.O., 2003. The envelope glycoprotein E2 is a determinant of cell culture tropism in ruminant pestiviruses. J. Gen. Virol. 84, 1269–1274.

Lin, M., Lin, F., Mallory, M., Clavijo, A., 2000. Deletions of structural glycoprotein E2 of classical swine fever virus strain alfort/187 resolve a linear epitope of monoclonal antibody WH303 and the minimal N-terminal domain essential for binding immunoglobulin G antibodies of a pig hyperimmune serum. J. Virol. 74, 11619–11625.

Liu, S., Tu, C., Wang, C., Yu, X., Wu, J., Guo, S., Shao, M., Gong, Q., Zhu, Q., Kong, X., 2006. The protective immune response induced by B cell epitope of classical swine fever virus glycoprotein E2. J. Virol. Methods 134, 125–129.

Rasmussen, T.B., Uttenthal, A., Reimann, I., Nielsen, J., Depner, K., Beer, M., 2007. Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus. J. Gen. Virol. 88, 481–486.

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

Reimann, I., Depner, K., Trapp, S., Beer, M., 2004. An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. Virology 322, 143–157.

Reimann, I., Blome, S., Beer, M., 2016. Chimeric pestivirus experimental vaccines. Methods Mol. Biol. 1349, 239–246.

Rice, C.M., 1996. Flaviviridae: The Viruses and Their Replication. In: Knipe aPH, B.N.F.D.M. (Ed.), Fundamental Virology, 3rd ed. Lippincott, Raven, Philadelphia, pp. 931–959.

Richter, M., Reimann, I., Schirrmeier, H., Kirkland, P.D., Beer, M., 2014. The viral envelope is not sufficient to transfer the unique broad cell tropism of Bungowannah virus to a related pestivirus. J. Gen. Virol. 95, 2216–2222.

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

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

Risatti, G.R., Holinka, L.G., Fernandez Sainz, I., Carrillo, C., Kutish, G.F., Lu, Z., Zhu, J., Rock, D.L., Borca, M.V., 2007a. Mutations in the carboxyl terminal region of E2 glycoprotein of classical swine fever virus are responsible for viral attenuation in swine. Virology 364, 371–382.

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

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

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

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

van Gennip, H.G., van Rijn, P.A., Widjojoatmodjo, M.N., de Smit, A.J., Moormann, R.J., 2000. Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. Vaccine 19, 447–459.

van Gennip, H.G., Bouma, A., van Rijn, P.A., Widjojoatmodjo, M.N., Moormann, R.J., 2002. Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E(rns) or E2 of CSFV. Vaccine 20, 1544–1556.

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

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

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

Weiland, F., Weiland, E., Unger, G., Saalmuller, A., Thiel, H.J., 1999. Localization of pestiviral envelope proteins E(rns) and E2 at the cell surface and on isolated particles. J. Gen. Virol. 80 (Pt 5), 1157–1165.