

The Pestivirus Glycoprotein E^{rns} Is Anchored in Plane in the Membrane via an Amphipathic Helix*

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Birke Andrea Tews and Gregor Meyers¹

From the Institut für Immunologie, Friedrich-Loeffler-Institut, D-72001 Tübingen

E^{rns} is a structural glycoprotein of pestiviruses found to be attached to the virion and to membranes within infected cells via its COOH terminus, although it lacks a hydrophobic anchor sequence. The COOH-terminal sequence was hypothesized to fold into an amphipathic α -helix. Alanine insertion scanning revealed that the ability of the E^{rns} COOH terminus to bind membranes is considerably reduced by the insertion of a single amino acid at a wide variety of positions. Mutations decreasing the hydrophobicity of the apolar face of the putative helix led to reduction of membrane association. Proteinase K protection assays showed that E^{rns} translated *in vitro* in the presence of microsomal membranes was protected, whereas a mutant with an artificial transmembrane region and a short cytosolic tag was shortened by the protease treatment. A tag fused to the COOH terminus of wild type E^{rns} was not accessible for antibodies within digitonin-permeabilized cells, but the variant with the tag located downstream of the artificial transmembrane region was detected under the same conditions. These results are in accordance with the model that the COOH-terminal membrane anchor of E^{rns} represents an amphipathic helix embedded in plane into the membrane. The integrity of the membrane anchor was found to be important for recovery of infectious virus.

Bovine viral diarrhea virus (BVDV)² belongs to the genus *Pestivirus* that also includes the animal viruses classical swine fever virus and border disease virus of sheep. The genus *Pestivirus* is part of the family *Flaviviridae*, which also comprises the genera *Flavivirus* and *Hepacivirus* (1).

Pestiviruses are positive strand RNA-viruses with a single-stranded genome of ~12.3-kb length that contains a single open reading frame coding for a polyprotein of about 4000 amino acids (2). The polyprotein is co- and posttranslationally processed by cellular and viral proteases into at least 12 mature proteins (3–12), arranged in the polyprotein in the following

order: NH₂-N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B-COOH. C, E^{rns}, E1, and E2 are part of the virion (13, 14), with C forming the capsid and E^{rns}, E1, and E2 representing glycoproteins associated with the virus envelope. E^{rns} and E2 elicit neutralizing antibody responses that can lead to protective immunity (15–18).

E^{rns} forms a disulfide-linked dimer of ~90 kDa, about half of which is due to glycosylation (8, 13, 19). The protein displays homology to the RNases of the T₂/S superfamily (20, 21). In different test systems, it was shown that E^{rns} indeed has RNase activity, a feature that is unique among viral glycoproteins (22–25). The protein is essential for virus growth (26), but the RNase activity is dispensable. Viruses in which the RNase activity had been knocked out were shown to be clinically attenuated (27, 28).

A role for E^{rns} in immune evasion has been proposed (28–30). This function could be based on the interception of the interferon answer and seems to be linked to the RNase activity of E^{rns}. A role with regard to the prevention of an interferon response was also shown for the nonstructural protein N^{pro} (31–36).

E^{rns} can be found in virus-free cell culture supernatant of infected cells and in the blood of infected animals (8, 37, 38). It lacks a hydrophobic region that could serve as a transmembrane anchor, and there is also no consensus sequence for GPI anchor addition. Nevertheless, E^{rns} is bound to the virion and is found associated with membranes when expressed within cells (8, 13, 14, 26, 37). We could show before that E^{rns} of BVDV CP7 interacts with membranes through its COOH terminus and that truncation of the COOH terminus leads to abrogation of the membrane association and nearly complete secretion of the protein (37). The sequence of the E^{rns} COOH terminus contains only about 50% hydrophobic amino acid residues. For the E^{rns} protein of classical swine fever virus (CSFV), it was proposed that the COOH terminus is able to form an amphipathic helix (39). Amphipathic helices are known to be able to act as membrane anchors, as was shown for a variety of cellular and viral proteins (40–48). All proteins discovered to be anchored by amphipathic helices so far are cytosolic proteins. In the present report, we analyzed the nature of the E^{rns} membrane anchor in more detail. The results allow conclusions on the secondary structure of the carboxyl-terminal end and its interaction with membranes. To the best of our knowledge, we report for the first time evidence for a glycoprotein anchored in plane in the membrane by an amphipathic helix.

EXPERIMENTAL PROCEDURES

Cells and Viruses—MDBK and PK15 cells were obtained from the American Type Culture Collection (Manassas, VA). SK6 cells were obtained from A. Summerfield (IVI, Mittel-

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¹ To whom correspondence should be addressed. Tel.: 49-7071-9670; Fax: 49-7071-967303; E-mail: gregor.meyers@fli.bund.de.

² The abbreviations used are: BVDV, bovine viral diarrhea virus; BHK, baby hamster kidney; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; CSFV, classical swine fever virus; DI, defective interfering virus; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; HA, hemagglutinin; HCV, hepatitis C virus; MDBK, Madin-Darby bovine kidney; PK, porcine kidney; SK, swine kidney; TM, transmembrane; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.

häusern, Switzerland). BHK-21 cells were kindly provided by T. Rümenapf (Universität Giessen, Germany). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids. Vaccinia viruses MVA-T7 (49) and vTF 7-3 (50) were kindly provided by B. Moss (National Institutes of Health, Bethesda, MD).

Immunofluorescence Assay—For immunofluorescence assays using selective permeabilization, cells were grown in μ -dishes (Ibidi, München, Germany), fixed with paraformaldehyde (4% in phosphate-buffered saline (PBS)) for 20 min at 4 °C, washed twice with PBS, permeabilized with 5 μ g/ml digitonin in permeabilization buffer (20 mM HEPES, pH 6.9, 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM EDTA) for 15 min at 4 °C or 1% Triton in PBS for 30 min at room temperature, and washed again twice with PBS. The cells were then incubated with the appropriate antibodies. After three washes with PBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-species antibodies (Dianova, Hamburg, Germany) were used to detect antibodies bound to viral antigens in the immunofluorescence assays.

For immunofluorescence assays after electroporation or infection of cells, cells were grown on coverslips. Cells were fixed with paraformaldehyde and permeabilized with 0.1% saponin (in PBS without Mg²⁺ and Ca²⁺). Cells were then incubated as described with the appropriate primary and secondary antibodies. For double labeling experiments, both primary antibodies were mixed. In this case, also secondary antibodies (TRITC-labeled) (Sigma) and FITC-labeled secondary antibodies were mixed.

The rabbit anti-E^{rns} serum was kindly provided by R. Stark and H.-J. Thiel (Universität Giessen). Fluorescence analysis was done with an Axiovert microscope using ApoTome technology (Zeiss, Göttingen, Germany).

Construction of Plasmids—Construct pB-E^{rns} was described before (37). All single codon insertion or substitution constructs used in this study were generated by PCR-based site-directed mutagenesis using synthetic primers and pB-E^{rns} as template. Mutants containing insertions of more than one alanine were generated using synthetic linkers ligated into the respective single insertion construct, which were cut with the appropriate enzyme (AfeI or HindIII, depending on the construct). The sequences of primers and linkers are available on request.

Mutations in the E^{rns} gene were incorporated in the infectious clone pA/BVDV (51) through a two-step cloning strategy. The sequence containing the respective mutations was transferred into a plasmid containing the BVDV sequence from a XhoI site to a NotI site using BamHI and NdeI. A larger sequence now containing the mutation was transferred into the infectious clone using restriction enzymes XhoI and NcoI.

Plasmid HHBiI “w”UbiNS3 was a very kind gift of Norbert Tautz (Universität Giessen) and is a modified version of plasmid Bi-NS3 described in Ref. 52. This bicistronic plasmid contains the pestivirus 5'-untranslated region followed by the gene for β -glucuronidase. Downstream of this is located an encephalomyocarditis virus internal ribosome entry site (EMCV IRES) that initiates translation of an open reading frame composed of a ubiquitin gene and the BVDV CP7 cDNA sequence from the NS3 gene to the 3'-untranslated region. Sequences for E^{rns} variants were amplified using primers containing BssHI and FseI

restriction sites and cloned into the plasmid HHBiI “w”UbiNS3, replacing the β -glucuronidase gene.

Plasmid pCSFV/ Δ E^{rns} is based on a variant of the infectious clone for CSFV Alfort/Tübingen pA/CSFV (53) and contains an SP6 RNA polymerase promoter. pCSFV/ Δ E^{rns} contains the full-length genome with a deletion of codons 268–490, thus lacking the E^{rns} coding sequence.

The cloned constructs were all verified by nucleotide sequencing with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Weiterstadt, Germany). Sequence analysis and alignments were done with Genetics Computer Group software (54). Further details of the cloning procedure are available from the authors upon request. Expression plasmids pTM/E1-HA and pTM/E2-HA were a kind gift of L. Cocquerel and J. Dubuisson and are described in Ref. 55.

Transient Expression of Proteins in Transfected Cells—BHK-21 cells were infected with vaccinia virus MVA-T7, subsequently transfected with plasmid DNA using SuperFect (Qiagen, Hilden, Germany) and labeled with ³⁵S-labeled amino acids as described earlier (37).

Membrane Fractionation of Infected and Transfected Cells—The supernatants (fraction 1) of infected and transfected cells (about 2 \times 10⁶ in 3.5-cm dishes) were removed. The cells were fractionated essentially as described previously (37, 56). The cells were harvested by scraping them into 1.5 ml of PBS and then passaged 12 times through a 27-gauge needle. Nuclei and cell debris were removed by centrifugation at 700 \times g for 3 min. The pellet of this centrifugation step was collected (fraction 2), and from the supernatant, the membrane fraction was recovered by centrifugation at 125,000 \times g (55,000 rpm; rotor TLA100.3; Beckman TL100 centrifuge) for 25 min (fraction 3) (includes also mitochondria). The supernatant of this centrifugation step (fraction 4) contained the water-soluble proteins. All pellets were resuspended in 1 \times RIP buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 2 mg/ml bovine serum albumin, 1% Triton X-100, 0.1% deoxycholic acid, 0.1% SDS, pH 7.6). All samples were denatured by the addition of 1% SDS and heating to 95 °C for 5 min before they were cooled on ice. Samples were sonicated and brought to the final concentration of 0.3% SDS by the addition of 1 \times RIP buffer before using them in immunoprecipitation experiments.

Proteinase K Protection Assay—Proteins were translated *in vitro* using rabbit reticulocyte lysate (Promega) and canine microsomal membranes (Promega) according to the manufacturers' protocols in a total volume of 25 μ l. Proteins were labeled with [³⁵S]methionine (MP Biochemicals, Eschwege, Germany). For *in vitro* translation, RNA was generated by *in vitro* transcription using T7 RNA polymerase (New England Biolabs) essentially as described before (53).

Aliquots of 10 μ l of the *in vitro* translation samples were treated with 0.3 units of proteinase K in the presence or absence of 1% Triton X-100 for 10 min on ice. Samples were inactivated by the addition of 2 μ l of phenylmethylsulfonyl fluoride (100 mM), treated with SDS-PAGE buffer, heated to 95 °C, and cooled on ice. Samples were separated by SDS-PAGE.

Immunoprecipitation—Immunoprecipitation was carried out essentially as described before (37). One-tenth of each fraction was used in immunoprecipitation with 5 μ l of rabbit anti-

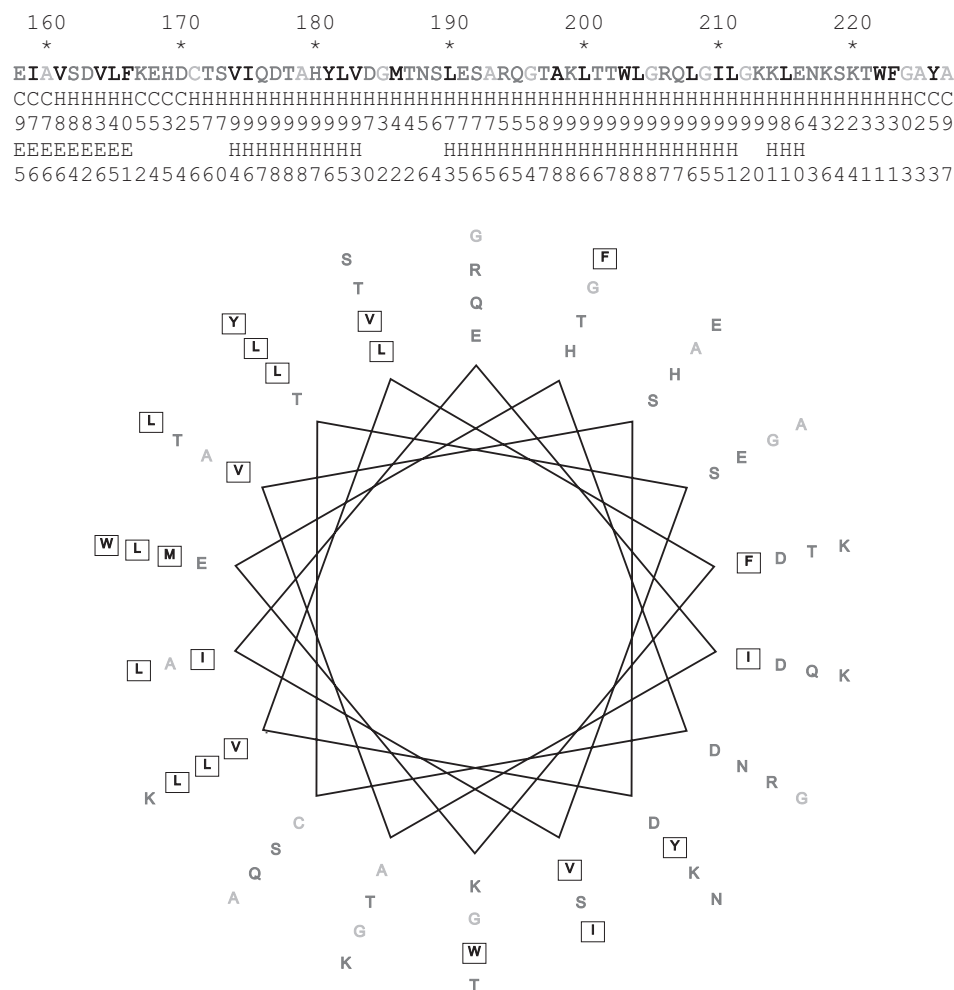


FIGURE 1. **Sequence and putative structure of the E^{ms} COOH terminus.** The sequence of the COOH terminus in *one-letter code* is shown at the *top*, with secondary structure predictions and their respective confidence value *below* (*H*, helix; *C*, coil; *E*, β -sheet; *1*, low probability; *9*, high probability). Predictions were made using the PSIPRED (64–66) (*top*) and PredictProtein (67, 68) (*bottom*) servers. *Below* the sequence, a helical representation of that sequence is shown. Hydrophobic residues are shown as *black letters* (*framed in helical representation*). For a better overview, amino acids Asp, Glu, Ser, Thr, Asn, Lys, Arg, Gln, and His (*D, E, S, T, N, K, R, Q*, and *H*) are shown in *dark gray*. Amino acids Ala, Gly, and Cys (*A, G*, and *C*) are shown in *light gray*.

E^{ns} serum. After incubation at 37 °C for 1 h and 4 °C for another 1 h, Formaldehyde-fixed *Staphylococcus aureus* was added, and the antibody-bound proteins were recovered by centrifugation (57). SDS sample buffer was added, and the complete sample was analyzed by gel electrophoresis under reducing conditions and subsequent quantification on a phosphor imager (Fujifilm imaging plate (Raytest, Straubenhardt, Germany) and Fujifilm BAS-1500 phosphor imager (Raytest)). Computer-aided determination of the intensities of the respective signals was carried out with TINA 2.0 software (Raytest). To test for quantitative precipitation, representative samples were precipitated a second time. We had already shown before that this leads to a quantitative precipitation of E^{ns} with our E^{ns}-specific antiserum (37). The distribution of the proteins between the fractions was almost constant, with only a slight increase in the supernatant after the double precipitation. The difference between single and double precipitation was negligible for the membrane-bound protein, so that for the current project regarding only the relation of the amount of the

membrane-bound protein, double precipitation was not deemed necessary.

Electroporation—Electroporation was used to transfect cells with RNA. RNA was generated by *in vitro* transcription using T7 (for pA/BVDV based plasmids) or SP6 polymerase (for pA/CSFV-based plasmids and HHBl1 “w”UbiNS3-based plasmids) essentially as described before (53). All plasmids were linearized beforehand using SmaI. SK6 (for all complementation experiments) or MDBK (for pA/BVDV-based plasmids) cells were freshly seeded into a 10.0-cm diameter dish and left to grow overnight to 100% confluence. On the day of electroporation, cells were harvested using trypsin, washed once with PBS, and resuspended in 1.6 ml of PBS. 0.5 ml of this cell suspension were mixed with the appropriate RNA and electroporated using 2-mm gap cuvettes by two pulses at 200 V and 500 microfarads in a gene pulser (Bio-Rad). Cells were resuspended in 5.5 ml of medium, 2 ml were seeded in a 3.5-mm diameter dish containing a coverslip, and the rest were seeded in a second 3.5-mm diameter dish. Cells seeded onto the coverslip were incubated 20–22 h. Afterward, the supernatant was removed, and the cells were washed, fixed, and used in immunofluorescence experiments.

cells was incubated for 60 h, harvested through freeze/thaw cycles, and used for infection of fresh cells.

Infection of Cells—To detect the formation of infectious particles, MDBK (for pA/BVDV-based viruses) and PK15 cells (for complementation experiments) were grown to 60% confluence on coverslips. Cells were washed with standard medium without fetal calf serum or antibiotics and infected with 0.5 ml of supernatant/lysate of electroporated cells in 1 ml of medium without fetal calf serum or antibiotics. Cells were incubated for 1 h at 37 °C and washed twice with PBS. Afterward, cells were incubated for 48 h under standard conditions before being used in immunofluorescence assays.

RESULTS

Insertion of a Single Alanine Residue at Different Positions in the E^{tns} COOH Terminus Leads to Severely Reduced Membrane Association—The pestivirus E^{tns} protein has long been known to be secreted from infected cells and also to be associated with the virion (8, 13, 26). We could show before that the COOH

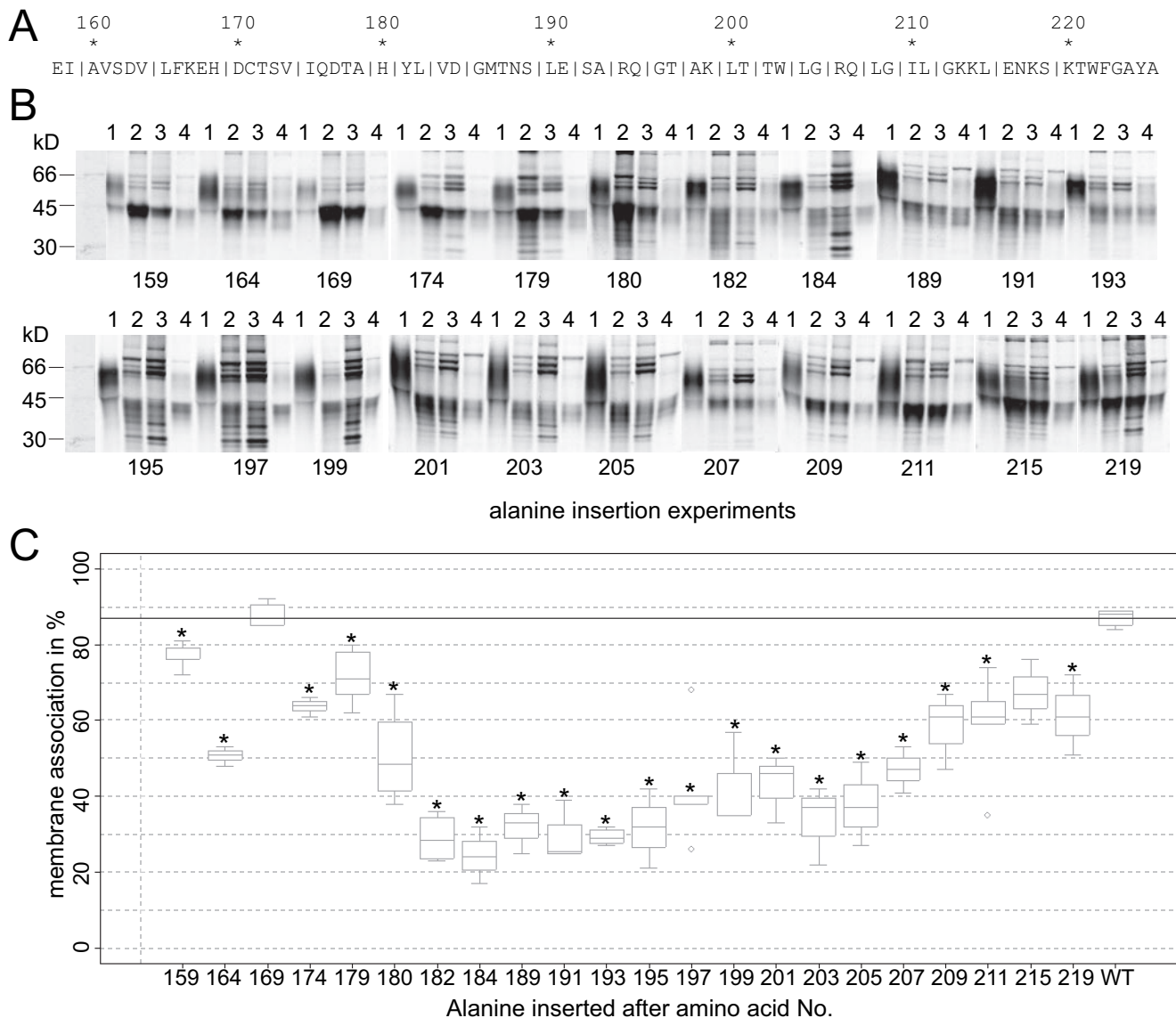


FIGURE 2. Membrane association of different alanine insertion mutants. A, the COOH-terminal sequence of E^{ns} is given, and positions at which alanine residues were inserted in the different mutants, are marked by slashes. B, example of SDS-PAGE under reducing conditions of the proteins precipitated from different fractions of cells transfected with expression plasmids with an alanine codon inserted after the position indicated below the respective lanes. Fractions are marked 1 for secreted proteins, 2 for debris, 3 for the membrane fraction, and 4 for the soluble proteins. C shows for each construct the calculated level of membrane association as a percentage. All constructs were tested several (at least three) times, and the median, upper, and lower percentile are shown. Proteins are arranged according to the position of the insertion. As a reference, the wild type membrane association is given at the right-hand corner of the graph, and the horizontal line depicts the mean of wild type membrane association. All mutated proteins, with the exception of the proteins containing insertions at positions 169 and 215, where judged to have a significantly different membrane association compared with the WT E^{ns} using the Welch test for unequal variances and a confidence interval of 95% (marked by an asterisk in the graph).

terminus of E^{ns} is responsible for membrane association of the protein and is also able to tether a foreign protein to membranes (37). The mechanism by which this association is accomplished remained unclear. Secondary structure predictions suggest the formation of a helix in the BVDV E^{ns} COOH-terminal region, and a look at the distribution of polar versus hydrophobic residues in this putative helix shows that it would display amphipathic characteristics (Fig. 1). Thus, E^{ns} could be anchored in the membrane by an amphipathic helix.

To test this hypothesis, a series of single alanine insertion mutants of E^{ns} was established. The insertion of an alanine in the region of the putative helix would introduce a 100° turn of the region downstream of the inserted residue with respect to

the region preceding the insertion. Dependent on the position of the inserted residue within the helix, the amphipathic character of the polypeptide structure would be destroyed (central location of insertion) or at least weakened (distal location of extra alanine).

Expression plasmids coding for E^{ns} proteins with single alanine insertions between amino acid positions 159 and 219 were constructed (Fig. 2A). The viral sequence was inserted downstream of an IRES under control of a phage T7 promoter. The mutated proteins were expressed in BHK-21 cells using the vaccinia MVA-T7 system. Distribution of the E^{ns} protein in the cell and supernatant was analyzed by fractionation via differential centrifugation and subsequent immunoprecipitation, fol-

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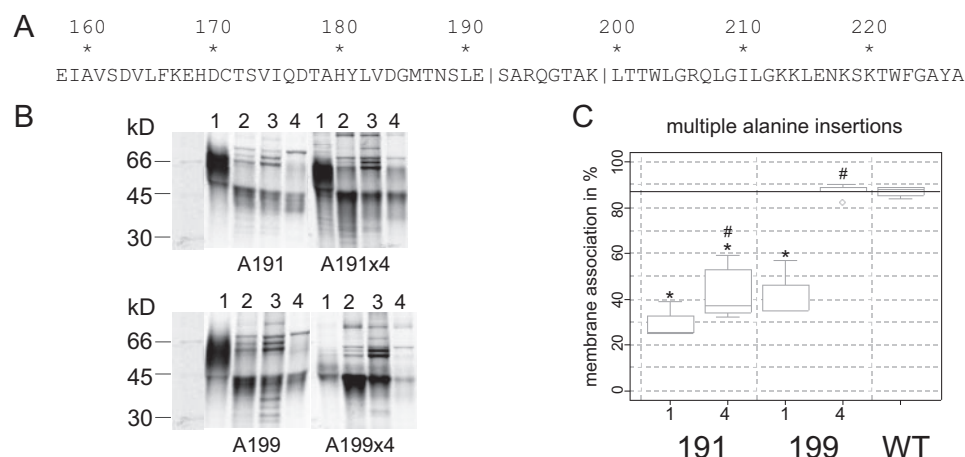


FIGURE 3. Membrane association of proteins with insertions of 1 or 4 alanine residues at individual positions. A, the positions of the insertions are marked by slashes in the sequence. SDS-PAGE of the proteins containing the insertions are shown in B. As before, the numbers at the tops of the lanes indicate the fraction (1, soluble protein; 2, debris; 3, membranes; 4, soluble proteins), whereas the position of the insertion and the number of alanine residues inserted is given below the lanes pertaining to one protein. C shows the membrane association as a percentage. The membrane association of the wild type protein is given as a reference, and the mean of the wild type membrane association is shown as a reference line across the graph. Constructs leading to a significantly different membrane association compared with the WT are marked with an asterisk, and constructs with a significantly different membrane association compared with the respective single insertion mutant are marked with a number sign.

lowed by protein quantification. First, the cell culture supernatant containing secreted proteins (fraction 1) was removed. Then the cells were scraped from the dish into PBS and disrupted by passage through a 27-gauge needle. From the cell lysate, the gross cell debris and nuclei were pelleted by low speed centrifugation, leading to fraction 2. From the cleared lysate the membranes, mitochondria and associated proteins (fraction 3) were collected by high speed centrifugation, leaving water-soluble proteins in the supernatant (fraction 4). E^{rns} was precipitated from equivalent amounts of the individual fractions with a specific anti- E^{rns} rabbit serum and detected after PAGE by autoradiography or phosphor imager analysis (Fig. 2B). The amount of E^{rns} in each fraction was determined via measurement of the total amount of labeled E^{rns} present in all four fractions and calculation of the percentage present in the individual fraction. As shown before, fraction 2 does not contain detectable amounts of soluble proteins (37), so that the total amount of membrane-bound proteins was determined by combining the values of fractions 2 and 3. Wild type (WT) E^{rns} shows a high degree of membrane association and a rather low but consistently observed level of secretion. The apparent molecular weight of the secreted protein is higher due to the changes in glycosylation that occur on the way through the ER and Golgi. On the average, 87% of WT E^{rns} protein remained membrane-bound. In contrast to the results obtained for the WT protein, the majority of the insertion mutants showed much higher rates of secretion and in consequence reduced rates of membrane binding. Again, only low amounts of soluble intracellular protein were found (Fig. 2B). All experiments were done at least in triplicate, and the results were statistically validated. A graph summarizing the data for the membrane-bound fraction is shown in Fig. 2C. The constructs are arranged in the order of the insertion position from NH_2 - to $COOH$ -terminal. The values range from only 30% membrane-bound

proteins for mutants with a single alanine inserted in the central region (positions 184–207) to ~60% when the additional alanine was introduced close to the carboxyl terminus. These results are in agreement with the hypothesis that an amphipathic helix serves as a membrane anchor, since an insertion in the central region of such a helix would lead to a severe disruption of its amphipathic character, whereas insertions made close to the end of the helix would result in a less pronounced effect.

Insertion of 4 Alanine Residues Close to the COOH Terminus Has Only Moderate Effects on the Membrane Association of E^{rns} —Insertion of 4 additional amino acids into a helix results in the addition of one complete helix turn and a twist along the axis of 40° and would therefore disturb the hypothesized

amphipathic character much less than the addition of a single amino acid. Two constructs containing 4 alanine residues after codon 191 or 199, respectively, were established to compare the effects of multiple insertions with those of single insertions (Fig. 3A). As before, the proteins were transiently expressed, labeled, and analyzed for membrane association (Fig. 3B). The protein expressed from pB-A191x4 showed a slightly higher level of membrane association than the single insertion mutant (Fig. 3C). The effect with regard to the restoration of membrane binding was much more pronounced for the protein expressed from pB-A199x4 that showed a membrane association similar to that of the WT protein, whereas the corresponding single residue insertion reduced membrane association to less than 50% of the WT level.

Optimizing of the Amphipathic Character of the COOH Terminus Leads to an Increased Membrane Association—To further validate the importance of the proposed amphipathic structure formed by the E^{rns} COOH terminus, selected amino acids were replaced by alanine, and the effect of these mutations on the membrane association was tested (Fig. 4). These substitution mutations had varied effects on the membrane association of the respective proteins. Considering the influence of the insertion position on the efficiency of membrane association revealed some interesting correlations (Fig. 5). Nearly all substitutions of polar or charged amino acids on the hydrophilic face of the helix did not lead to significant changes of membrane association. The proteins containing the mutations E168A, Q176A, G185A, S192A, Q195A, K199A, T201A, R206A, N217A, and S219A all showed a wild type-like membrane association of 80–90%. Exceptions to this general rule were observed for mutants E191A and E216A that exhibited a greatly reduced membrane association. The effect is especially pronounced for the mutation at position 191. To determine if the loss of the acidic character of glutamate is responsible for

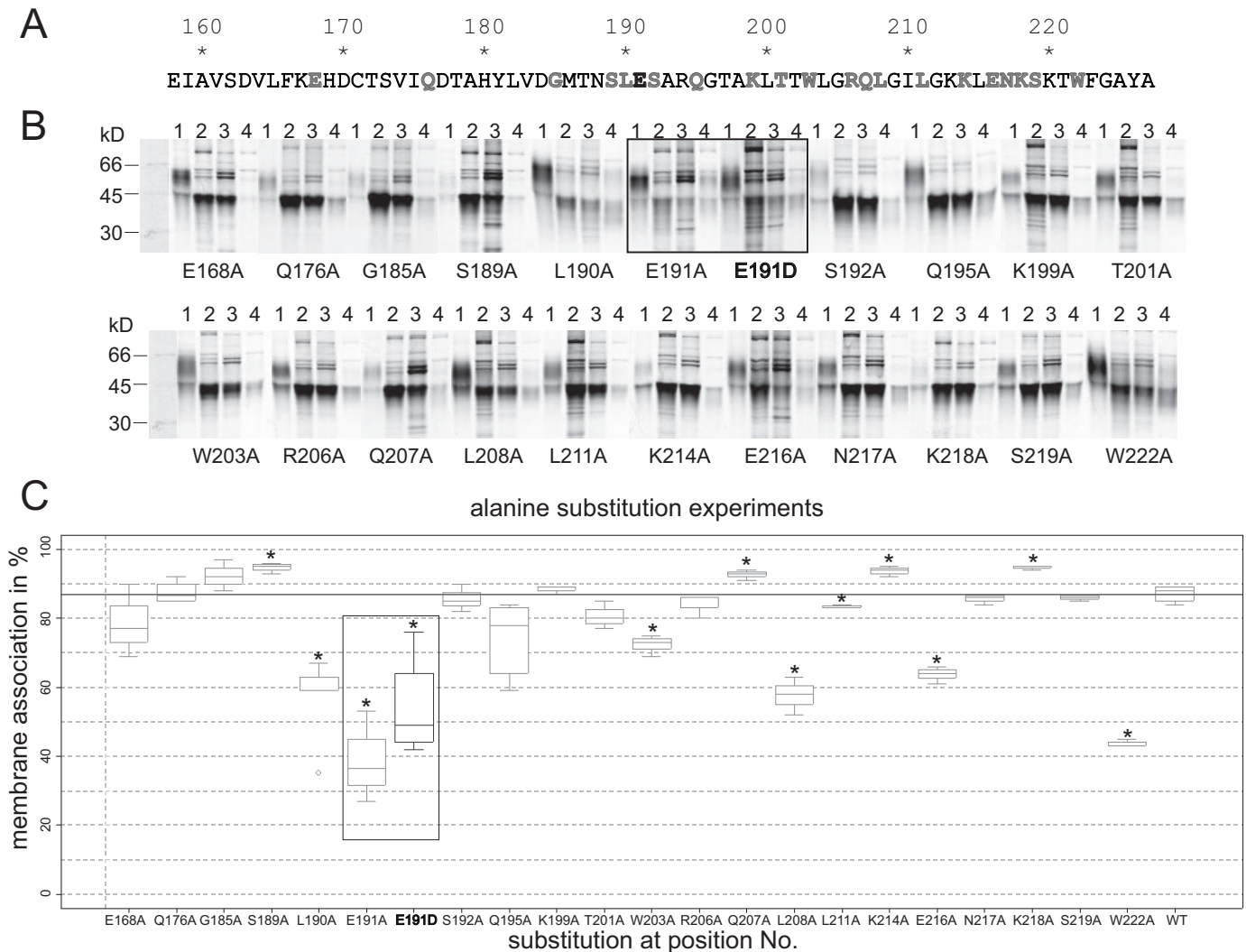


FIGURE 4. Membrane association of different substitution mutants. In the sequence in *A*, the amino acids replaced by alanine are marked in *boldface type*. The glutamate at position 191 was substituted with alanine and with aspartate. *B*, typical SDS-PAGE of the proteins after immunoprecipitation. At the *tops* of the *lanes*, the fractions are given (1, soluble protein; 2, debris; 3, membranes; 4, soluble proteins). *Below* the *gels*, the position of the substitution is indicated. The gel showing the proteins containing the substitution at position 191 is framed (E191A and E191D). The membrane association as a percentage is shown in *C*. The substitution mutants are arranged according to their substitution position. As in *B*, the mutants at position 191 are highlighted. Mutants with a significantly different membrane association compared with the WT are marked with an *asterisk*.

this effect, a more conservative substitution mutant was established. The exchange of aspartate for glutamate at position 191 (E191D) still had a severe effect on the membrane association (Fig. 4).

A substitution of alanine for a large hydrophobic residue on the hydrophobic face of the helix led to a significantly reduced membrane association of the mutant protein compared with the wild type. This could be seen for proteins carrying the mutations L190A, W203A, L208A, L211A, and W222A.

Substitution of the few polar or charged amino acids situated on the hydrophobic face of the helix led to elevated levels of membrane association. The proteins containing the mutations S189A, Q207A, K214A, and K218A all showed a membrane association of about 93% that is significantly higher than that of WT E^{rns}.

E^{rns} in Vesicles Is Protected against Degradation by Proteinase K Treatment—A typical hydrophobic helix that spans the membrane has a length of about 20 amino acids. A single

amphipathic helix is not suited to span the helix because of the exposition of its polar face to the hydrophobic core of the lipid bilayer. However, it could interact in a dimer to form a super-helix where polar and charged residues located inside of the structure would interact to allow binding of the monomers. The presence of identically charged residues at equivalent positions argues against such a dimer made from two monomers of the same protein. Alternatively, the E^{rns} COOH terminus could be inserted in the lipid bilayer in plane on the side facing the ER lumen, so that the polar and charged residues would point toward the aqueous compartment and the hydrophobic side would reach into the lipid bilayer making contacts to the fatty acids. This would mean that the utmost carboxyl-terminal region of the protein would be on the luminal side of the membrane or could be hidden in the membrane but would not be exposed in the cytoplasm.

Given the typical length of a membrane-spanning helix and the fact that the alanine insertion mutants defined the ~40

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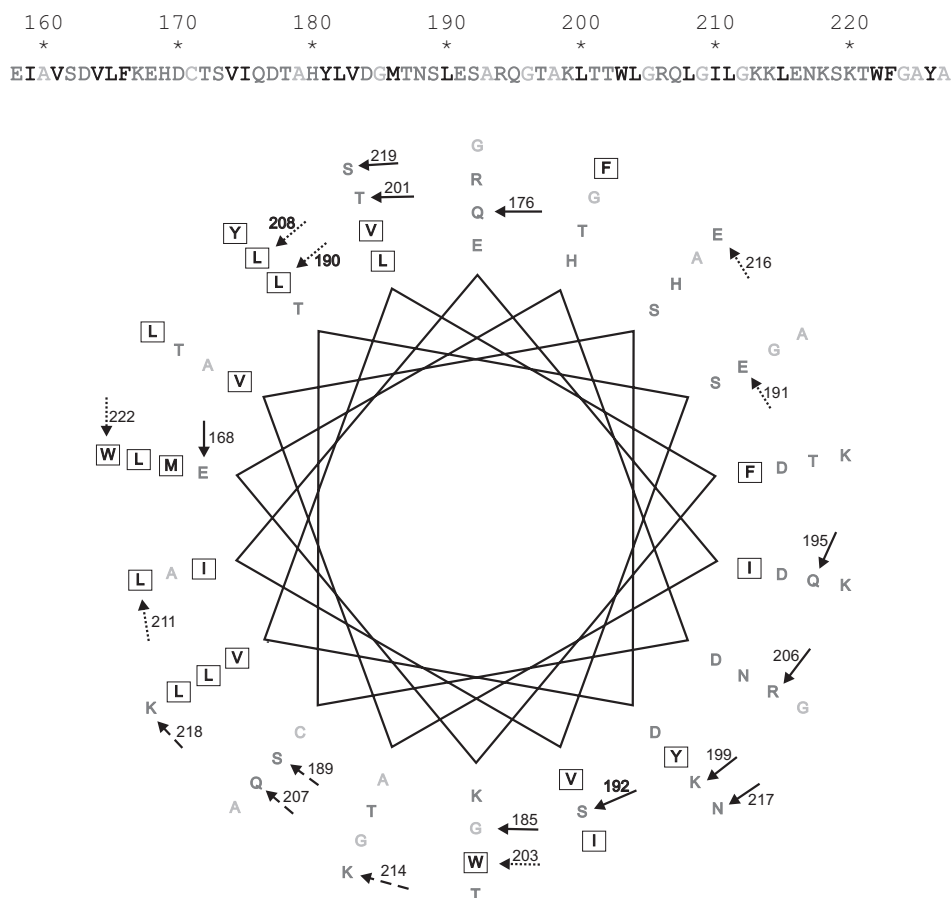


FIGURE 5. **Helical representation of the E^{rns} COOH terminus.** Hydrophobic residues are shown as framed letters; the arrows point at the amino acids that were replaced by alanine. The style of the arrows indicates the change in membrane association that results from the mutations. Dotted line, reduced membrane association; dashed line, increased membrane association; solid line, results similar to the wild type.

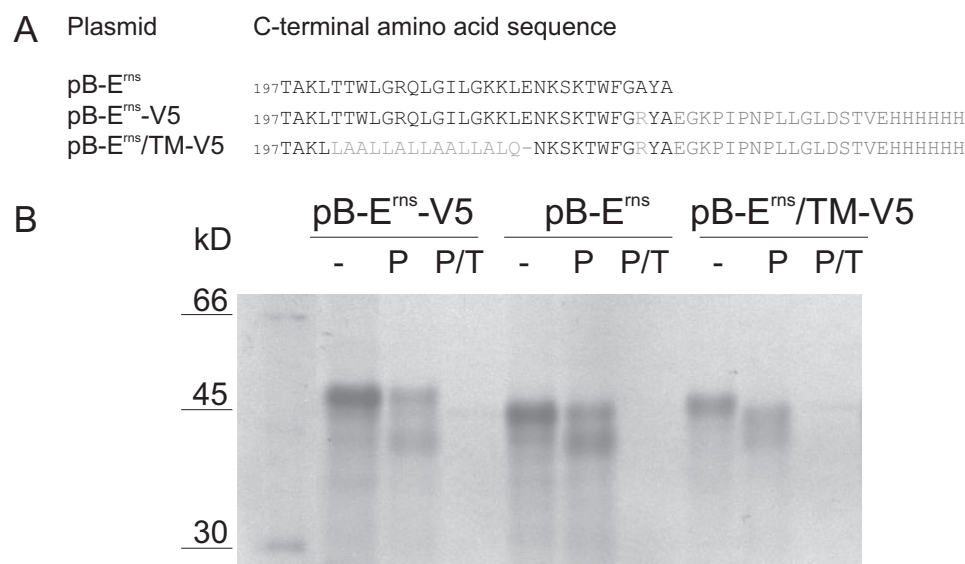


FIGURE 6. **Proteinase K protection assay.** A, the COOH-terminal sequence of the proteins used in this assay and the names of the plasmids encoding them. pB-E^{rns} expresses the wild type protein; pB-E^{rns}-V5 expresses a variant that contains a COOH-terminal V5 tag; pB-E^{rns}/TM-V5 expresses a variant containing a hydrophobic region as well as the V5 tag. B, proteins were expressed *in vitro* in the presence of canine microsomal membranes and left untreated (-), treated with proteinase K (P), or treated with proteinase K in the presence of 1% Triton X-100 (P/T). The proteins were separated using SDS-PAGE. The band of about 40 kDa present in different intensity in all lanes with samples treated with proteinase K in the absence of detergent is nonspecific.

carboxyl-terminal amino acids to be essential for membrane binding, the E^{rns} membrane anchor could also span the membrane twice and form a hairpin-like structure, probably exposing a short cytosolic loop. To test which of these two hypothetical membrane topologies is adopted by the E^{rns} COOH terminus, a proteinase K protection assay was conducted. Proteins were expressed using rabbit reticulocyte lysate in the presence of canine microsomal membranes and [³⁵S]methionine. All samples were then treated with proteinase K in the presence or absence of 1% Triton X-100, which solubilizes all membranes and membrane-bound proteins. After inactivation of the proteinase, the samples were separated using SDS-PAGE (Fig. 6B). In addition to the wild type E^{rns}, a version with a COOH-terminal V5 tag was tested (Fig. 6A) as well as a construct containing a hydrophobic region and a COOH-terminal V5 tag, expected to form a transmembrane region and a short cytosolic domain. The separation of the resulting samples by SDS-PAGE showed that E^{rns} and E^{rns}-V5 were protected by membrane vesicles in the samples. For both constructs, the E^{rns} bands of proteinase K-treated and -untreated samples comigrated, showing that the complete polypeptides were protected by the vesicle membranes. The addition of Triton X-100 to the samples led to the total destruction of the proteins, since bands were no longer detected. In contrast, the tagged variant with the artificial transmembrane region (E^{rns}-TM-V5) showed a different result. The protein in the samples treated with proteinase K migrated faster than the untreated protein in the SDS-PAGE, indicating that part of the protein was not protected by the membranes of the vesicles and could be cleaved off by the proteinase in the absence of detergent.

E^{rns} with a COOH-terminal Tag Does Not Reach into the Cytosol—To further characterize and validate the topology suggested by the results of the proteinase K protection assay,

cells expressing different E^{rns} mutants were selectively permeabilized and analyzed in immunofluorescence experiments. This approach enabled us to specifically visualize those parts of the protein found on the cytosolic side of the ER membranes. Only the tagged variants of E^{rns} were used in these experiments, since their COOH-terminal tag can be recognized by specific antibodies. Cells were transfected with plasmids coding for BVDV E^{rns} -V5 and BVDV E^{rns} -TM-V5. As a control, a plasmid coding for the hepatitis C virus protein E1 fused to a COOH-terminal HA tag (E1-HA) was used. It had already been shown that the HCV E1 adopts a topology where the COOH-terminal tag reaches into the cytosol, whereas the main part of the protein is located in the lumen (55).

Cells were fixed and treated with digitonin to permeabilize only the plasma membrane (58) or with Triton X-100 to permeabilize all membranes. The proteins were detected using a polyclonal anti- E^{rns} serum or the monoclonal antibody A4 against HCV E1. For identification of the tags, a monoclonal antibody against the V5 tag or a polyclonal antiserum against the HA tag was used. The results of these experiments are shown in Fig. 7. All proteins and tags could be successfully detected after permeabilization with Triton X-100. The E1 and E^{rns} proteins could not be detected in digitonin-permeabilized cells, showing that the bulk of the proteins was hidden by ER membranes as expected. The COOH-terminal tags of E1-HA and E^{rns} -TM-V5 could be detected in digitonin-permeabilized cells as well as in the Triton X-100-treated cells, demonstrating that these sequences have a cytosolic localization. In contrast, the COOH-terminal tag of E^{rns} -V5 could not be detected after permeabilization with digitonin, but only after treatment with Triton X-100. Thus, the protein with the WT membrane anchor has a topology different from that of the mutant with the transmembrane region or HCV E1, since in contrast to the latter two, not only the bulk of the protein but also its COOH terminus is hidden by ER membranes.

Influence of Changes in the COOH-terminal Sequence of E^{rns} on the Viability of BVDV CP7— E^{rns} is a very peculiar protein, since it maintains a balance between secretion and membrane association. To analyze whether mutations in the E^{rns} COOH terminus that change the equilibrium between secretion and membrane association are tolerated by the virus, we established several full-length cDNA clones of BVDV CP7 with sequences coding for E^{rns} containing COOH-terminal alanine insertions. We chose mutated sequences that either lead to severely diminished membrane association or have only minor effects on membrane binding. Full-length cRNA was generated through *in vitro* transcription of these plasmids and the resulting RNA brought into the cell through electroporation. Successful replication of the RNA was detected by immunofluorescence and the observation of the cytopathic effect. Recovery of infectious progeny virus was tested by reinfection of cells with the lysates of the electroporated cells. Two single insertion mutants at positions 191 and 199, both with a severely diminished membrane association, were tested. In addition, the effect of the corresponding quadruple insertion mutants, one of which showed a membrane association comparable with that of the wild type (compare Fig. 3), was analyzed. All mutant viral genomes were tested in three separate experiments. They all

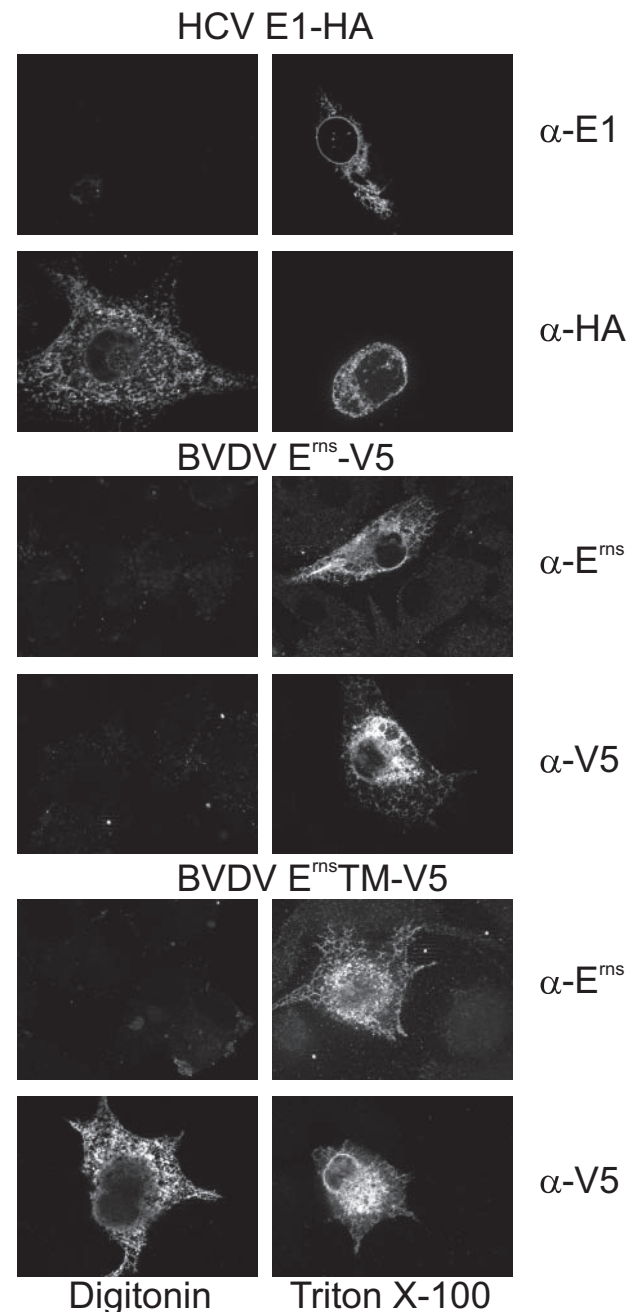


FIGURE 7. Topology of the membrane anchor of E^{rns} . pB- E^{rns} -V5, pB- E^{rns} /TM-V5, and pTM/E1-HA were used for transfection of BHK cells after infection with vaccinia virus MVA/T7. Cells were fixed with paraformaldehyde and permeabilized with digitonin or Triton X-100. The proteins were detected using antibodies against the HA tag and E1 (pTM/E1-HA) or V5 and E^{rns} (pB- E^{rns} -V5, pB- E^{rns} /TM-V5). The right panel shows images from cells treated with Triton X-100, and the left panel shows the results of cells treated with digitonin.

yielded positive immunofluorescence upon transfection (Fig. 8). Since translation of input RNAs does not lead to detectable fluorescence in our assay system, this result demonstrates replication of the mutated RNAs. Except for the wild type control, infection of fresh cells with extracts of the transfected cells did not result in detection of viral protein, showing that no infectious mutant viruses had been generated (Fig. 8).

The inability to rescue infectious particles from mutated full-length cDNA constructs can have multiple reasons. To ensure

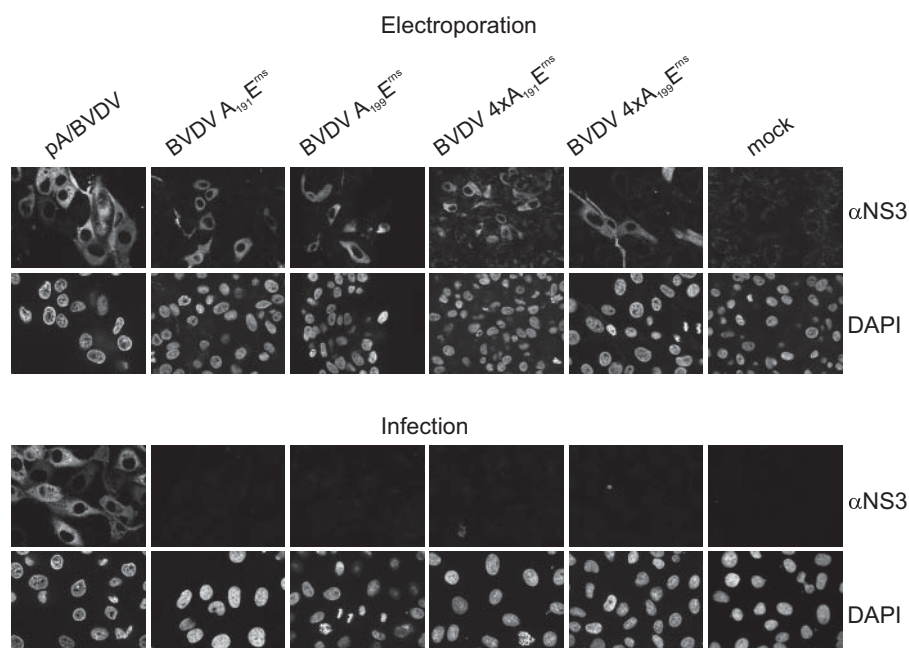


FIGURE 8. Immunofluorescence analysis of cells electroporated with RNA of different variants of the infectious clone pA/BVDV or infected with extracts of the electroporated cells. The full-length constructs contained insertions of one or four alanine codons at codon positions 191 or 199 of the E^{ns} coding sequence, respectively. RNA transcribed *in vitro* from these plasmids and the parental plasmid pA/BVDV (51) was used for electroporation of MDBK cells. The upper set of microscopic pictures shows the results of immunofluorescence analyses of cells ~20 h posttransfection. The cRNA used for transfection is indicated at the top of each panel. Only one-third of the electroporated cells were used for immunofluorescence. The other two-thirds were seeded in separate dishes and harvested ~60 h posttransfection. Freeze/thaw extracts of these cells were used for infection of fresh MDBK cells that were again analyzed by immunofluorescence ~48 h postinfection (lower set of images). All cells were fixed with paraformaldehyde, permeabilized, and stained with an NS3-specific monoclonal antibody and an FITC-labeled anti-mouse secondary antibody (shown in the upper rows of each set of images). Cell nuclei were stained using 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) (shown in the lower rows of each set). Mock-transfected cells were used as a control.

that in the present case the failure to rescue infectious progeny was indeed due to a lack of E^{ns} function, further experiments were conducted, in which wild type E^{ns} or mutations thereof were tested for their ability to *trans*-complement an E^{ns} -defective replicon. To ensure an independent formation of E^{ns} , a bicistronic DI complementation system was used. This was based on a full-length cRNA of CSFV Alfort/Tübingen (53) with an internal deletion of codons 268–490. This cRNA is able to replicate, but due to the lack of E^{ns} , it does not lead to infectious particle formation (Fig. 9). The CSFV DI-RNA was complemented with different variants of a bicistronic DI-RNA containing the BVDV CP7 5' region (52), followed by sequences coding for the signal sequence and the (mutated) E^{ns} and a translational stop codon. Downstream of this, the RNA contained an EMCV IRES, an open reading frame starting with a ubiquitin gene and containing the genes for all nonstructural proteins from NS3 on and the 3'-NTR. This DI-RNA is also able to replicate but unable to form infectious particles due to the lack of structural proteins C, E1, and E2 as well as P7 and NS2. Both RNAs were used for electroporation of SK6 cells. The formation of infectious particles was observed after reinfection of PK15 cells (Fig. 9). The different porcine cell lines were used, because SK6 can be transfected with higher efficiency, whereas PK15 cells give better results in immunofluorescence analyses. Autonomous replication of the transfected RNAs and the expression of E^{ns} could be clearly seen after electroporation

(Fig. 9). Recovery of infectious virus as demonstrated by infection of fresh cells with extracts of the transfected cells was only successful if the ΔE^{ns} genome was complemented with the wild type protein.

These results could be further validated by *trans*-complementation experiments with the ΔE^{ns} genome, in which E^{ns} or the respective mutants were expressed from transfected plasmids via T7 RNA polymerase provided by vaccinia virus vTF7-3 (50). In these experiments, the four mutants initially tested in the full-length constructs were analyzed in addition to a wild type construct. Expression of E^{ns} could be clearly shown for all five plasmids, but only the wild type protein led to the production of infectious virus from these cells (data not shown).

Taken together, expression of the mutated E^{ns} proteins tested here could not complement the ΔE^{ns} genome in *trans*.

DISCUSSION

The E^{ns} protein of pestiviruses is an essential virus protein. It represents a structural component of the

virion and is found as membrane-associated protein in infected or transfected cells (13, 37). However, a considerable percentage of the E^{ns} protein translated in cells is also secreted into the supernatant (8). E^{ns} lacks a hydrophobic membrane anchor, but we could show previously that the COOH terminus of the protein is able to bind to membranes (37). If folded in a helical conformation, the E^{ns} COOH terminus acquires amphipathic characteristics. Here we tested the hypothesis that E^{ns} may be bound in plane to the membrane by such an amphipathic helix.

Insertion of a single alanine residue in the E^{ns} COOH terminus results in significantly reduced membrane association of the protein regardless of the position at which the alanine is inserted. This finding argues for a structural effect rather than an effect based on the disruption of a specific primary sequence. In the context of the proposed amphipathic COOH-terminal helix, the influence of the alanine insertions on membrane binding would result from a ~100° turn of the helix downstream of the insertion with regard to the upstream part and, in consequence, the destruction of the amphipathic character.

Insertions at the end of the COOH terminus had a gradually less severe effect. This finding fits well with the amphipathic helix hypothesis, since an insertion in the last 15 amino acids of the protein would leave the major part of the proposed helix untouched and functional.

Concerning the structural aspect, the proposed helix would be much less disturbed by an insertion of four amino acids that

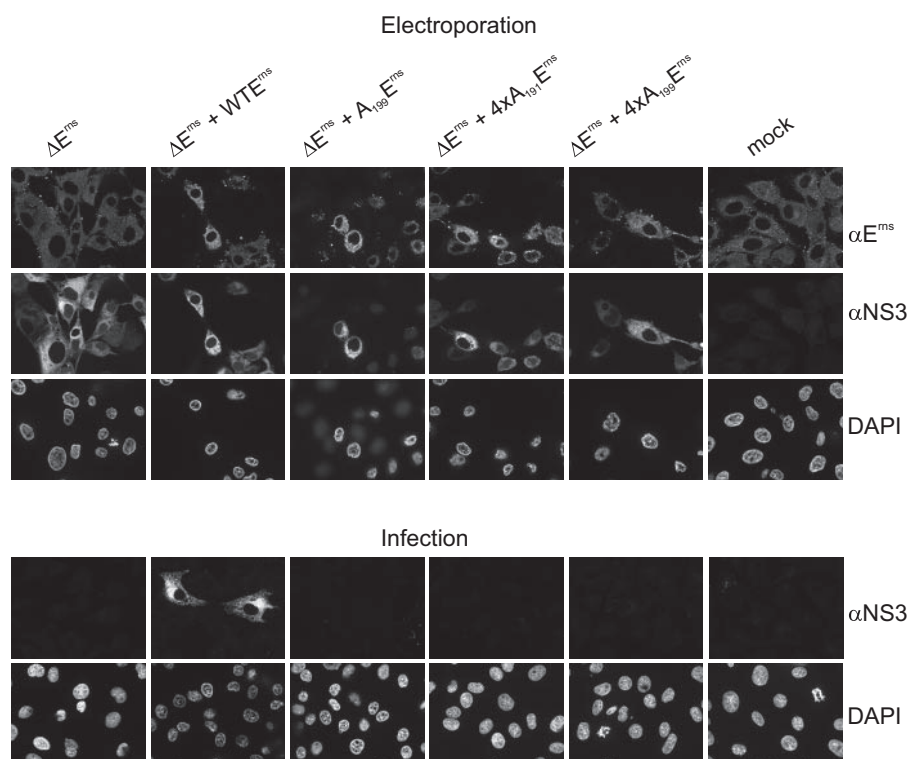


FIGURE 9. *trans*-Complementation analyses. cRNA of an E^{ms} -defective CSFV replicon was used for electroporation of SK6 cells in the absence or presence of bicistronic BVDV DI-RNAs. The bicistronic RNAs contained the sequences coding for WT E^{ms} or for mutants thereof with single or multiple alanine codon insertions after codon 191 or 199, respectively. The cRNA or combination of cRNAs used for transfection is indicated at the top of the panel. One-third of the electroporated cells were seeded for immunofluorescence analysis conducted ~20 h posttransfection (*upper set of images*). The other two-thirds were seeded and harvested ~60 h later. Freeze/thaw extracts of these cells were used to infect PK15 cells that were stained by immunofluorescence ~48 h postinfection. For immunofluorescence analysis, cells were fixed with paraformaldehyde, permeabilized, and incubated with an NS3-specific monoclonal antibody (*middle row in the set showing the results of the electroporation and upper row in the analysis of the infection experiment*). Antibody bound to NS3 in electroporated cells was detected with a TRITC-labeled secondary antibody, whereas an FITC-labeled secondary antibody was used for infected cells. Expression of E^{ms} was detected in electroporated cells with a polyclonal E^{ms} -specific antiserum and an FITC-labeled anti-rabbit secondary antibody (*top row in the first set of images*). The polyclonal anti- E^{ms} serum contains antibodies that react nonspecifically with the cells and lead to considerable background fluorescence. Nevertheless, the much brighter specific signals can be clearly differentiated from the background signal. Cell nuclei were stained with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) (*lower rows in each set of images*). Mock-transfected cells served as a control.

would introduce a bit more than a complete turn and thereby twist the helix by only about 40°. In fact, the insertions of four alanines clearly result in a higher degree of membrane association than the corresponding single insertions, arguing once again for the hypothesized helix. However, this effect varies considerably between the insertion positions. The insertion of four alanines at position 199 leads to the full recovery of membrane association, whereas an equivalent insertion at position 191 increases membrane association by only about 13% compared with the single insertion. As already pointed out, a complete reconstitution of membrane binding efficiency should not be expected, since the amphipathic character of the helix would not be fully restored, but the helix would only be less distorted by the insertion of 4 amino acids instead of one. The addition of one complete turn to the helix together with a considerable change of the sequence characteristics could result in different effects that are difficult to predict and could be dependent on the flanking sequence. Thus, the position effect observed here may be due to a combination of a specific sequence context, that allows for a slight distortion or not, and the length of the

helical structure that lies before and after the insertion. Position effects could also be seen with constructs containing 7 or 8 alanines (data not shown). The insertion of 7 amino acids at position 199 led to a membrane association only slightly reduced compared with the wild type, whereas the insertion of 7 alanines at position 191 resulted in an increase in membrane association by only 6% with respect to the single insertion mutant. The insertion of 8 alanines resulted in a reduced membrane association in both cases, but for the insertion construct at position 191 it was similar to that of the single insertion, whereas the mutant with 8 additional residues at position 199 bound to membranes with much higher efficiency than the single residue insertion mutant but was still reduced compared with that of the wild type protein or the proteins containing 4 or 7 alanines. As with the insertion of 4 amino acids, insertion of 7 or 8 alanines results in a considerable elongation of the helix and a substantial change of the biochemical characteristics of the sequence, since it lacks any hydrophobic residues on the hydrophobic face in these turns. The latter could have a considerable impact on membrane binding, as can be concluded from the pronounced reduction of membrane binding efficiency due to the loss of

hydrophobic residues that was seen in the alanine substitution scanning. All substitutions of hydrophobic residues on the apolar face of the helix led to a diminished membrane association, whereas substitution of polar residues on this side resulted in a higher membrane association of the mutated proteins. In contrast, substitution of polar or charged residues on the hydrophilic face of the helix had in nearly all cases no effect on membrane association. These results are once again in agreement with the hypothesis that an amphipathic helix is responsible for E^{ms} membrane association, since the hydrophobic face of such a structure would be most important for the membrane binding. This side mediates the contact with the core of the lipid bilayer that provides the majority of the binding energy.

Only two exchanges on the hydrophilic face of the proposed helix had considerable impact on membrane binding, namely those affecting the 2 glutamate residues at positions 191 and 216. Substitution of these 2 amino acids resulted in reduced membrane association. This effect was especially pronounced for glutamate 191 and was still clearly observed if aspartate was substituted for glutamate. These results indicate that properties

other than the acidic character of glutamate play a role for the membrane association.

The results of the alanine insertion and substitution scanning strongly support the hypothesis that the proposed amphipathic helix is crucial for the anchoring of E^{rn}s. To prove that the anchor is indeed oriented in plane in the membrane, experiments aiming at elucidation of the topology of the protein were carried out. Proteinase K protection assays revealed that E^{rn}s as well as a tagged variant of the protein remain protected by membranes if expressed *in vitro* in the presence of canine microsomal membranes. This result shows that the respective proteins do not span the membrane to expose residues on the cytosolic face of the membrane. In contrast, a variant containing a transmembrane anchor shows a slightly reduced apparent molecular weight after the treatment, most probably due to the loss of some carboxyl-terminal residues. Further experiments using detection of defined parts of the proteins after selective permeabilization of cells supported these results. Both variants of BVDV E^{rn}s and HCV E1 (55) as well as the tag sequences could be detected after treatment with Triton X-100, showing that all proteins were expressed successfully and recognized by the chosen antisera. With antisera directed against the ectodomains, E^{rn}s and HCV E1 could not be detected after permeabilization with digitonin, showing that the integrity of the ER membrane was preserved after digitonin treatment. However, the COOH-terminal tags of the transmembrane variant of E^{rn}s and of the HCV E1 control protein were found to be accessible after permeabilization of the plasma membrane with digitonin, demonstrating their location in the cytosol. In contrast, the COOH-terminal tag fused to the wild type E^{rn}s protein was only accessible after complete solubilization of all membranes, providing further evidence that the proteins' COOH terminus is not oriented toward the cytosolic side of the ER membrane.

Theoretically, these results would be in accordance with two possible conformations for the COOH-terminal sequence of the protein. E^{rn}s could be anchored in plane via an amphipathic helix, or it could be inserted straight into the membrane, without reaching the cytosol. The latter could be achieved by a superstructure in which the COOH terminus interacts with itself to form a kind of hairpin structure with an overall hydrophobic surface. Several arguments can be put forward against the latter model. First, the results of the alanine substitution scanning indicate that substitutions of polar amino acids in the COOH terminus, that would be important for the intramolecular interaction within the superstructure, have mostly no effect on the membrane association. Second, the COOH terminus contains many basic residues, some of which would be located in close vicinity and thus destabilize the superstructure. Third, some of the basic residues in the COOH terminus have been shown to be important for the interaction of E^{rn}s with glycosaminoglycans (59–62). This interaction is supposed to play an important role during infection. The basic residues would not be accessible if they were hidden inside of a hydrophobic structure in the membrane. In contrast, the solvent-exposed side of an in-plane amphipathic helix is known to serve as a surface for contacts with other molecules.

Viral genomes with E^{rn}s coding sequences containing alanine codon insertions were unable to direct the generation of infec-

tious particles, although they were shown to support RNA replication. Further experiments testing the ability of different E^{rn}s variants to *trans*-complement an E^{rn}s-defective replicon confirmed these findings, since only the co-expression of the wild type protein led to formation of infectious particles. This result should not be due to the fact that we used a combination of an E^{rn}s-defective CSFV and a bicistronic replicon (52) that was engineered to express BVDV E^{rn}s or mutants thereof, since efficient complementation of CSFV by BVDV E^{rn}s was published (63) and also found in further experiments with the CSFV strain used in our work (not shown). The defect with regard to the recovery of infectious viruses observed for the mutants is not due to a reduced membrane association of E^{rn}s alone, since the mutant with four alanines inserted at position 199 showed WT-like membrane association. One possible explanation could be that the inserted sequence disrupts the contact surface exposed to the outside. This point has to be addressed in future studies. The fact that all tested mutants were unable to produce infectious particles shows the importance of the COOH-terminal sequence. Indeed, the amphipathic character of the proposed E^{rn}s COOH-terminal helix is widely conserved among pestiviruses.

Taken together, all of our results indicate that E^{rn}s is anchored in plane in the membrane via an amphipathic helix. Several proteins are known to have amphipathic helices as membrane anchors. The NS5A proteins of both pestiviruses and hepaciviruses represent prominent examples of viral proteins anchored by amphipathic helices. Some cellular members of the family of tail-anchored proteins are also bound this way. However, it has to be stressed that to our knowledge, all of these proteins are bound to the cytosolic side of the membrane. E^{rn}s is the first translocated protein anchored in plane by an amphipathic helix.

E^{rn}s is still one of the especially enigmatic proteins of pestiviruses. On the one hand, it is a glycoprotein associated with the virion and essential for the production of infectious particles. On the other hand, this protein is secreted from infected cells and seems to play an important role as a virulence factor in the host via its RNase activity (27, 28). The latter activity is also important for the establishment of persistent pestivirus infections (30). E^{rn}s and especially the RNase activity have been reported to play a role in inhibiting the interferon response toward double-stranded RNA present in the extracellular space (29). Thus, the E^{rn}s protein has to fulfill two very different roles that in part demand different properties. As a structural protein, it needs to be associated with the virion, whereas its secretion is an important point in the hypotheses put forward to explain its role as a virulence factor and interferon antagonist. The different requirements that this protein must fulfill could be the reason behind the unusual membrane anchor, which could be able to establish a defined balance between secretion and membrane association, between structural protein and virulence factor.

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