Restoration of glycoprotein E<sup>rns</sup> dimerization via pseudoreversion partially restores virulence of classical swine fever virus

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Abstract

The classical swine fever virus (CSFV) represents one of the most important pathogens of swine. The CSFV glycoprotein E<sup>rns</sup> is an essential structural protein and an important virulence factor. The latter is dependent on the RNase activity of this envelope protein and, most likely, its secretion from the infected cell. A further important feature with regard to its function as a virulence factor is the formation of disulfide-linked E<sup>rns</sup> homodimers that are found in virus-infected cells and virions. Mutant CSFV lacking cysteine (Cys) 171, the residue responsible for intermolecular disulfide bond formation, were found to be attenuated in pigs (Tews BA, Schürmann EM, Meyers G. J Virol 2009;83:4823–4834). In the course of an animal experiment with such a dimerization-negative CSFV mutant, viruses were reisolated from pigs that contained a mutation of serine (Ser) 209 to Cys. This mutation restored the ability to form disulphide-linked E<sup>rns</sup> homodimers. In transient expression studies E<sup>rns</sup> mutants carrying the S209C change were found to form homodimers with about wt efficiency. Also the secretion level of the mutated proteins was equivalent to that of wt E<sup>rns</sup>. Virus mutants containing the Cys171Ser/Ser209Cys configuration exhibited wt growth rates and increased virulence when compared with the Cys171Ser mutant. These results provide further support for the connection between CSFV virulence and E<sup>rns</sup> dimerization.

INTRODUCTION

Classical swine fever virus (CSFV) is classified as one member of the genus Pestivirus within the family Flaviviridae that also comprises the genera Flavivirus, Hepacivirus and Pegivirus [1]. The genus Pestivirus includes also two types of bovine viral diarrhoea virus (BVDV-1 and BVDV-2), and Border disease virus (BDV) of sheep. All these viruses are important pathogens of livestock. To include also related viruses, a new classification concept with 11 species named pestivirus A to K has been proposed [2]. CSFV (proposed pestivirus C), induces a severe, sometimes haemorrhagic disease in pigs and wild boar. Nowadays, fatal CSFV in adult pigs is more rarely seen, due to the evolution of less virulent CSFV strains establishing longer lasting infections. Nevertheless, infection of pigs with CSFV still has enormous commercial impact due to reduced productivity and the induction of abortion and fetal malformation in pregnant sows [3].

Members of the Flaviviridae are enveloped RNA viruses with a single-stranded, positive-sense genome with one ORF. The pestiviral genomic RNA has a size of ~12,3 kb and codes for a polyprotein of about 4000 amino acids. Co- and post-translational processing results in 12 mature proteins [3, 4], among which four, C, E<sup>rns</sup>, E1 and E2, represent structural components of the virion [5]. E<sup>rns</sup>, E1 and E2 are found in the viral lipid envelope, with E<sup>rns</sup> and E2 being targets for neutralizing antibodies and inducers of protective immunity in infected animals [5–8]. The pestivirus envelope proteins form disulphide-linked dimers that are found in infected cells and virions. Homodimers of E<sup>rns</sup> and E2, as well as heterodimers of E1 and E2, have been described [3, 5].

The repression of the host’s innate immune system is a crucial factor for the fitness of viruses [9]. Pestiviruses express two unique proteins for which counterparts are missing in the other family members [3]. These two proteins, the non-structural protein N<sup>pro</sup> and the viral envelope protein E<sup>rns</sup>,...
are involved in repression of the host’s type 1 IFN (IFN-1) response, which can be regarded as an initial and crucial reaction to virus infection [10–22]. Npro represents a non-essential non-structural protein exhibiting protease activity [3, 23–27]. Npro is found in the cytoplasm of infected cells and blocks the type 1 IFN response to the infection by inducing degradation of IFN regulatory factor 3 [13–22].

In contrast to Npro, Ems represents an essential component of the virion. Viral RNAs lacking the Ems-coding region represent replicons unable to produce infectious virus particles [28–30], (G. Meyers, unpublished results). A unique feature of Ems among surface proteins is its intrinsic RNase activity. The Ems sequence contains a motif typical for members of the T2/S RNase superfamily [28, 31–35]. Ems lacks a typical transmembrane region but is bound to the viral envelope by a C-terminal amphipathic helix [36–39]. This type of membrane anchor is highly unusual for a surface protein and is responsible for the secretion of considerable amounts of Ems into the extracellular space [37, 39, 40]. A recent publication has provided evidence that the amphipathic helix of the Ems membrane anchor is also important for the release of infectious CSFV and can replace host-derived apolipoproteins during release of hepatitis C virus, whereas CSFV particle formation in the absence of Ems could partially be rescued by apolipoproteins [41].

Ems is an important factor for pestivirus virulence and pathogenicity since recombinant RNase-negative pestiviruses with a mutation of active centre residues in Ems are clinically attenuated in the natural host even though their replication efficiency in tissue culture is not significantly affected [12, 37, 39, 40, 42, 43]. We found that not only RNase activity but also the formation of Ems homodimers is important for pestivirus virulence. Prevention of dimerization by mutations affecting the cysteine (Cys) 171 resulted in significant attenuation of CSFV [44]. We report here on the identification and characterization of pseudorevertants that regained the ability to establish homodimers via a mutation of serine (Ser) at position 209 to Cys. Our results provide further evidence for the importance of Ems homodimer formation for pestivirus virulence.

RESULTS
Identification and characterization of pseudorevertants of CSFV dimerization-negative mutants

In earlier work we established different CSFV mutants with alterations affecting Cys 171 of the Ems protein [44]. This amino acid is responsible for Ems homodimer formation. The different virus mutants displayed either a deletion of codon 171 or exchanges for phenylalanine or Ser codons and were all deficient in Ems homodimerization. In tissue culture, the recovered mutants showed only a slight growth retardation in comparison to the wt virus but proved to be significantly attenuated in the natural host [44].

Sequence analyses of the Ems-coding region of viruses reisolated from blood of the infected animals between days 6 and 13 revealed the presence of mixed sequences composed of TTC and TGC/TGT/TCC for codon 171 of viruses isolated from animals infected with the V-C171F mutant (the sequence of the wt CSFV is TGT) (Fig. 1). This finding indicated a tendency for reversion. However, there was no increase of the reverted sequence over time indicating that the viruses with the altered sequences had at least no dramatic selective advantage. To our surprise, we found an additional alteration affecting codon 209 in some cDNA sequences determined with samples from animals infected with either the V-C171F or the V-C171d mutant. The wt codon 209 is AGT (Ser) and was in part replaced by TGT resulting in a S209C mutation (Fig. 1). Again, this change

Fig. 1. Graphs of sequences determined with RNA extracted from cells infected with viruses reisolated from pigs in the course of the animal studies described before [44]. On the left, sequences around codon 171 obtained from animals infected with the V-C171F variant are shown. The graph on top displays the sequence of the original virus mutant, whereas the middle graph presents an isolate with a mixture of a phenylalanine-coding triplet and a reversion to a Cys codon. A reversion to the latter is found in the bottom graph together with a variable wobble base of codon 170. On the right, sequence variation for codon 209 is demonstrated with the original AGT (top), mixture of AGT and a lower level of TGT (middle) and almost exclusive pseudoreversion to TGT (bottom). The codons of interest are marked by a box.
was not becoming dominant over time. We inserted E\textsuperscript{rns}-coding cDNA from these samples into a plasmid vector, and determined the sequence of individual clones. As expected, some of the cloned plasmids clearly displayed the S209C mutation together with the original change at position 171.

The reintroduction of a Cys into the E\textsuperscript{rns} membrane anchor region but within a distance of 38 residues from the position of the original C171 raised the question of whether E\textsuperscript{rns} can form homodimers linked via a Cys at position 209. We therefore expressed different variants of E\textsuperscript{rns} transiently and looked for homodimers. We analysed constructs with codons 171C/209C, 171D/209C, 171S/209C, 171D/209S and wt 171C/209S (2091A, 2091B, 2091C, 2091D, 2091E and pI1, respectively). For all constructs containing a Cys codon in the carboxyterminal region, E\textsuperscript{rns} dimers could be detected in addition to monomers (Fig. 2a). For construct 2091A that contains two Cys codons (171Cys/209Cys) higher molecular weight bands were observed indicating formation of E\textsuperscript{rns} oligomers (Fig. 2a). The monomer/dimer ratio of the E\textsuperscript{rns} proteins expressed from constructs 2091B or C (171D/209C or 171S/209C, respectively) showed no significant difference compared to the wt protein (Fig. 2b). Thus, the presence of Cys at position 209 allows for efficient E\textsuperscript{rns} homodimer formation in transient expression studies.

Dimers formed via Cys 209 in E\textsuperscript{rns} derived from p2091B and p2091C migrated faster on the gel. Also after peptide-N-glycosidase F (PNGase F) treatment the dimers expressed from these two constructs migrated faster than the wt protein in non-reducing PAGE (Fig. 3a). Moreover, the monomeric E\textsuperscript{rns} derived from p2091B and C was visible as a double band on the gel. E\textsuperscript{rns} with two Cys in the carboxyterminal region (from p2091A), displayed double bands for both the monomeric and dimeric form, whereas the proteins without Cys (constructs D and E) migrated as a single monomeric band after PNGase F treatment (Fig. 3a). The double bands of monomeric E\textsuperscript{rns} might result from different arrangements of intramolecular disulfide bonds involving Cys209 since the different position of this residue might allow alternative interactions. If this hypothesis was true, only single bands should be detected under reducing conditions. Indeed, all E\textsuperscript{rns} proteins comigrated as single bands after treatment with PNGase F and \(\beta\)-mercaptoethanol showing that double band formation is a result of alternative folding employing disulfide bond formation (Fig. 3b).

Mutations within the carboxyterminal long amphipathic helix serving as a membrane anchor of E\textsuperscript{rns} can result in significantly increased secretion of this protein [37, 39, 45]. Quantification of the products of the transient expression studies showed that the secretion levels of the pseudorevertants (no Cys at position 171 and S209C) was in the same range as wt. Similarly, the double Cys mutant (2091A) and the two mutants deficient in dimerization (2091D and E) exhibited no significant difference with regard to secretion rates (Fig. 3c).
Establishment of viruses with a S209C mutation

Since the virus preparations recovered from the animals represented mixtures of the original mutants, revertants and pseudorevertants we established defined mutants via our infectious cDNA clones. We introduced the S209C mutation into the CSFV wt context as well as into the mutant plasmids containing already the changes C171D, C171F or C171S. From all four plasmids, infectious viruses could be recovered that were named V-C/C (171wt/209C), V-Cd/SC (C171D/S209C), V-CF/SC (C171F/S209C) and V-CS/SC (C171S/S209C). The presence of the desired mutations was proven by RT-PCR and nucleotide sequencing. Growth curves were recorded that showed a slightly reduced performance of the virus mutants compared to the wt virus (Fig. 4a and data not shown for virus V-C/C and V-CF/SC).

To show that the transient expression results obtained for dimer formation of E\textsuperscript{rns} proteins containing the S209C mutation were also true in the context of the replicating virus we infected SK6 cells with V-CS/SC and analysed the E\textsuperscript{rns} protein in a Western blot (Fig. 5). The wt virus and the engineered dimer-negative CSFV V-C171S [44] served as controls. Both the wt virus and the S209C mutant expressed E\textsuperscript{rns} homodimers in addition to the monomeric protein. As shown before, the mutant V-C171S was not able to generate dimers.

Animal experiment

In an animal study we compared the pseudorevertant V-CS/SC (C171S/S209C) with the parental virus mutant V-C171S and wt CSFV. The growth curves revealed that the pseudorevertant from passage 5 and the wt virus behaved very similarly, whereas the dimerization-negative virus V-C171S (passage 7) exhibited a significantly impaired growth with the end titre reduced by about 1 log (Fig. 4b). The reduced growth rate of the latter virus was already reported before [44].

Twelve pigs with a weight of about 20 kg were used. The first group of four animals was infected with the dimerization-negative virus V-C171S. The pseudorevertant V-CS/SC and the wt virus were tested in groups 2 and 3, respectively. Inoculation was done with 10\textsuperscript{6} TCID\textsubscript{50} of the respective virus on day 0. The inoculum was administered i.m. in the brachiocephalic muscle. The animals were monitored daily for general health status. Clinical scores [44, 46], and rectal temperatures were recorded from day –2 post infection (p.i.) until day 20 and 21 p.i., respectively.

EDTA blood was taken on days –1, 2, 5, 7, 9, 12, 15 and 21, except for animals from group 3 that had to be killed prematurely for welfare reasons (see below). From these animals a last blood sample was taken on the day of slaughter.

The attenuated virus V-C171S (group 1) induced only mild symptoms as reported before [44]. Only animal no. 4 developed more distinctive symptoms. The general health status of this animal was reduced for a couple of days along with thin faeces. These symptoms resembled the symptoms of group 2 (V-CS/SC). For all animals infected with the pseudorevertant a reduction of attentiveness and feed intake as well as a change of defeaetion quality were observed on 6–13 days p.i. All animals infected with the wt virus
developed CSFV-specific symptoms and were culled when the symptoms reached end point criteria. Animal no. 7 developed severe symptoms already by 7 days p.i. and was culled. The pathological examination confirmed a pre-existing lung condition which in combination with the infection with wt CSFV led to the faster onset of severe symptoms. In the course of the trial animal no. 5 had to be culled 16 days p.i. and animals no. 6 and no. 8 19 days p.i. The health status of all animals and the signs of disease were recorded in the form of a clinical score (Fig. 6a).

Body temperatures above the physiological level over several days were only observed for the animals in group 3, whereas members of groups 1 and 2 showed only limited elevation for short times for individual animals leading to only slight peaks in the curves presented in Fig. 6(b). Similarly, reduction of white blood cell (WBC) numbers was only temporarily observed for the animals in groups 1 and 2 with a slightly more pronounced drop in group 2. In contrast, the WBC counts did not recover in animals infected with wt virus (Fig. 6c).

A clear difference between groups 1 and 2 was determined via qRT-PCR concerning the amount of viral RNA in the blood, with higher values (lower cycle threshold) in group 2 compared to group 1 (Fig. 6d). The highest RNA levels were determined for the wt group. The animals of this group also showed the highest number of virus positive buffy coat preparations (all tested samples positive from day 5 until slaughter). Also, attempts to reisolate the virus from the buffy coat samples of group 2 animals gave more positive results than the samples from group 1 (18/24 wells positive instead of 11/24, respectively). Analysis of the E<sub>ns</sub>-coding sequences of the reisolated viruses revealed the instability of codon 210 (variation of ACA=T and AAA=K), whereas the Cys codon at position 209 showed no variation so that the newly generated sequence at codon position 209 was stable once the pseudoreversion had occurred.

The results described above were supported by a further animal study (see supplementary information and Fig. S1, available in the online version of this article). Taken together, the results of the studies showed that restoring the ability to form E<sub>ns</sub> dimers leads to partial reversion to...
virulence despite the different location of the Cys residue forming the disulfide bond.

**DISCUSSION**

The E<sup>rns</sup> protein of pestiviruses is one of the enigmatic molecules viruses developed during evolution. E<sup>rns</sup> is a highly glycosylated envelope protein and represents an essential component of the virus particle. Even though E1 and E2, the other two pestivial envelope proteins, are sufficient for infection of cells by pseudotyped viruses [47, 48], pestiviral RNAs lacking the E<sup>rns</sup>-coding region were only functional as repli-cons but could not support the production of infectious viruses [29, 30]. In addition to its function as a structural protein, E<sup>rns</sup> is also an important factor for the interplay of pestiviruses with the host organism. The intrinsic RNase activity of E<sup>rns</sup> was shown to be involved in blocking the innate immune response of the host organism. To achieve this, secretion of part of the E<sup>rns</sup> molecules translated in the infected cells is regarded as crucial. *In vivo* tests provided evidence that E<sup>rns</sup> residing in the extracellular space is able to prevent the type 1 IFN response to exogeneous double-stranded RNA [10, 11], and can be internalized into cells and stay active in endosomal compartments for several days able to destroy RNA that otherwise would activate an IFN response via Toll-like receptors [15]. Whether such a mechanism is working in the natural host is still an open question, but a connection between the E<sup>rns</sup> RNase activity and the IFN system was found in animal studies, providing evidence that this function of the protein represents an important factor for establishment of persistent infection [12].

E<sup>rns</sup> forms disulfide-linked homodimers via Cys 171 in addition to the monomeric protein [5]. Both forms are present within infected cells, in pestivirus virions and secreted in the supernatant of infected cells [37, 39, 40, 45]. There are (very few) naturally occurring pestiviruses lacking C171 [44], and we provided final evidence that formation of disulfide-linked E<sup>rns</sup> homodimers is not necessary for pestivirus viability in experiments with different recombinant dimerization-negative pestivirus mutants [44]. These virus mutants were stable during tissue culture passages and exhibited only a slight growth retardation. However, significant attenuation of three different CSFV mutants lacking C171 was observed in pigs, indicating that homodimer formation plays a role in the natural host. In fact, prevention of dimerization had a similar attenuating effect as abrogation of E<sup>rns</sup> RNase function via deletion of one of the active site histidines [44]. Therefore, the idea was put forward, that
homodimerization of E\textsuperscript{ns} is a prerequisite for its biological function as a virulence factor.

Results of the animal studies revealed that the amount of the dimerization-negative CSFV mutants detectable in the blood of the infected animals was similar to that of the RNase-negative mutant and considerably lower than wt virus. Even though both virus mutants induced a significant neutralizing antibody response (not shown) one can speculate that they replicate at reduced levels in the animal. Accordingly, a revertant would have a considerable advantage compared to the original mutant. For the RNase-negative mutant we have never seen reversion but it has to be kept in mind that most animal studies were conducted with a mutant deficient of a histidine codon essential for RNase activity (deletion of codon 346 in CSFV/349 in BVDV). Of course, a deletion cannot be easily reverted and the complex structure of the active centre of an enzyme should prevent the possibility to restore enzymatic activity via a second side mutation. In line with the former argument, we also did not detect reversion at position 171 for the deletion variant V-C171d whereas clear indication for reversion was found for the point mutant V-C171F.

The detection described above of true revertants is not surprising when expecting enhanced growth in consequence of the change. The unexpected finding in our analyses was the recovery of pseudorevertants with a Cys at a position quite far away from the mutated original site. Importantly, this was the case not only for the codon 171 deletion mutant not able to truly revert but also for the point mutant V-C171F, which indicates that the pseudoreversion represents a real alternative to reversion. However, we never found indications for pseudoreversion in the V-C171S mutant, suggesting that the amino acid at position 171 has at least an important influence on the tendency for reversion or pseudoreversion. Apparently, the similar biochemical character of Ser and Cys reduces not only the pressure towards reversion but also pseudoreversion. As V-C171S also shows a growth reduction that is apparently compensated when S209 is converted to C, this finding is interesting but cannot be explained at the moment.

The S209C mutation restores the ability to form E\textsuperscript{ns} homodimers in mutants lacking C171. This outcome is independent of the nature of the alteration affecting position 171. The monomer/dimer ratio of proteins with C171 or C209 was very similar indicating that there is no significant structural or steric hindrance with regard to disulfide bond formation between two Cys at position 209. This is surprising since position 209 is located within the central part of the amphipathic helix serving as a membrane anchor for E\textsuperscript{ns} [36, 39]. In contrast, position 171 is located outside of the membrane anchor region, rather close to the aminoterminal end of the amphipathic helix. Also the growth characteristics of the recovered viruses support the conclusion that dimers formed via C209 have no disadvantage since impaired growth rates seem not to be connected with the exchange at position 209 but with the change at position 171 (more significantly reduced growth rates for the C171F and C171Δ mutations). Even V-C/SC, the virus with two Cys in the anchor region that expresses oligomers of E\textsuperscript{ns}, replicates with wt efficiency. It has been published that pestiviruses need not only a pH shift but probably also a reduction step for infection [49]. It therefore might be that E\textsuperscript{ns} dimers or even oligomers are reduced to monomers during infection, and, therefore, the presence of dimers or oligomers in the virion has no influence on virus performance in tissue culture.

Nuclear magnetic resonance CLEANEX spectra revealed that residue 209 is part of a long stretch of amino acids unable to exchange protons with water so that one can speculate that these residues are buried in the lipid bilayer [36]. In a helical wheel, position 209 is located within the amphipathic helix close to the hydrophobic side. Accordingly, disulfide formation between two Cys at position 209 should require either parallel or antiparallel aligning of the membrane-associated helices of the two monomers. The requirements with regard to the positioning of the Cys might be responsible for the fact that we never found pseudorevertants with the Cys at other positions than 209. Another explanation might be that position 209 is one of very few where variation is detected in the highly conserved membrane anchor. More than 20 independent CSFV sequences in the NCBI protein data bank show 100 % identity of the residues from C171 to the carboxyterminal end of E\textsuperscript{ns}. Many variants with one exchange in this conserved sequences exhibit alterations at position 209. Further variable residues are found at position 210 and, when also BVDV and BDV are included, 211, demonstrating that this region provides some flexibility. In addition to possible conformational constraints and the intrinsic variability of the residue at position 209, it must also be kept in mind that the exchange of Cys for Ser does not change so much with regard to the biochemistry of the protein.

An important result of our work concerns the connection between E\textsuperscript{ns} dimer formation and CSFV virulence. We have shown before that different alterations of the residue at position 171 prevented formation of stable dimers and led to significant attenuation of the virus [44]. Even though these data provided evidence for attenuation in consequence of abrogation of dimerization an effect just because of exchanging the residue at position 171 could not be generally excluded. We now show that despite preserving the change at position 171 the restauration of E\textsuperscript{ns} dimerization via a second site mutation leads to an increase in virulence compared to the original mutant. Even though these results do not fulfill all requirements for statistical significance due to the low number of pigs that can be used considering animal welfare, the data clearly support the conclusion of an increase in virulence since the animals infected with the pseudorevertant showed higher clinical scores, slightly stronger initial drop of WBC counts and higher viral loads. It has of course to be mentioned that the pseudorevertant did not regain the virulence of the wt virus but it is not really surprising that introduction of two exchanges into a highly conserved and functionally
important sequence element has effects on the fitness of the virus in its natural host, even when one important defect of the original mutant has been eliminated by restoration of E\textsuperscript{mns} dimerization. It therefore can be concluded that the regain of function in consequence of the pseudoreversion finally proves the importance of E\textsuperscript{mns} dimer formation for CSFV virulence.

We have shown before that abrogation of the RNase activity in the E\textsuperscript{mns} protein of BVDV cannot prevent transmission of virus to the fetus upon infection of pregnant heifers but results in significant induction of a type 1 IFN response in the fetus [12]. The mechanism behind this finding is still not well understood, and the role of E\textsuperscript{mns} dimerization in this context is even more obscure since also the monomeric protein shows RNase activity [44]. There are examples of RNases that show a biological activity resulting in cytotoxicity only when present as dimers [50–53]. It would therefore be highly interesting to test in future work whether viruses with active E\textsuperscript{mns} RNase but defective in dimerization of the protein are also unable to block the type 1 IFN response as efficiently as the wt virus.

**METHODS**

**Cells and viruses**

SK6 cells were obtained from A. Summerfield (IVI, Mittelhäusern, Switzerland), BHK-21 cells from T. Rümenapf (Veterinary University Wien, Austria). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10 % fetal calf serum and non-essential amino acids. CSFV strain Alfort/Tübingen was described in [54]. Vaccinia virus MVA-T7 [55] was kindly provided by B. Moss (NIH).

**Infection of cells and immunofluorescence assay**

Freeze/thaw lysates of infected cells stored at –70 °C were used for infection with a m.o.i. of 0.1.

CSFV infection in SK6 cells was monitored by indirect immunofluorescence (IF) analysis with monoclonal antibody (MAb) a18 directed against glycoprotein E2 [7]. The cultures were washed twice with PBS, fixed with ice-cold methanol-acetone (1:1) for 15 min, air-dried, rehydrated with PBS and incubated with anti-CSFV mAb a18 and afterwards with FITC-conjugated goat anti-mouse serum (Dianova, Hamburg, Germany) [56].

**Construction of plasmids**

Plasmids for transient expression of E\textsuperscript{mns} and mutants thereof were based on construct SSeqErns [57]. It contains the E\textsuperscript{mns} coding sequence from CSFV, a T7 promoter and a picornaviral internal ribosomal entry site. Mutations were introduced into SSeqErns by PCR-based site-directed mutagenesis using synthetic primers (QuickChange mutagenesis protocol, Stratagene, Heidelberg, Germany). The same primers and method were used to introduce mutations into plasmid 578 [43]. The XhoI/BglII insert of 578 carrying the desired changes was used to replace the wt sequence of pA/CSFV [58]. The presence of the desired mutations and the absence of second site changes were determined by nucleotide sequencing (BigDye terminator cycle sequencing kit, Applied Biosystems, Weiterstadt, Germany). Sequence analysis was done with Genetics Computer Group software [59] or the Geneious software package (Biomatters ApS, Silkeborg, Denmark). The primer sequences are available on request.

**Expression, metabolic labelling and immunoprecipitation of proteins**

For transient expression of proteins the respective plasmids were transfected into BHK-21 cells infected with vaccinia virus MVA-T7. Metabolic labelling of proteins 0.25 mCi ml\textsuperscript{–1} of [\textsuperscript{35}S] Met-label, (Hartmann- Analytic, Braunschweig, Germany) for 16–20 h was done as described before [44, 55]. The medium was used to detect secreted proteins. Cell extracts were prepared in buffer RIPA (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % DOC, 0.1 % SDS, 1 mg l\textsuperscript{–1} BSA, pH 7.6), sonicated and insoluble debris pelleted at 45 000 r.p.m. in a TLA45 rotor (Beckmann) at 4 °C. Cell extracts were incubated with 100 µl of undiluted mAb 24/16 [6] and cross-linked *Staphylococcus aureus*. Where specified in the text, the precipitated proteins were treated before electrophoresis with 1 µl PNGase F for 1 h at 37 °C as suggested by the supplier (New England Biolabs, Schwalbach, Germany). Analysis of the precipitated proteins was done by SDS-PAGE using Tricine-buffered gels [60]. The gels were fixed (30 % methanol and 10 % acetic acid for 1 h, rinsed in water containing 20 % methanol and 3 % glycerol for 3 h, vacuum-dried at 60 °C, and exposed to BioMax X-ray films (Kodak, Stuttgart, Germany), or quantified with a phosphorimager (Fujifilm imaging plate (Raytest, Straubenhardt, Germany) and Fujifilm BAS-1500 phosphorimager (Raytest)). TINA 2.0 software was used for analysis (Raytest).

**Western blot**

Separation of the proteins was done with the same gel system as described above. After the run, gels were equilibrated in transfer buffer (0.25 M Tris, 1.925 M Glycin, 0.1 % SDS, 18 % ethanol, pH 8.3) before blotting onto nitrocellulose membranes at 100V for 1 h in the same buffer. Ponceau red was used to strengthen the colour of marker proteins. Membranes were blocked with 5 % non-fat milk in PBS-T (PBS 0.05 % Tween 20), washed with PBS-T, incubated overnight at 4 °C with the anti-E\textsuperscript{mns} mab 24/16 diluted 1 : 10 in PBS-T. Membranes were washed with PBS-T, incubated for 1–2 h at room temperature with a peroxidase-coupled anti-rabbit antibody diluted 1 : 10 000 in PBS-T and washed with PBS-T. Staining of the blot was done with SuperSignal West Pico chemoluminescent substrate (Pierce, Rockford, IL).

**Recovery and characterization of engineered virus mutants**

*In vitro* transcription of RNA from the engineered full-length plasmids and RNA transfection of cells via electroporation was done as described [44, 58]. The recovered viruses were passaged at least once before titre determination and recording of growth curves.
**Animal studies**

12 pigs, German landrace hybrid, average weight of 20 kg, randomly allocated to three groups of four animals were infected after 6 days of acclimatization with CSFV mutants V-C171S [44] (group 1), V-CS/SC (group 2) or CSFV wt strain Alfort/Tübingen (recovered from the infectious cDNA clone) (group 3). Each animal received 10⁶ TCID₅₀ virus in 4.1 ml DMEM via the intramuscular route into the muscle brachiocephalicus. To check for titre reduction on the way to or in the stable samples of the diluted virus suspensions carried back from the stable were titrated and found to contain the desired amounts of virus. After infection, animals were monitored daily. Rectal temperatures were recorded daily from –2 to 20 days p.i. On these days, also a clinical score according to Mittelholzer was recorded but with some modifications as described before (soft faeces, normal amount=0; reduced amount of faeces, dry/thin faeces=1; only small amount of dry, fibrin-covered faeces, or diarrhoea=2; no faeces, mucus in rectum, or watery or bloody diarrhoea=3) [44, 46].

Buffy coat preparation and virus isolation on SK6 cells were done essentially as described before [12, 42, 43, 56]. WBC numbers were determined with the SYMSEX XT-2000i blood analyzer (SYMSEX DEUTSCHLAND GMBH, Norderstedt, Germany).

Virus detection via real time RT-PCR (qRT-PCR) was done according to the published protocol using the primer pair CSF 100-F and CSF192-R and an EGFP-specific internal control [61]. Viral RNA from the EDTA blood samples was purified with the KingFischer 96 flex work station (Thermo Fisher, Dreieich, Germany) using the Qiagen MagAttract Virus Mini M48 Kit (Qiagen, Hilden, Germany). RNA was purified of 100 µl EDTA blood. RNA-extraction controls were implemented. The real time rTPCR was conducted using the primers and probes described before [61] and the QuiaGen Quantitect Probe RT-PCR Kit. A positive control and a no template control were also amplified on the 96-well plate. In total, 42 cycles were performed using a Bio-Rad CFX 96 cycler (Bio-Rad, München, Germany).

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