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Dimerisation of glycoprotein E^{rns} of classical swine fever virus is not essential for viral replication and infection

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Summary. The pestivirus glycoprotein E^{rns}, a ribonuclease, is expressed on the surface of virions and in infected cells as a disulfide-linked homodimer. E^{rns} is involved in the infection process and its RNase activity is probably involved in viral replication and pathogenesis. The most C-terminal cysteine residue forms an intermolecular disulfide bond with another E^{rns} monomer, resulting in an E^{rns} dimer. To study the function of dimerisation of E^{rns} for viral replication, the cysteine residue at amino acid position 438 was mutated into a serine residue. The mutated C438S gene was cloned into a vector containing an infectious cDNA copy of the CSFV C-strain genome. Using reverse genetics, a mutant virus was generated that only expressed monomeric E^{rns}, confirming that Cys 438 is essential for homodimerisation. Characterization of this mutant virus and of a baculovirus-expressed C438S mutant protein indicated that the loss of the dimeric state of E^{rns} reduced the affinity of binding of virions and E^{rns} to heparan sulphate (HS), the receptor for E^{rns} on the cell surface of SK6 cells. This suggests that interaction of virus-bound E^{rns} homodimers with membrane associated HS may be a joined action of the two HS-binding domains (one in each monomer) present in the homodimer.

Introduction

Classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus belong to the genus *Pestivirus* of the *Flaviviridae* family [27], which infect pigs, cattle, and sheep. Classical swine fever is characterized by fever and haemorrhage and can run an acute or chronic course. Outbreaks of CSF and BVD can cause high economic losses. Like other members of the family, pestiviruses are plus-strand RNA viruses whose genome comprises one long open reading frame [2, 17, 27]. Translation into a polyprotein is accompanied by processing into mature proteins. The structural proteins include a nucleocapsid protein, C, and three envelope glycoproteins, E^{rns}, E1, and E2 [22].

Glycoprotein E^{rns} is encoded by 226 amino acids and the protein backbone has a mass of 25,4 kDa. In the ER-Golgi compartment the quaternary structure of E^{rns} is established by intramolecular disulfide linkages and intermolecular disulfide linkages between two monomers [22]. In these compartments there are also 6 to 7 N-linked glycosyl groups attached to the protein, forming the other half of the apparent total protein mass of monomeric E^{rns}, 44–48 kDa. Homodimerisation of the E^{rns} leads to a final protein mass of approximately 100 kDa [20]. E^{rns} lacks a trans-membrane spanning domain but results from co- and sequential immunoprecipitation studies between E2 and E^{rns} suggested that E^{rns} is attached to the pestivirus envelope through a direct interaction with E2 [14]. The E^{rns} protein contains two short regions that are homologous to the active-site domains of ribonucleases of the Rh/T2/S RNase superfamily. Indeed, these two regions express RNase activity [5, 21]. E^{rns} is an endoribonuclease with unusual base specificity, cleaving 5′of uridine residues irrespective of the preceding nucleotide (Np/U). Whereas the initial NpU cleavage is specific, the second step was unspecific degradation [3]. Between CSFV, BVDV and BDV few differences are found regarding the amino acid sequence of E^{rns}. The two RNase domains and the cysteine residues (involved in the folding of the E^{rns}) are highly conserved. Within the *Pestivirus* genus, most differences are found in the C-terminal part of the protein. In this region the CSFV E^{rns} contains positively charged arginines where the BVDV E^{rns} mostly contains positively charged lysines. For both viruses it was demonstrated that this positively charged region is important for interaction with heparan sulphate (HS) [7, 9], a glycosaminoglycan (GAG) present on the cell surface of virtually all types of eukaryotic cells. This positively charged region probably binds with high affinity to the negatively charged sulphate groups clustered over the HS polysaccharide chains. This initial attachment process of E^{rns} with HS facilitates virus entry but is not decisive, as was shown with pseudotyped retroviruses [26].

The three dimensional structure of the E^{rns} protein of CSFV remains, as yet, unknown. However, based on multiple proteolytic enzyme analysis of E^{rns}, followed by liquid chromatography-electrospray ionisation mass spectrometry, a three dimensional model was built by homology modelling based on alignments with members of the Rh/T2/S RNase family [13]. In the same study, the arrangement of the nine cysteines present in the mature dimer was established. Out of 9 cysteine residues, 8 residues form intra-molecular disulfide bonds. The most C-terminal located cysteine residue at amino acid position 438 (position in polypeptide) is responsible for intermolecular disulfide bond formation with another E^{rns} monomer, resulting in the forming an E^{rns} homodimer (Fig. 1; [13]). This dimeric state of E^{rns} is a unique feature, and although multimerization is universal for viral surface proteins, for RNases it is described only for the cytotoxic bovine seminal RNase (BS-RNase), a member of the mammalian superfamily of "pancreatic-type" RNases [31]. Like BS-RNase, E^{rns} shows immunosuppressive activity since it induced apoptosis in concanavalin A-stimulated T cells of several species [1]. *In vivo* studies with a CSF virus in which the RNase activity of E^{rns} in a recombinant virus was inactivated showed that the virus was attenuated

and was not able to induce B-cell depletion. It was postulated that E^{rns} might be responsible for the decrease in the B-cell count in infected pigs, most probably due to induction of apoptosis [16]. However, the function of the RNase activity remains elusive. Because an extracellular protein with RNase activity most probably has an intracellular target, it was anticipated that E^{rns} had some kind of way to enter the cell. Previously it was shown that E^{rns} was indeed able to translocate into cells, and the translocation activity was mapped on the C-terminal domain [12].

In this report the role of dimerisation of E^{rns} for CSFV replication and infection was studied. The cysteine residue at amino acid position 438 in CSFV C-strain was mutated into a serine. The mutant E^{rns} gene was inserted into the full-length infectious cDNA generated from the RNA genome of CSFV C-strain. By reverse genetics, a mutant virus was generated expressing E^{rns} monomers. This virus was able to grow to nearly the same titer as wt C-strain virus, but when grown under methylcellulose, the plaque size increased compared to wt C-strain. This indicated that the affinity for HS might be affected by the abrogation of the dimeric state of E^{rns}. This was confirmed by analysis of a C438S E^{rns} mutant gene expressed in the baculovirus-insect cell system.

Methods

Cells and viruses

Swine kidney cells (SK6, [11]) were maintained as described previously [18]. SK6.T7 cells were maintained as described [23]. The recombinant viruses derived from an infectious DNA copy of CSFV strain C were propagated as described previously [18]. FBS and cells were free of BVDV, and FBS was free of anti-BVDV antibodies. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV were propagated in the *Spodoptera frugiperda* cell line Sf21 as described previously [4]. Sf21 cells were grown as monolayers in either TC100 medium (Gibco-BRL) supplemented with 10% FBS and antibiotics or SF900II serum-free medium (Gibco BRL) plus antibiotics.

Construction and selection of baculovirus recombinant viruses

In a first PCR, a part of the E^{rns} gene was amplified in a 35-cycle reaction with Pwo polymerase (Roche) using a baculovirus transfer vector containing the wild-type C-strain E^{rns} gene [5] as template DNA. In this reaction, primer C438S (5′-CTG GAG CAA ACT TCC GGA CTC ATG ATC CCC-3′) in which the cysteine (TGC) was substituted for a serine codon (TCC) was used as reverse primer. Together with forward primer p1198 (5′ GTA GAA TTC CAT CAC CAT CAC CAT CAC GAA AAT ATA ACT CAA TGG) a PCR fragment was generated with a mutation at position 438 in the polyprotein of CSFV strain C. This PCR fragment was used in a second PCR to recover the complete E^{rns} gene (amino acids 268 to 494 in the polyprotein), together with reverse primer p1194X (5′-CTG ATC TAG ATT AGG CAT AGG CAC CAA ACC AGG TTT T-3′). This PCR fragment was cloned into pFastBac Dual vector downstream the polyhedrin promotor of AcNPV using *Eco*RI (underlined in primer p1198) and *Sal*I (underlined in primer p1194). This construct, named pfbE^{rns} C438S, also contained an N-terminal signal sequence of pseudorabies virus glycoprotein gX as described [5], followed by a 6× Histidine tag. Recombinant baculoviruses (AcNPV + E^{rns} C438S) were generated using the baculovirus expression system (Invitrogen, The Netherlands).

Characterisation of E^{rns} expressed in insect cells

After 48 h of infection with AcNPV + E^{rns} C438S or AcNPV + wt-E^{rns}, Sf21-cells were lysed in 5 ml lysisbuffer (30 mM Tris pH 7.4, 10 mM MgAc, 1 mM PMSF and 1% N-P40-Nonidet). The lysate was centrifuged for 10 min at 2500 g and the supernatant was stored at −20 °C until further use. Samples were analysed for the presence of E^{rns} as decribed [5] and the ELISA titer was determined (the dilution of the sample at which it has an OD of 1). The purified proteins were analyzed under reducing and nonreducing conditions by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 30 µl lysate was mixed with 10 µl reducing protein loading buffer (containing β-mercaptoethanol) or 10 µl non-reducing protein loading buffer (without β-mercapthonol). The samples were boiled for 3 min at 100 °C and loaded on the SDS-PAGE gel, which was run at 150 V. The gel was incubated for 30 min in blotting buffer and then blotted onto a nitrocellulose membrane for 1 h with a current of 200 mA. The blot was washed 3 times 5 min in PBS and blocked for 1 h in PBST (PBS + 0.1% Tween) + 4% horseserum and 5% ELK powder. Then incubated for 1 h with HRPO conjugated anti C-strain E^{rns} mAb C5 [29] (1:1000 in PBST + 4% Horse serum), washed 3 times 5 min with PBST and stained with AEC.

The C438S and not mutated (wt [5]) E^{rns} recombinant proteins were purified from insect cells by immuno-affinity chromatography as described in Hulst et al. [5].

Determination of the RNase activity of the purified E^{rns} samples

The RNase activity of purified E^{rns} was determined following the protocol as described in Hulst et al. [5]. The RNase activity was expressed as A_{260} units min^{-1} ml^{-1} E^{rns} ELISA $titer^{-1}$.

Binding of E^{rns} to immobilized heparin

The fractions containing the highest concentration of immuno-purified E^{rns} were diluted 5 times with 10 mM phosphate buffer (pH 7.0) and loaded on prepacked and pre-eluted heparin columns (1 ml; Hitrap-Sepharose [Pharmacia]) as described [8]. Fractions were collected (1 ml) and assayed for E^{rns} in an E^{rns}-specific ELISA as described previously [5]. The concentration of NaCl in the fractions was determined by measuring the osmolarity with an osmometer (model 3D3; Advanced Instruments Inc.). Hitrap columns of the same lot number were used for all experiments.

Construction of full-length DNA of the C-strain with mutated E^{rns} gene C438S

The mutated E^{rns} C438S gene was amplified by PCR as described above. The baculovirus transfer vector pfbE^{rns} C438S was used as template DNA, and p935 as forward primer (5′-CCG AAA ATA TAA CTC AAT GG-3′) and p925 as reverse primer (5′-CAT AAG CGC CAA CCC AGG TT-3′). The obtained fragment was cloned into vector pPRKC5 [6] containing N^{pro}, and the structural proteins C, E1, and E2. From this clone a *Cla*I-*Ngo*MIV fragment was isolated and inserted into the low copy vector pPRKflc34 containing the full-length CSFV (C-strain) genome (pPRKflc34 is a derivative of pPRKflc33, [18]. The obtained construct, carrying the C438S mutated E^{rns} gene, was named pPRKflc49 E^{rns} C438S.

Generation of recombinant virus vflc49 E^{rns} C438S

Plasmid DNA from pPRKflc49 E^{rns} C438S was purified on columns (Qiagen) and linearized with *Xba*I. The DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water. Linearized DNA (250 ng) was transfected to SK6.T7 cells, which constitutively express the T7 DNA dependent RNA polymerase, grown in 24 wells plates

($5 * 10^5$ cells/well) as described [23]. After growth for 1 day at 37 °C, cells were washed twice with Optimem. Fresh Optimem (160 µl) was added, followed by the DNA transfection mixture. After 16 h of incubation at 37 °C, the transfection mixture was removed and the wells were supplied with complete medium. The cells were incubated for 4 days at 37 °C, after which the medium was stored at −70 °C. Cells were immunostained with C-strain specific anti-E^{rns} MAb C5 [29]. The medium collected from wells in which E^{rns} expression was observed was used to infect SK6 cells. After three additional passages in SK6 cells, two virus stocks of vflc49 E^{rns} C438S (clones 1 and 2) were prepared as described previously [18]. The titers ($TCID_{50}$ per milliliter) of the virus stocks were determined by end-point dilution.

Characterization of vflc49 E^{rns} C438S

The E^{rns} gene of recombinant virus vflc49 E^{rns} C438S was sequenced. For this test, confluent monolayers of SK6 cells grown in 25-cm^2 tissue culture flasks were infected with vflc49 E^{rns} C438S as described above. After 72 h of growth, total RNA was isolated using the RNeasy mini kit (Qiagen) and used as template in a standard RT-PCR. The amplified DNA fragments, covering the complete E^{rns} genes of vflc49 E^{rns} C438S, were isolated from agarose gels and directly sequenced with E^{rns} flanking and internal primers by use of an ABI Ready Reaction Dye Terminator cycle sequencing kit (PE Applied Biosystems) and an ABI Prism 310 genetic analyzer (PE Applied Biosystems).

The size of infectious centres (hereafter denoted as plaques) after infection of SK6 cells with viruses vflc34 (wt C-strain) and vflc49 E^{rns} C438S, and growth of these cells under methylcellulose was determined in a plaque assay [8]. Confluent monolayers of SK6 cells grown in 2-cm^2 tissue culture wells were infected with appropriate dilutions of virus stocks for 30 min at 37 °C. The virus was removed from the cells, the cells were washed twice, and supplied with 0.5 ml EMEM/methylcellulose (1:1) with 1% L-Glutamine, 5% FBS and 2% antibiotics (overlay medium). After 48 h incubation at 37 °C the cells are washed 3 times with PBS before the cells were fixed and immunostained with E2-specific Mab V3 [28]. The relative plaque size was determined at the same magnification and was expressed as large (>) or small (<)[8].

Virus neutralization index

The virus neutralization index (log reduction of virus titer [TCID50/milliliter] by a neutralizing serum) was determined at a 1:250 dilution of serum 716 specifically directed against E^{rns} of CSFV strain C and at a 1:1,000 dilution of pig serum 539 specifically directed against E2 of CSFV strain Brescia [6]. The virus stocks of vflc34 (wt C-strain) and vflc49 E^{rns} C438S were subjected to titer determination by endpoint dilution in the presence or absence of these CSFV neutralizing antibodies.

Dose dependent heparin assay

Dose dependent inhibition of infection of SK6 cells by heparin of mutant virus vflc49 E^{rns} C438S and wt C-strain vflc34 was performed as described [8].

Characterisation of viral glycoproteins

The E^{rns} and E2 products of recombinant viruses were analysed by immunoprecipitation. Monolayers of SK6 cells were infected for 1.5 h with a multiplicity of infection of 2 with vflc34, vflc49 or mock infected. Forty hours after infection, cells were labelled for 24 h with 150 µCi S^{35}-labeled cysteine. Cells were lysed in PBS-TDS (1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS] in PBS) and aliquots of

the lysates were immunoprecipitated as described [5]. Wild-type CSFV E2 glycoproteins were precipitated with α-E2 mAbs V3 [28] and E^{rns} wt or mutated proteins were precipitated with a mix of mAbs C5 [29], 140.1 [19] and Pab 716 [6]. The precipitated proteins were incubated for 5 min at 100 °C in sample buffer under nonreducing (without 2-mercaptoethanol) conditions, and analyzed on a 12% SDS-polyacrylamidegel, fixed, dried under vacuum, and exposed to Hyper-MP® film (Amersham). The X-ray film was developed in a Photo develop machine CP-345 (G.E.R. Benelux B.V., The Netherlands).

Results

Rescue and characterization of mutant CSF virus vflc49 E^{rns} C438S

Through analysis of proteolytically cleaved recombinant E^{rns} proteins, Langedijk et al. [13] established the arrangement of the nine cysteines present in E^{rns} (Fig. 1). They showed that the cysteine at position 438 is involved in dimerization of E^{rns}. To study the function for dimerization of the E^{rns} protein for infection and growth of CSFV *in vitro*, the cysteine residue (TGC) at position 438 (polyprotein sequence) was mutated to a serine residue (TCC). This mutated E^{rns} C438S gene was inserted into an infectious DNA copy of the C-strain. The resulting full-length cDNA pPRKflc49 E^{rns} C438S was linearised with *Xba*I and transfected to SK6.T7 cells. Four days after transfection, infected cells were detected by immunostaining with an E2 specific mAb V3. SK6 cells were also efficiently infected with medium collected from wells in which virus-infected cells were detected. These results indicated that the mutant virus named vflc49 E^{rns} C438S is able to replicate efficiently in SK6 cells. The presence of E^{rns} molecules on the surface of the virion is mandatory for infection of cells at the apical membrane [30]. The observation that vflc49 E^{rns} C438S was able to infect SK6 cells efficiently through the medium

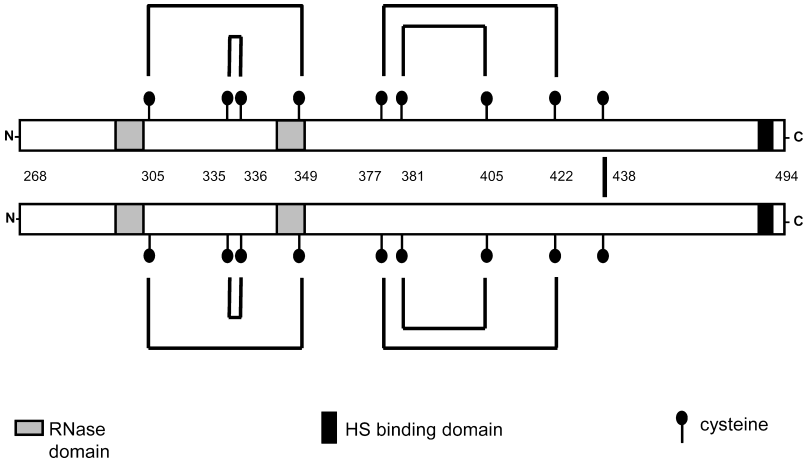

Fig. 1. Schematic representation of the E^{rns} homodimer showing the disulfide bridge connection [13]. Cysteine residues are numbered and the two active RNase domains are shaded. The HS binding domain in the C-terminus of E^{rns}, which was determined for BVDV [10], is depicted as a black box

Table 1. Neutralization of CSF viruses by antibodies

Virus	Virus neutralization reduction (log $TCID_{50}$/ml) with serum[a]	
	716 (directed against E^{rns})[b]	539 (directed against E2)[c]
vflc49	2.65	1.7
vflc34	2.5	1.5

[a]Log TCID $_{50}$/ml reduction of CSFV titers due to the presence of serum
[b]Rabbit serum prepared against E^{rns} of CSFV strain C
[c]Pig serum specifically directed against E2 of CSFV strain Brescia

suggests that mutant E^{rns} molecules are exposed on the surface of vflc49 E^{rns} C438S virions.

To determine whether E^{rns} was incorporated into the viral envelope, virus stocks of vflc49 E^{rns} C438S and wt C-strain vflc34 were subjected to titer determination in the presence of CSFV neutralizing antibodies (Table 1). Neutralization of virus vflc49 E^{rns} C438S by E^{rns}-specific polyclonal rabbit serum 716 indicated that the E^{rns} C438S protein was incorporated in the viral envelope. Virus vflc49 E^{rns} C438S was neutralized to the same extent as the parent virus vflc34 with both the E^{rns}-specific and E2-specific neutralizing polyclonal antibodies.

The mutation seemed to have a slight effect on growth of virus vflc49 E^{rns} C438S compared to wt C-strain vflc34. The virus titers of vflc49 E^{rns} C438S were about 2-fold lower than that of wt C-strain vflc34 at 1, 2 and 7 days p.i. (data not shown).

After each passage of the mutant vflc49 E^{rns} C438S and vflc34 wt C-strain on SK6 cells the sequence of the E^{rns} gene in the viral RNA was determined by RT-PCR. After five successive passages no changes were detected in the sequence of the E^{rns} gene of the mutant and the wt virus. This suggested that the cysteine to serine mutation at position 438 is stable.

To study whether the E^{rns} C438S mutation lead to loss of the dimeric state of E^{rns} in the mutant virus vflc49 E^{rns} C438S, ^{35}S-labeled E^{rns} proteins were immunoprecipitated from vflc49- and vflc34 (wt)-infected SK6 cells and analysed on SDS-PAGE under non-reducing conditions (Fig. 2). The pattern of bands that appeared after immuno-precipitation with E2-specific Mab V4 were equal for both infections, suggesting that growth of both vflc49 E^{rns} C438S and vflc34 wt C-strain viruses were similarly efficient. All forms of the E2 protein were present, the monomer (51–54 kDa, Fig. 2, arrow 3), the homodimer (∼105 kDa, Fig. 2, arrow 5) and the E2-E1 heterodimer (∼85 kDa, Fig. 2, arrow 4). E^{rns} homodimers were only detected when SK6 cells were infected with vflc34 wt C-strain (Fig. 2, arrow 2). No E^{rns} homodimers were detected when SK6 cells were infected with vflc49 E^{rns} C438S, although for both infections E^{rns} monomers were detected (Fig. 2, arrow 1). These results indicated that during infection in SK6 cells the recombinant vflc49 E^{rns} C438S is unable to link two E^{rns} monomers by forming an intermolecular disulfide bridge, while the vflc34 wt C-strain E^{rns} monomers is

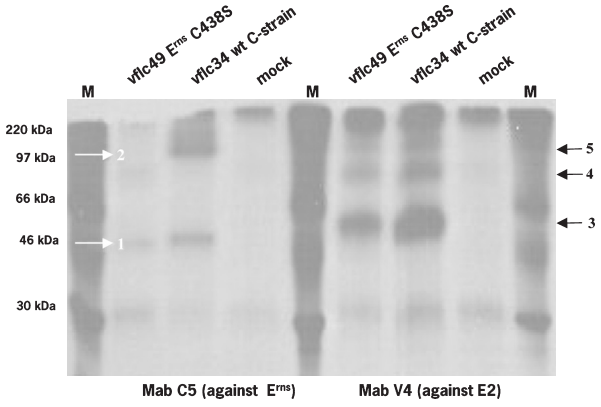

Fig. 2. SDS-PAGE under non-reducing conditions of ^{35}S labelled E^{rns} and E2 after immunoprecipitation E^{rns} with a mix of mAbs C5 [29], 140.1 [19] and Pab 716 [6] or E2 with V3 [28] in lysates of SK6 cells infected with: vflc49 E^{rns} C438S, C-strain vflc34 (wt), uninfected SK6 cells, *M* = rainbow marker. Numbered arrows indicate different forms of E^{rns} and E2. (*1*) E^{rns} monomers, (*2*) E^{rns} homodimers, (*3*) E2 monomers, (*4*) E1/E2 heterodimers, (*5*) E2 homodimers

able to form this disulfide bridge. The mutant E^{rns} migrated slightly faster than the wt E^{rns} protein, probably due to the amino acid mutation or altered post-translational processing. The mutation had no detectable effect on the structure of monomeric E^{rns}, since the binding domains for several antibodies and binding capacities were not altered (data not shown).

Wt-E^{rns} of CSFV strain C, present as homodimers on the viral envelope, binds with high affinity to HS polysaccharide chains. The positively charged C-terminal amino-acid domain of E^{rns} interacts with the negatively charged sulfate-rich clusters of these polysaccharide chains. Interaction of this domain of E^{rns} with HS on the surface of cells facilitates attachment of viral particles to the cell surface [8, 10]. Therefore, the effect of heparin on the infection of SK6 cells with vflc49 E^{rns} C438S and wt C-strain was tested in a dose dependent manner (Fig. 3).

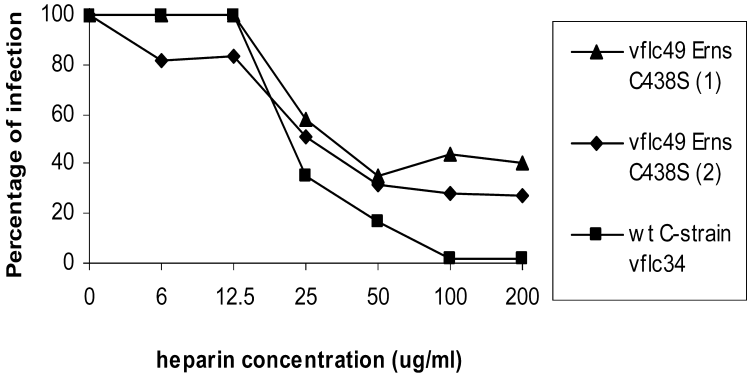

Fig. 3. Dose dependent inhibition of infection of SK6 cells by heparin of mutant virus vflc49 E^{rns} C438S (clones 1 and 2), and wt C-strain vflc34 [18]

The level of infection for vflc49 E^{rns} C438S decreased to a minimum at 35% at a concentration of 200 μg/ml heparin, whereas infection of SK6 cells with wt C-strain vflc34 was inhibited completely at this concentration of heparin. Although vflc49 E^{rns} C438S still uses E^{rns} monomers to attach to, and enter SK6 cells, these results show that the affinity of the mutant E^{rns} C438S protein for heparin/HS type polysaccharide chains is significantly lower than that of wt-E^{rns}.

Hulst et al. [8] demonstrated that CSFV variants with a high affinity for HS, when grown under methylcellulose, form relatively small plaques compared to variants with a low affinity for HS. Virus particles with a high affinity for HS expose a relatively higher overall or locally net positive charge at their surface than particles with a low affinity for HS. Due to stronger electrostatic interactions with the negatively charged sulfated polysaccharides present in the methyl-cellulose their spread is reduced more. Therefore, mutant virus vflc49 E^{rns} C438S and wt C-strain vflc34 were grown under methylcellulose to determine their plaque size. Virus vflc49 E^{rns} C438S displayed a relative large (>) plaque size compared to a small (<) plaque phenotype observed for wt C-strain (Fig. 4). These results indicate that the surface of vflc49 E^{rns} C438S virions bears a lower overall or locally net positive charge than wt C-strain virions, most likely due to the loss of the dimeric form of E^{rns}.

Characterization of the E^{rns} C438S protein expressed in insect cells

The E^{rns} C438S gene was expressed the baculovirus-insect cell system. Protein lysates prepared from *Spodoptera frugiperda* 21 (Sf21) cells infected with the here generated AcNPV + E^{rns} C438S recombinant virus and cells with the baculovirus that expressed unmutated E^{rns} of CSFV strain C (AcNPV + wt-E^{rns}; [6] and were analysed by SDS/PAGE and western blotting (Fig. 5).

Under non-reducing circumstances (Fig. 5a) the mutant E^{rns} C438S protein migrated as monomers (44–48 kDa), whereas the majority of the wt E^{rns} protein migrated as homodimers (approximately 97 kDa).

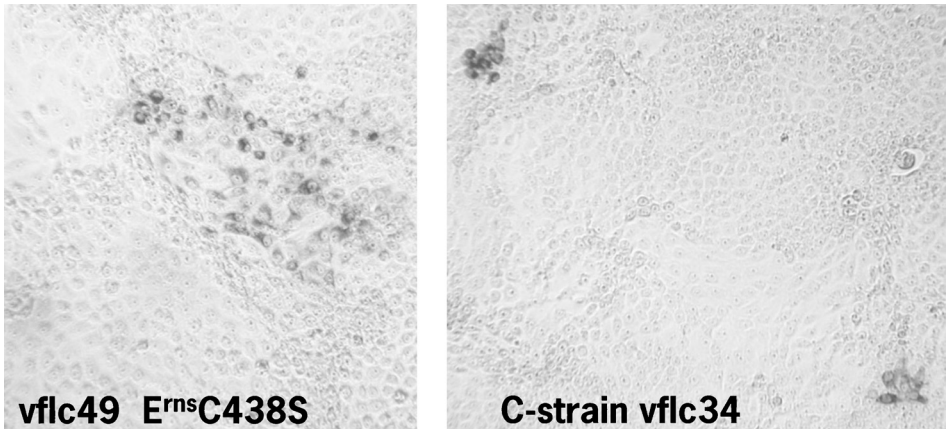

Fig. 4. Relative plaque size of C-strain vflc34 (wt) and mutant vflc49 E^{rns} C438S after two days of growth under methylcellulose overlay medium

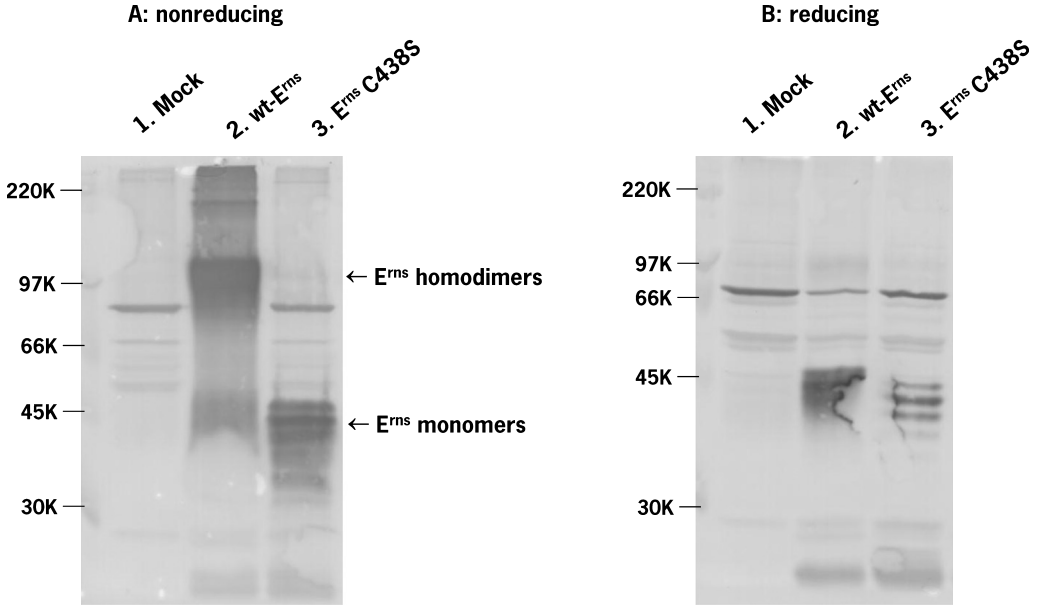

Fig. 5. Western blot detection of E^{rns} proteins with Mab C5 [29] in lysates of Sf21 cell infected with: *1* Mock-infected, *2* AcNPV + E^{rns} (wt), *3* AcNPV + E^{rns} C438S. Results are shown under non-reducing (**A**) and reducing (**B**) conditions

Under reducing circumstances (Fig. 5b) the mutant E^{rns} monomers migrated slightly faster than the wild-type monomers. The Cys-to Ser may influence post-translational processing (e.g. glycosylation) of the E^{rns} protein. Thus, in two different eukaryotic cell systems the mutant E^{rns} C438S protein is unable to form homodimers by disulphide-bridging. This confirmed the finding of Langedijk et al. [13] that the cysteine residue at amino acid position 438 is involved in covalently linking two E^{rns} monomers.

Mutations in the RNase domain or conformational changes in E^{rns} can abrogate its enzyme activity. Therefore, the RNase activity of purified E^{rns} C438S and E^{rns} wt proteins was determined. The E^{rns} proteins were purified by immuno-affinity chromatography and the fractions were assayed for E^{rns} using the E^{rns}-specific ELISA and for their RNase activity (Fig. 6). The RNase activity co-eluted precisely with the E^{rns}-ELISA titer. This suggested that the cysteine to serine mutation did not abrogate the RNase activity of E^{rns} [28]. However, the RNase activity of the fraction that contained the majority of E^{rns} C438S protein had a significantly lower specific activity than that of the E^{rns} wt peak fraction (fraction 14; see Fig. 6).

The purified wt and mutant E^{rns} proteins were tested on their ability to bind to heparin. E^{rns} proteins bound to immobilized heparin were washed and than eluted by increasing the NaCl concentration. Each fraction was tested for the presence of E^{rns} in the E^{rns}-specific ELISA and the osmolarity (Fig. 7).

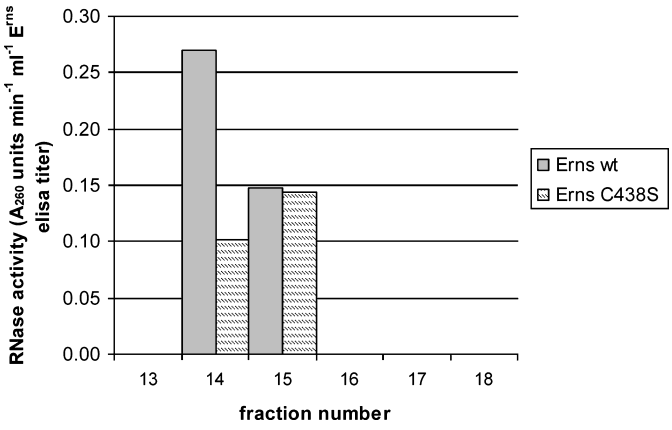

Fig. 6. RNase specific activity of purified E^{rns} proteins. The RNAse specific activity of fractions containing the majority of the immuno-purified wt-E^{rns} and E^{rns} C438S proteins was determined and expressed as A_{260} units min^{-1} ml^{-1} E^{rns} ELISA $titer^{-1}$ [6]

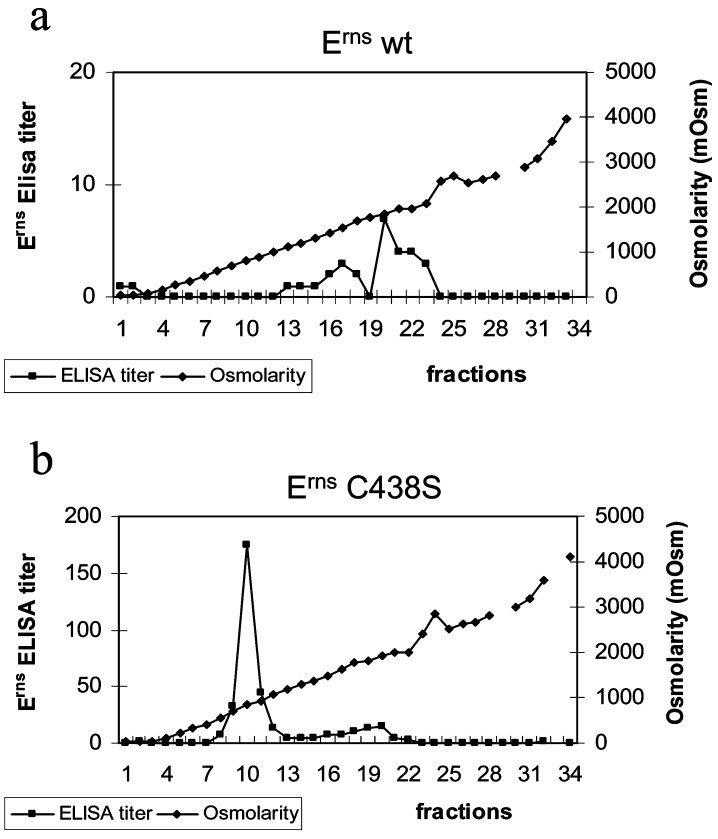

Fig. 7. Binding of purified (**a**) wt-E^{rns} and (**b**) E^{rns} C438S baculo-expressed proteins to immobilized heparin. The E^{rns} ELISA titers and the osmolarity of the fractions that were eluted from the heparin column, by increasing the NaCl concentration, were measured

The wt-E^{rns} protein eluted from the heparin column at a NaCl concentration of 925 mM. The E^{rns} C438S protein eluted from the heparin column at a NaCl concentration of 425 mM. These results indicated that the interaction between the E^{rns} C438S protein and heparin/HS-type polysaccharide chains is weaker than the interaction between E^{rns} wt and heparin.

Discussion

In this study we studied the function of dimerisation of E^{rns} for virus replication and infection *in vitro*. Langedijk et al. [13] demonstrated that the first eight cysteine residues in E^{rns} form intramolecular disulfide bridges. The most C-terminally located cysteine at position 438 (in polyprotein) is involved in covalently linking two E^{rns} monomers through disulfide bonding. This was confirmed in our study by analysis of baculovirus-expressed mutated E^{rns} C438S proteins. The mutated E^{rns} C438S proteins were unable to form homodimers, whereas wt-E^{rns} of C-strain formed homodimers in insect (Sf21)-cells. The mutated E^{rns} C438S gene was also inserted in an infectious cDNA copy of the C-strain. Through reverse genetics mutant virus vflc49 E^{rns} C438S was rescued from SK6.T7 cells.

Surprisingly, the C438S mutation appeared to be stable during five rounds of amplification on SK6 cells. However, there was little growth disadvantage for vflc49 E^{rns} C438S, thereby eliminating selection of faster growing revertants. Furthermore, the choice of cells can determine the absence/presence of revertants [25]. Analysis of ^{35}S-radiolabeled proteins showed that vflc49 E^{rns} C438S expressed the monomeric form of E^{rns}, whereas wt C-strain vflc34 expressed E^{rns} homodimers. Our results show that at least one cysteine at position 438 is needed in covalently linking two E^{rns} monomers. Also, the possibility that two monomers interact by non-covalent bonding is still possible. Such non-covalent complexes may even be exposed on the surface of virions. To investigate this properly we first have to detect these non-covalent dimer complexes on the surface of the mutant virus or in insect cells. Dissociation of two E^{rns} molecules by changing the physiological conditions may then prove that two monomers are associated with each other by a non-covalent interaction. We have not been able to investigate this due to low virus titers, which make subsequent experiments difficult.

Further characterization of mutant virus vflc49 E^{rns} C438S on SK6 cells showed that it had a large relative plaque size compared to wt C-strain vflc34, when grown under methylcellulose. It was shown that the plaque size of CSFV particles is correlated with the net charge of the E^{rns} amino acid backbone [8]. Furthermore, infection of virus vflc49 E^{rns} C438S on SK6 cells could not be completely inhibited by heparin (maximum inhibition 65%) compared to 100% inhibition of infection for wt C-strain vflc34. These results indicated that the mutation in E^{rns} C438S somehow lowered the overall (or local) net positive charge of the virus particle, leading to a reduced affinity for heparin/HS type polysaccharide chains. Several explanations may be plausible for this reduced affinity. The mutation C438S, located in the C-terminus of E^{rns}, could directly affect the conformation of E^{rns},

or the absence of its monomeric partner may provoke conformational changes in specific protein domains. In addition, the incorporation/assembly of monomeric E^{rns} in the viral envelope is, most likely, different from that of homodimers. Even when equal numbers of E^{rns} C438S monomers are incorporated in the envelope as homodimers, the molarity of the positively charged E^{rns} HS-binding domains exposed on the surface of the mutant virus will be half of the domains exposed by wt virus. The HS-binding domain is located directly behind amino acid 438, which, as was shown here and by Langendijk et al. [13], covalently links the two monomers in proximity of each other. Binding of homodimeric E^{rns} to HS might be a joined action of these two domains. The loss of one of these two HS-binding domains may account for the reduced affinity of the E^{rns} C438S mutant virus for HS.

For CSFV Brescia E^{rns} SST with amino acid residues ^{276}S, ^{476}S, ^{477}T it was demonstrated *in vitro* that within 3 rounds of replication the virus mutates from a heparan sulphate non-sensitive strain to a HS sensitive strain [7]. After at least five of the vflc49 E^{rns} C438S rounds of replication no viruses that contained a reverted cysteine residue were observed. Interestingly, C438 is the only cysteine that is not completely conserved in E^{rns}. A few pestivirus strains of BVDV type 1 and two CSFV strains have no cysteine residue at position 438 and contain only eight cysteine residues (acc. no. O11993, O11994, Q91WA6, P19711, AF352565 and Q98426). However, the sequence data deposited at genbank concerning CSFV strains Riems and LPC show discrepancies for this cysteine residue at position 438. The first deposited sequence of CSFV strain Riems has one amino acid sequence with a cysteine residue at position 439 [15], whereas the second amino acid sequence of CSFV strain Riems (accession number AAA86908) lacks the cysteine at position 438. After re-examination of CSFV strain Riems in this region we unequivocally found a cysteine residue at position 438 (genbank accession number AY845225). CSFV strain LPC has 2 genbank submissions (AAS20416 and AAA79140) which have a cysteine at position 438, whereas the two other genbank submissions (AF352565 and AAB57701) for this strain lack the cysteine at position 438. Surprisingly, after re-examination of BVDV strain NADL we also found a cysteine residue at position 438 (genbank accession number AY5226). Although sequence artefacts can never be excluded, other explanations for the discrepancies of sequences within these strains other than differences in laboratory handling (e.g. selection of virus variants by passaging on different cell lines and/or animals) are hard to find. Verification of sequences of authentic viral material would be favourable. When the sequence artefacts are excluded, it still remains a fact that *in vitro* pestivirus strains occur which lack the cysteine residue at position 438, which are viable in cell culture just as we found for virus vflc49 E^{rns} C438S. Whether there are field strains with the cysteine mutation at position 438 remains unclear and therefore the results presented here might still be relevant for a situation *in vivo* as discussed below.

The BVDV/BDV strains and the CSFV strains differ in the sequence at the HS domain, which was determined for BVDV as ^{480}KKLENSKSK^{487} [10], whereas amino acid residue 476 in CSFV was found to be involved in the determination for heparin after adaptation to cells in tissue culture [7]. The pestivirus

strains which lack the cysteine at position 438 seemed to have found another mechanism to compensate, if necessary, for the advantage of the dimeric state of E^{rns}. Langedijk et al. [13] proposed alternative dimerization through possible domain-swapping.

The function of E^{rns} and the importance of its dimeric nature are not understood. The only other known dimeric RNase members of the Rh/T2/S RNase family is BS-RNase, which belongs to the monomeric "pancreatic-type" family. The dimeric nature is crucial for the cytotoxic and immunosuppressive activity of BS-RNase. Although E^{rns} and BS-RNase are from different RNase families, they share two unique features: the dimeric nature and probably the vicinal disulfide [13]. Like BS-RNase, E^{rns} shows immunosuppresive activity since it induced apoptosis in ConA-stimulated T-cells of several species [1] and *in vivo* tested RNase negative CSFV mutants did not show a decrease in peripheral B cells [16, 25]. This issue was not addressed in this study, but it would be very interesting to know whether the mutated E^{rns} C438S with abrogated dimeric state is able to induce apoptosis in ConA-activated T-cells.

In vivo the situation might be different with respect to HS binding, because significant differences exist between CSFV vaccine strain C and virulent CSFV strain Brescia (CoBrB SST) and even between *in vivo* and *in vitro* generated virus preparations of CSFV strain Brescia [8]. Further analysis of *in vitro*- and *in vivo*-generated CoBrB SST strain with a mutation C438S in E^{rns} might give further insight in this subject. In another study, *in vitro* and *in vivo* comparison of genetically related virulent and cell-adapted avirulent CSFV Brescia variants suggested that acquirement of positively charged amino acid residues in E^{rns} and E2 are correlated with virulence [24]. The acquirement of positively charged amino acids results in an overall (or locally) increase of the positive net charge of the virus particle. Maybe, *in vivo* studies with recombinant viruses that express monomeric E^{rns} will help to understand the mode of interaction between CSFV and the HS receptor, and the role of this RNase in immunosuppression of its host.

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