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The Carboxy-Terminal Sequence of the Pestivirus Glycoprotein E^{rns} Represents an Unusual Type of Membrane Anchor

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The E^{rns} protein is a structural glycoprotein of pestiviruses that lacks a typical membrane anchor sequence and is known to be secreted from the infected cell. However, major amounts of the protein are retained within the cell and attached to the virion by a so far unknown mechanism. Transient-expression studies with cDNA constructs showed that in a steady-state situation, 16% of the protein is found in the supernatant of the transfected cells while 84% appears as intracellular protein. We show here that E^{rns} represents a membrane-bound protein. Membrane binding occurs via the carboxy-terminal region of E^{rns}. By fusion of this sequence to the carboxy terminus of green fluorescent protein (GFP), the subcellular localization of the reporter protein switched from cytosolic to membrane bound. A core sequence of 11 amino acids necessary for membrane binding was elicited in truncation experiments with GFP constructs. However, this peptide is not sufficient to confer membrane anchoring but needs either upstream or downstream accessory sequences. Analyses with different extraction procedures showed that E^{rns} is neither easily stripped from the membrane, like a peripheral membrane protein, nor as tightly membrane bound as a transmembrane protein.

The three animal viruses classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus of sheep compose the genus *Pestivirus* within the familiy *Flaviviridae*, which also contains the genera *Flavivirus* and *Hepacivirus* (16). Pestiviruses are single-stranded, positive-sense RNA viruses with genomes ~12.3 kb in length that contain one long open reading frame coding for a polyprotein of about 4,000 amino acids, which is co- and posttranslationally processed into at least 12 mature proteins (6, 7, 13, 14, 31, 45, 50, 51, 62, 64). The four proteins C, E^{rns}, E1, and E2, are structural components of the virion (53, 59). Both E^{rns} and E2 induce neutralizing antibodies in infected animals (2, 9, 57, 58) and elicit protective immunity (23, 28, 44, 54).

The envelope protein E^{rns} has the unique feature of containing an intrinsic RNase activity (15, 18, 47, 61) whose active site exhibits sequence homology with RNase Rh, a member of the T$_2$/S RNase superfamily (17). The protein forms a disulfide-linked homodimer of about 90 kDa, nearly half of which are due to glycosylation (28, 45). It lacks a typical transmembrane (TM) region and accomplishes its association with the viral envelope by a yet unknown mechanism (20, 45). The protein is not only part of the viral envelope, but is also secreted in considerable amounts into the extracellular space (45).

A role for E^{rns} in virulence and pathogenicity is strongly suggested by the fact that recombinant pestiviruses in which the RNase activity of E^{rns} is knocked out are clinically attenuated (36, 37). A role for E^{rns} and its RNase in the interaction of the virus with the immune system of the host or the host cell has been proposed (26, 36, 37). Notably, E^{rns} would have to be internalized into the cytosol or even be transported to the nucleus in order to act on intracellular RNA, meaning that it would need to attach itself to the target cell and to translocate across the cell membrane. Recent research has shed some light on the mechanisms by which E^{rns} may achieve this goal: studies with purified recombinant E^{rns} showed that the protein binds to a variety of culture cell types (20), most likely to glycosaminoglycans on the cell surface (21, 22, 24). Recombinant E^{rns} dimers are internalized into epithelial cells and accumulate around the nucleus (33). The binding site for glycosaminoglycans was found to be located close to the C terminus of the protein (25). The C-terminal stretch, which is highly heterologous between CSFV, BVDV, and border disease virus (20), harbors many basic residues and is predicted to build an amphipathic helix (33). A synthetic peptide representing 27 amino acids from the C terminus of CSFV E^{rns} was internalized into different culture cells, localized to membranous parts in the cytosol, and accumulated in the nucleoli. The same peptide was able to transport chemically coupled proteins across the membrane (33).

In this study, we show that E^{rns} is a membrane protein that is bound to the membrane less strongly than a typical transmembrane protein. Biochemical and immunofluorescence analyses were used to identify the carboxy-terminal sequence of E^{rns} as a membrane anchor and to determine the sequence for membrane interaction with E^{rns} mutants and a set of green fluorescent protein (GFP) constructs.

MATERIALS AND METHODS

Cells and viruses. MDBK cells and PK15 cells were obtained from the American Type Culture Collection (Manassas, Va.). 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 virus MVA-T7 (33, 63) was kindly provided by B. Moss (National Institutes of Health, Bethesda, Md.).

Immunofluorescence assay. For immunofluorescence assays, cells were fixed with paraformaldehyde (4% in phosphate-buffered saline [PBS]) for 20 min at

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4°C, washed twice with PBS, permeabilized with 0.1% saponin in PBS for 5 min at 4°C, and washed again twice with PBS. The cells were then incubated with the appropriate antibodies. After three washes with PBS, fluorescein isothiocyanate-conjugated goat anti-species antibodies (Dianova, Hamburg, Germany) were used for detecting bound viral antigen in the immunofluorescence assays.

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. The GFP5 fusion constructs were based on plasmid pBlueKS-GFP5, which was obtained by cloning a GFP5 PCR product kindly provided by H. Kolmar (Institute of Microbiology and Genetics, University of Göttingen, Germany) into the XhoI/SmaI sites of pBluescript KS(−). pBlueKS-GFP5 contains the GFP coding sequence (codons 1 to 235, followed by the nucleotide sequence CATTCTGGGTAC, followed by the SmaI-SacI sites of the polylinker) without the stop codon and allows the construction of C-terminal fusions. The region coding for nucleotides 1692 to 1958 of BVDV strain NCP7 was obtained from plasmid pA-BVDV/Ins− (38) by PCR with primers BVD32III (AAATCTCTGCTGTACATGGCACATG) and CM13 (AGCTGG AGCTCTCATGCATATGCCCCAAACCATGTCT) and was cloned into pBlueKS-GFP5 after restriction with SpeI and SacI, yielding plasmid pK1. PCR was carried out with *Tfl* Polymerase (Promega, Mannheim, Germany) following the manufacturer's recommendations and using 50 to 100 ng of DNA template and 25 pmol of each primer. The fusion product was excised from this plasmid with XbaI, made blunt ended, and then cut with SalI and transferred into the $SacI^{blunt}$/XhoI sites of pCITE2c(+) (Novagen), resulting in plasmid pK2.

The GFP5 sequence was excised from pK1 with KpnI and XhoI and transferred into the expression vector pCI (Promega) cut with the same enzymes, leading to plasmid pK13. Expression plasmid pK16, which contains the initial GFP-E^{rns} fusion product, was generated by cloning the EcoRI/$HindIII^{blunt}$ fragment from pK10 into pCI. Expression vector pK19 containing the final GFP5-E^{rns} fusion was generated by transferring the AflIII/EagI fragment from pK16 to pK13.

Cloning of shorter fragments of the C-terminal part of the E^{rns} sequence was generally conducted as follows. The desired fragment was obtained by PCR, using pK19 as the template, and cloned into pK13. In the case of shortening the E^{rns} coding sequence from its 3′ end, the entire insert of pK19 was amplified with a 5′ primer annealing upstream of the GFP sequence and a 3′ primer that introduced an *opal* stop codon behind the last desired codon and half a SmaI site at its end. The resulting PCR product was then restricted with XhoI and cloned into the XhoI/SmaI sites of pCI. In the case of shortening the sequence coding for the C-terminal end of E^{rns} from its 5′ end, a PCR product of the desired E^{rns} sequence fragment was generated, using pK19 as the template, with a 5′ primer that introduced a KpnI site (pK27 and pK37) or a BsrGI site (pK46 and pK48) and a 3′ primer that annealed downstream of the stop codon. The resulting PCR products were restricted with EagI and the enzyme corresponding to the introduced restriction site and cloned into the KpnI/EagI sites of pK13.

For transient expression, the E^{rns} coding region of BVDV strain NCP7 (nucleotides 1119 to 1859) was amplified from the infectious clone pA/BVDV/Ins− (38) with primers Ol-CM107 (GGAATTCCATGGAGAAAGCCCTATTGGC CTGGG) and Ol-CM106 (GCTCTAGAATCATGCATATGCCCCAAACCA TGT) and cloned into the NcoI/XbaI sites of pCITE2c(+), yielding plasmid pB-$E^{rns.}$ To be able to transiently express an E^{rns} protein with a truncated C terminus, a cDNA fragment was amplified with oligonucleotides Ol-CM107 and Ol-E03T1 (GACTCTAGACTTTCGCGGTCCCTTGCCTGGC) (template pA/BVDV/Ins−), cut with NcoI and XbaI, and inserted into pCITE2c(+), yielding plasmid pB-E^{rns}/dCT.

Plasmid pB-E^{rns}/TM contains an artificial sequence coding for a hydrophobic transmembrane sequence that replaces the E^{rns} sequence coding for the core region of a putative membrane anchor. The most 3′-terminal end of the E^{rns} gene coding for several charged amino acids, which we expected to act as part of a stop transfer sequence, was preserved with as little change of the protein as possible. pB-E^{rns}/TM was constructed by amplifying the leucine-alanine-rich region of pK93D (see below) with primers BT-11 (CAAGGTACCGCCAAGC TTCTGG) and BT-12 (TGCATATGCACCAAACCATGTTTTGC) and amplifying E^{rns} of pB-E^{rns} with primers CM107 and BT-7 (CGCGGTACCTTGCCT GGCACT); both were cut with KasI, ligated, and cloned into the NcoI/NdeI sites of pB-E^{rns}. Plasmid pK93D was derived from pK92, which encodes a BVDV NewYork '93-derived E^{rns} with 17 codons of the C-terminal part exchanged for a TM sequence, was generated as follows. First, a PCR product was amplified from pK40A (36) with primers CM14 (CTCGTATATGGATTGGACGTC AAC) and CM184 (GAATTCAAGCTTGGCCGTCCCTACCCTTGC) and cloned into the XhoI/HindIII sites of plasmid pRc/CMV (Invitrogen), yielding plasmid pK90. Then, a second PCR product was generated from template pK40A with primers CM22 (CCTGTTATTAGCATCAGCCACA) and CM185 (GAATTCTCGAGAACAAAAGCAAAGCATGGT). This PCR product was restricted with XhoI and NdeI; pK90 was cut with HindIII and NdeI, and both were ligated with the hybridized oligonucleotides CM186 (AGCTTCTGGCAG CTTTACTGGCACTTCTGGCAGCTTTACTGGCACTTC) and CM187 (TCG AGAAGTGCCAGTAAAGCTGCCAGAAGTGCCAGTAAAGCTGCCAGA) at the same time. The resulting plasmid was termed pK92. Then, the BsrGI/NdeI fragment from this plasmid was transferred into pK22A, a plasmid containing the 5′-terminal 2 kb of BVDV-2 strain New York '93, resulting in plasmid pK89C. For transient expression, the E^{rns} insert of pK89C was amplified and cloned into pCITE2c(+), resulting in pK93D.

To obtain constructs pB-dCo1, pB-dCo2, and pB-dCo3 with internal deletions in the core region of the membrane anchor, small 3′-terminal fragments of the E^{rns} gene were amplified by PCR with oligonucleotides Ol-dCore1 (ATGGCGCC GCGAAACTAACAACTATACTAGGAAAGAAACTGGAAAAC), Ol-dCore2 (ATGGCGCCGCGAAGAAACTGGAAAACAAGAGTAAG), or Ol-dCore3 (A TGGCGCCGCGATACTAGGAAAGAAACTGGAAAAC) and Ol-pCITErev (C AGCTATGACCATGATTAC), cut with NarI and XbaI, and inserted into pB-E^{rns}/TM cut with the same enzymes. The constructs contain deletions of codons 203 to 209, 199 to 212, and 199 to 209, respectively. In addition, codon 197 was changed from a glycine- to an alanine-coding triplet for cloning purposes in all three constructs.

The constructs pB-E^{rns}/d1, pB-E^{rns}/d2, pB-E^{rns}/d3, and pB-E^{rns}/d4 were obtained by ligation of pCITE-2a/NcoI-XbaI with PCR fragments amplified from template pB-E^{rns}, with sense primer Ol-pCITE, together with antisense primers Ol-dCTp1 (AGTCTAGATCACCATGTCTTACTCTTGTTTTCCAG), Ol-dCTp2 (AGTCTAGATCATATCCCAAGCTGCCTGCCCAGCC), Ol-dCTp3 (AGTCTAGATCACCCAAGCTGCCTGCCCAGCCAAG), and Ol-dCTp4 (A GTCTAGATCACCTGCCCAGCCAAGTTGTTAGTTTC), respectively, cut with NcoI and XbaI.

The cloned PCR products 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 (8).

Further details of the cloning procedure, e.g., the sequences of the primers used for generating fusions between the GFP5 sequence and different portions of the sequence coding for the C terminus of E^{rns}, are available on request from the authors.

Transient expression of proteins in transfected cells. BHK21 cells were infected with vaccinia virus MVA-T7 and subsequently transfected with SuperFect (QIAGEN) as described earlier (37).

PK15 cells were transfected with Lipofectamine reagent (Invitrogen, Karlsruhe, Germany); 2 μg of each plasmid was diluted with 100 μl OptiMEM (Invitrogen). For each sample, 15 μl of Lipofectamine were mixed with 100 μl OptiMEM, added to the plasmid solution, and incubated at room temperature for 20 min. In the meantime, the cells (ca. 50% confluent) were washed once with OptiMEM. The transfection mixture was diluted with 800 μl OptiMEM and added to the cells. After 4 h of incubation, 500 μl of Dulbecco's modified Eagle's medium with 30% fetal calf serum was added, and the cells were incubated for another 15 h.

Labeling of cells with ^{35}S amino acids was done as described before (37).

Membrane fractionation of infected and transfected cells. The supernatants (fraction 1) of infected and transfected cells (about 2×10^6 in 3.5-cm dishes) were removed and cleared by low-speed centrifugation ($100 \times g$; 5 min). The cells were fractionated essentially as described previously (48). 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 $107{,}000 \times g$ (55,000 rpm; rotor TLA100.3; Beckmann TL100 centrifuge) for 25 min (fraction 3). The supernatant of this centrifugation step (fraction 4) contained the water-soluble proteins. All pellets were resuspended in 1× 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% sodium dodecyl sulfate [SDS], pH 7.6), and appropriate volumes of 10× RIP buffer were added to the supernatants so that they also contained the ingredients in 1× concentration.

For Western blot analysis of the GFP fusion proteins, cells were treated as described above. Pellets were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 6 M urea, 5% mercaptoethanol, 0.01% bromphenol blue, and 0.01% phenol red), and SDS-PAGE loading buffer was added to the supernatants. All samples were heated to 95°C for 5 min and cooled on ice prior to being loaded on the gel.

TABLE 1. C-terminal sequences of E^{rns} proteins encoded by expression constructs[a]

Name	C terminus of E^{rns} or indicated mutant
	*180 *190 *200 *210 *220
pB-E^{rns}	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTWFGAYA
pB-E^{rns}/dCT	TSVIQDTAHYLVDGMTNSLESARQGTAKV
pB-E^{rns}/TM	TSVIQDTAHYLVDGMTNSLESARQGTAKLLAALLALLAALLALQ-NKSKTWFGAYA

[a] Asterisks mark amino acid positions.

To further separate the peripheral and integral membrane proteins by Triton X-114 extraction, the membrane pellet (fraction 3) was dissolved in ice-cold cell lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-114 and incubated for 1 h at 4°C. The mixture was centrifuged for 15 min at 10,000 × *g* and 4°C. The supernatant was transferred to a fresh tube and incubated at 37°C for 3 min. After centrifugation (10,000 × *g*; 1 min; room temperature), the upper (aqueous) phase was transferred to a fresh tube and reextracted with 100 μl of cell lysis buffer. After centrifugation, 700 μl of the aqueous phase was recovered and the lower phase was discarded. The detergent phase of the first centrifugation step was reextracted with 1 ml of TNE buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA). After centrifugation, the aqueous phase from this step was completely removed and discarded.

Similarly, isolated membrane fractions were extracted with PBS, 1 M NaCl, 0.1 M Na_2CO_3, pH 11.5, 4 M urea, 6 M urea, and 2% Triton X-100. The pellet obtained after ultracentrifugation was dissolved in 100 μl of the respective solution precooled to 4°C, incubated on ice for 20 min, and ultracentrifuged as described above. The pellet was dissolved in 250 μl 1× RIP buffer. The supernatant was diluted with 1× RIP buffer to a final volume of 1.1 ml. Both fractions were subjected to immunoprecipitation as described above.

For detergent-based fractionation of whole-cell proteins, Triton X-114 phase separation of transfected cells was done with a 3.5-cm dish containing ca. 2×10^6 transfected cells. The culture supernatant was removed, and the cells were washed once with cold PBS buffer and then incubated with 1 ml ice-cold cell lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-114) for 1 h at 4°C. The mixture was centrifuged for 15 min at 10,000 × *g* and 4°C. The supernatant was transferred to a fresh tube, an aliquot was removed to serve as a nonfractionated control, and the remaining sample was incubated at 37°C for 3 min. Centrifugation and the further steps of the phase separation procedure were done as described above.

Immunoprecipitation. Immunoprecipitation was carried out essentially as described before (37). Where cell culture supernatants were analyzed, 300 μl of the supernatant was diluted with 10× RIP buffer to a final concentration of 1× RIP buffer, and 5 μl of rabbit anti-E^{rns} serum was added. After incubation at 37°C for 1 h and 4°C for another hour, *Staphylococcus aureus* was added, and the antibody-bound proteins were recovered by centrifugation (27). A further aliquot of the antiserum was added to the supernatant of this centrifugation step, and a second cycle of precipitation was performed in order to obtain quantitative recovery of the target protein. The two-cycle precipitation was assumed to be quantitative, since a third cycle of precipitation did not yield detectable amounts of the target protein. The pellets of both precipitations were pooled and diluted with SDS sample buffer to a final volume of 50 μl; 20 μl was analyzed by gel electrophoresis and subsequent quantification on a phosphorimager (Fujifilm imaging plate [Raytest, Straubenhardt, Germany] and Fujifilm BAS-1500 phosphorimager [Raytest]). Computer-aided determination of the intensities of the respective signals was carried out with TINA 2.0 software (Raytest).

Western blot analysis. For Western blot analysis, the proteins were separated by PAGE in a 10% Jagow gel (46) and blotted onto a nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH, Dassel, Germany) at 100 V for 1 h. The membrane was blocked with Blotto buffer (PBS buffer, 5% milk powder, 0.05% Tween 20). The blots were washed four times with PBS-Tween (0.05% Tween 20). The membranes were incubated with the appropriate first antibody (GFP polyclonal antibody sc-8334 [Santa Cruz Biotechnology Inc. Santa Cruz, CA], calnexin polyclonal antibody spa-860 [Stressgen Biotechnologies Corp., Victoria, Canada], GAPDH [glyceraldehyde-3-phosphate dehydrogenase] monoclonal antibody CSA-335 [Stressgen Biotechnologies Corp.], and aldolase polyclonal antibody sc-12061 [Santa Cruz Biotechnology Inc.]) in PBS-Tween overnight at 4°C. The blots were washed four times with PBS-Tween, and the corresponding secondary antibody (α-rabbit peroxidase-conjugated antibody, α-goat peroxidase-conjugated antibody, α-mouse peroxidase-conjugated antibody; all from Dianova) was added in PBS-Tween for 2 h at room temperature. The membranes were washed four times with PBS-Tween and then incubated with SuperSignal West Pico chemoluminescent substrate (Pierce, Rockford, Ill.). The signal was detected with Kodak Biomax films.

RESULTS

The C-terminal end of BVDV E^{rns} is important for membrane interaction. The pestivirus E^{rns} protein is known to be secreted from the infected cell (45). However, a considerable amount of the E^{rns} protein synthesized in a pestivirus-infected cell has to be retained within the cell at the site of virus budding. When the virion is formed, E^{rns} is attached to the particle, since it is found on the surfaces of pestiviruses. An important element for achievement of both these features in viral glycoproteins is usually a membrane anchor. The carboxy terminus of E^{rns} is able to mediate translocation from the outside through lipid membranes into the cell (33). In order to test whether this sequence could confer binding of E^{rns} to intracellular membranes, we constructed two different expression plasmids that encoded either the complete BVDV CP7 E^{rns} preceded by the internal signal sequence of the virus (plasmid pB-E^{rns}) or a variant of this sequence with the 3′-terminal 27 codons deleted (plasmid pB-E^{rns}/dCT). As a control, a further construct was established that coded for an E^{rns} protein with major parts of the C-terminal sequence replaced by a hydrophobic sequence that is supposed to form a TM sequence and to ensure stable membrane anchoring (construct pB-E^{rns}/TM) (Table 1). All three constructs contained a phage T7 promoter. The vaccinia virus MVA-T7 system (63) was used for protein expression after transfection of BHK-21 cells with plasmid DNA. In immunofluorescence analyses with a rabbit serum specific for BVDV E^{rns}, the proteins were detected only after cell permeabilization, but not by surface staining, indicating intracellular localization (not shown). With permeabilization, signals were obtained that indicated differences with regard to the intracellular localization of the three proteins. A condensed signal with brilliantly fluorescing granula was observed for wild-type (wt) E^{rns} (Fig. 1A). The fluorescence was predominantly detected in the perinuclear region. Similar results were obtained after expression of the major viral glycoprotein E2, which represents a typical membrane protein with a carboxy-terminal transmembrane sequence (not shown). The carboxy-terminally truncated E^{rns} expressed from construct pB-E^{rns}/dCT seemed to be present in the cells in lower concentration, since the signal was always much less intense. It seemed that the protein detected in the cell was also mainly present in the perinuclear region, but the staining was more uniform with a less condensed signal and hardly any granula (Fig. 1A). For construct pB-E^{rns}/TM, a brilliant condensed signal organized in a network surrounding the nucleus and extending into the cell was detected.

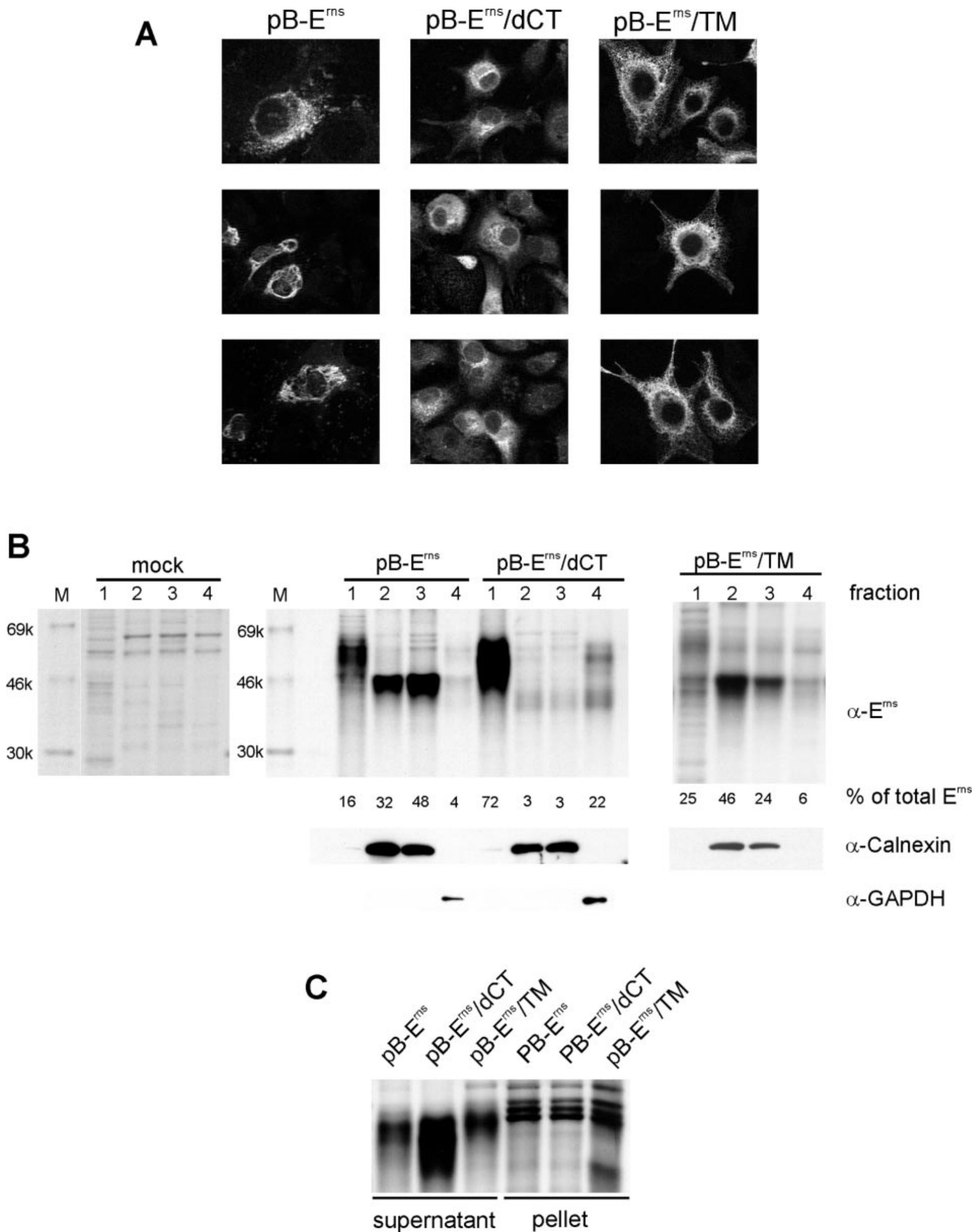


FIG. 1. Transient expression of cDNA constructs coding for the BVDV CP7 E^{rns} protein (pB-E^{rns}) or variants thereof with the C-terminal 27 amino acids deleted (pB-E^{rns}/dCT) or replaced in part by a hydrophobic sequence (pB-E^{rns}/TM) (Table 1). Expression was done in BHK-21 cells via vaccinia virus MVA/T7. (A) Transfected cells were analyzed by immunofluorescence with an E^{rns}-specific serum. (B) Transfected cells were labeled with ^{35}S amino acids. The cultures were fractionated in supernatant (fraction 1), cell debris after cell destruction (fraction 2), membrane pellet after high-speed centrifugation (fraction 3), and supernatant of high-speed centrifugation containing non-membrane-bound soluble proteins (fraction 4). E^{rns} was precipitated with a specific antiserum and analyzed via PAGE and fluorography (top). On the left side, the results obtained in a parallel experiment with mock-transfected cells (infected with vaccinia virus MVA-T7) are shown. The gel with the mock control was

To further investigate the putative differences with regard to the localization of wt and mutant E^{rns}, radioactively labeled cell cultures were fractionated after transient expression of the constructs. The cell culture supernatant and cells were harvested separately. The supernatant (fraction 1) was cleared from the cells and cell debris by centrifugation. The cells were harvested from the culture dishes by scraping and were disrupted by being passaged through a 27-gauge needle. Residual intact cells, gross cell debris, and nuclei were removed by low-speed centrifugation (fraction 2). The supernatant was subjected to high-speed centrifugation, resulting in the pelleted membrane fraction (fraction 3) and the supernatant containing soluble proteins and low-molecular-weight compounds (fraction 4). For control purposes, aliquots of each fraction were subjected to PAGE and Western blotting with antibodies directed against marker proteins for cellular membranes (calnexin) and the cytoplasm (GAPDH) (GAPDH analysis not shown for pB-E^{rns}/TM). The analysis proved that secreted, membrane-bound, and soluble proteins were properly separated, since GAPDH was found almost exclusively in fraction 4 and calnexin was found only in the membrane fraction (fraction 3) and in fraction 2, which contained the pellet with gross cell debris and nuclei (Fig. 1B).

To identify E^{rns}, the different fractions were analyzed by immunoprecipitation with the E^{rns} antiserum, separation of the precipitates by PAGE, and visualization of the protein bands by autoradiography. The electrophoretically separated proteins were also subjected to phosphorimager quantification. The determined counts of all fractions were taken as 100%, and the recovery of proteins in the individual fractions was calculated. At least three independent experiments were conducted to determine the distribution of the protein in the different fractions. (Fig. 1B). Except for fraction 2, which contained 32% of the total wt E^{rns}, the wt protein was almost exclusively detected in the cell culture supernatant (fraction 1; 16%) and the membrane fraction (fraction 3; 48%). Since fraction 2 contained calnexin but no GAPDH, it is obvious that the debris is predominately composed of membranous material. Thus, a total of 80% of the wt E^{rns} is found in membranes. In contrast to the intracellular protein of about 44 to 48 kDa, the secreted wt protein was detected as a smear located between the 46- and 69-kDa marker bands. The higher molecular mass of the secreted protein is due to changes in glycosylation. This increase in molecular mass was also observed with budded virions and secreted E^{rns} (45, 53). Since only minimal amounts of the protein were detected in the fraction with the soluble proteins (fraction 4), it is obvious that intracellular wt E^{rns} is strongly associated with membranes.

Interestingly, the E^{rns} protein with the artificial transmembrane sequence expressed from pB-E^{rns}/TM was also found in the supernatant of the cells (25% of the total protein), where it was present in two forms with different electrophoretic mobilities (Fig. 1B). This finding stands in contrast to the distribution of the calnexin control, which was hardly detectable in the supernatant. Almost the entire remainder of the E^{rns}/TM protein was recovered from the membrane fractions (46% in fraction 2 and 24% in fraction 3).

In contrast to the other two proteins, the C-terminally truncated E^{rns}/dCT was hardly detectable in the membrane fractions (3% each in fraction 2 and fraction 3). The majority of the mutated protein was detected in the cell culture supernatant (72%), and some protein was found in the soluble protein fraction (fraction 4; 22%). In the case of the truncated protein, fraction 4 contained a considerable amount of the high-molecular-weight form of E^{rns}. This finding probably reflects the increased secretion rate, which leads to a higher proportion of protein with fully processed carbohydrate side chains within the cells. Taken together, these data show that the C-terminal 27 amino acids of E^{rns} are important for both membrane interaction and retention of the protein.

To investigate whether the secreted protein was indeed soluble and not present in vesicles or viruslike particles, we subjected the cell culture supernatant to ultracentrifugation at 107,000 × *g* and subsequently looked for E^{rns} in the pellet and the supernatant. Neither wt E^{rns} nor E^{rns}/dCT was detected in the pellet. For E^{rns}/TM, the lower band detected in the supernatant of the transiently transfected cells was recovered entirely from the pellet, while the larger form was present only in the supernatant (Fig. 1C).

E^{rns} is not as tightly membrane bound as a transmembrane protein. Based on the analyses described above, the wt E^{rns} and the mutant with TM sequence are associated with the membrane to similar extents. To obtain more information on the nature of the membrane interaction of wt E^{rns}, aliquots of fractions 3 of the above-described experiment were subjected to extraction with Triton X-114. Extraction with this detergent is known to strip soluble or loosely membrane-bound proteins from the membrane while tightly membrane-bound (integral) proteins remain attached (3). After this treatment, the wt E^{rns} was found to be distributed almost evenly between the water and the detergent fractions (51% and 49%, respectively) (Fig. 2A). In contrast, most of the E^{rns} variant with the artificial transmembrane sequence remained membrane bound (84%).

In further experiments, we analyzed the E^{rns} membrane interaction by extraction of pelleted membranes of E^{rns}-expressing cells with different solutions. In addition to controls treated with PBS, membranes were incubated with 1 M NaCl, 0.1 M Na_2CO_3, pH 11.5, 4 M urea, 6 M urea, and 2% Triton X-100. As before, cells transfected with pB-E^{rns}/TM served as a control. After incubation at 4°C for 20 min, the membranes

overexposed to visualize all nonspecific bands. For control purposes, aliquots of the different fractions of the E^{rns}-expressing cells were analyzed by Western blotting with antisera against the cellular membrane protein calnexin (1:2,000 in PBS-Tween) and the cytoplasmic protein GAPDH (1:2,500 in PBS-Tween) (bottom; only samples from cells expressing pB-E^{rns} and pB-E^{rns}/dCT). For the upper gel, the location of size marker protein bands (M) are indicated and the molecular masses of the proteins (in kilodaltons) are given on the left. On the right, the different antisera used for the analyses are indicated. The amounts of the proteins recovered in the different fractions were quantified by phosphorimager analysis. Quantitative recovery of the target protein was ensured by two cycles of precipitation (see the text for further information). The counts determined for all four fractions were taken as 100%. Below the upper gel, the amounts of the protein detected in the individual fractions are indicated as percentages of the total recovery. The values represent the averages of at least three independent experiments. (C) Analysis of cell culture supernatant obtained from cells transiently expressing the indicated constructs by ultracentrifugation and subsequent immunoprecipitation of E^{rns}.

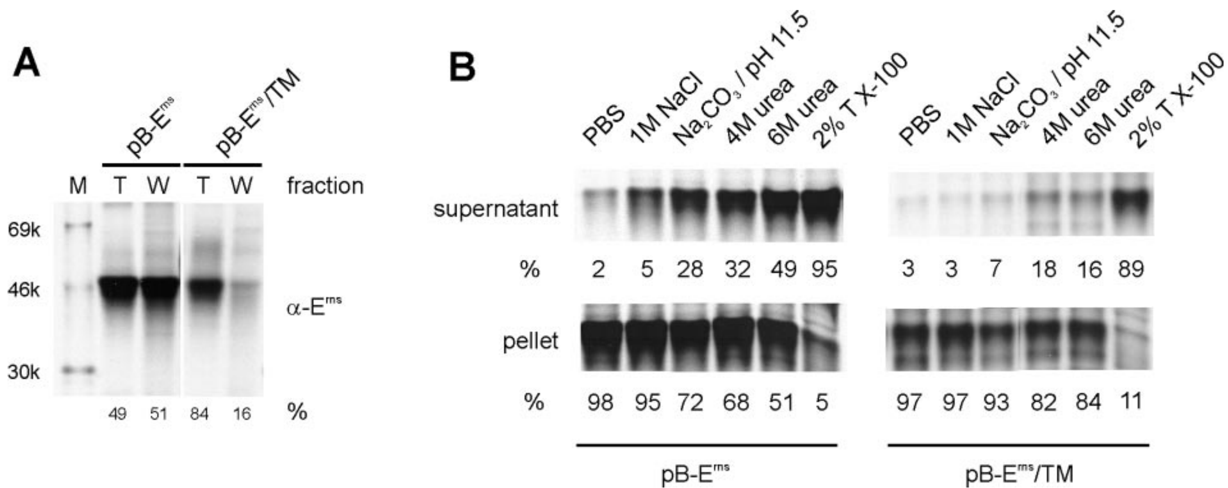


FIG. 2. Analysis of the nature of the E^{rns} membrane association. (A) The membrane fraction of cells transiently expressing E^{rns} or E^{rns}/TM, the variant with a hydrophobic transmembrane sequence, was further fractionated by extraction with Triton X-114. Both the Triton X-114 (T) and aqueous (W) fractions were further analyzed by immunoprecipitation and phosphorimager quantification (see the legend to Fig. 1). The counts determined for both fractions were taken as 100%. (B) Similar to panel A, but the membrane pellet was resuspended in the indicated solutions and, after incubation, subjected to a further ultracentrifugation step.

were pelleted and the pellets and supernatants were analyzed for the presence of E^{rns}. As expected, treatment with PBS did not solubilize significant amounts of the membrane-bound proteins, whereas Triton X-100 led to recovery of ~90% of the protein in the supernatants for both constructs. Under high-salt conditions (1 M NaCl), charges are shielded, which results in weakening ionic interactions that can bind peripheral proteins to membranes either directly or indirectly via other membrane proteins. Thus, extraction with high salt would result in solubilization of typical peripheral membrane proteins that are bound by ionic interactions. This is not the case for E^{rns}, which stays membrane bound to the same extent as the variant with the transmembrane sequence (Fig. 2B). Extraction with sodium carbonate, pH 11.5, treatment is known to transform microsomes into membrane sheets and to extract a variety of peripheral proteins from the membrane. In contrast to the high-salt conditions, treatment with 0.1 M sodium carbonate, pH 11.5, resulted in a clear difference between the wt E^{rns} and the TM variant, even though only part of the wt protein was solubilized. Similarly, a 6 M concentration of the strong chaotropic agent urea solubilized a considerable percentage of the wt E^{rns} but only small amounts of the TM variant. Once again, extraction of the wt protein was not complete, and the use of 4 M urea had a much smaller effect. It therefore can be concluded that on the one hand wt E^{rns} is not as tightly membrane bound as the TM variant, but on the other hand, it does not behave like a typical peripheral membrane protein.

Fusion of the E^{rns} C-terminal sequence to green fluorescent protein alters the subcellular localization of the reporter protein. The above-described experiments showed that the pestivirus E^{rns} represents a membrane-associated protein. The carboxy-terminal 27 amino acids are crucial for membrane anchoring. An interesting question was whether the carboxy-terminal region of E^{rns} is sufficient to confer membrane binding on a soluble protein and thus represents the E^{rns} membrane anchor. We therefore fused the C-terminal sequence of E^{rns} to the carboxy terminus of GFP. To do so, the cDNA construct pK19, which contained the 56 3′-terminal codons of the E^{rns} coding sequence fused to the 3′ end of the GFP gene, was established. After expression in BHK-21 cells, the fusion protein gave a condensed signal with brightly fluorescing granula, whereas wt GFP expressed from plasmid pK13 resulted in uniform staining of the cytoplasm and, in some cells, a strong signal in the nuclei (Fig. 3A). It was therefore obvious that fusion of the pestivirus sequence to GFP resulted in a different cellular localization of the indicator protein. To prove that this different localization was due to membrane binding, transfected cells were fractionated as described above. Because of the absence of a signal sequence, the transiently expressed proteins were not secreted into the cell culture supernatant (not shown). Therefore, only fractions 3 and 4 were analyzed by Western blotting. The wt GFP was recovered only in the fraction with the soluble proteins. In contrast, the fusion protein was detected almost exclusively in the membrane protein fraction, showing that the E^{rns} C terminus is indeed sufficient to hook a cytoplasmic protein to intracellular membranes (Fig. 3B).

Identification of the core membrane binding sequence. The above-described experiments showed that the 56 carboxy-terminal amino acids of E^{rns} function as a membrane anchor when fused to the carboxy terminus of a foreign protein. To track down the sequence that is responsible for this activity, the pestivirus sequence fused to the GFP reporter was truncated from the N and C termini. Table 2 gives an overview of the construct names and the included BVDV E^{rns} C-terminal sequences. All constructs were transiently expressed in BHK-21 cells. The transfected cells were fractionated in order to find out whether the GFP reporter was membrane bound or soluble. Both amino-terminal and carboxy-terminal truncation of the BVDV sequence in the fusion proteins resulted in reduced recovery in the membrane fraction (Fig. 4A). The ability of the stretch of E^{rns} residues to confer membrane association seemed to be reduced consecutively with increasing truncation

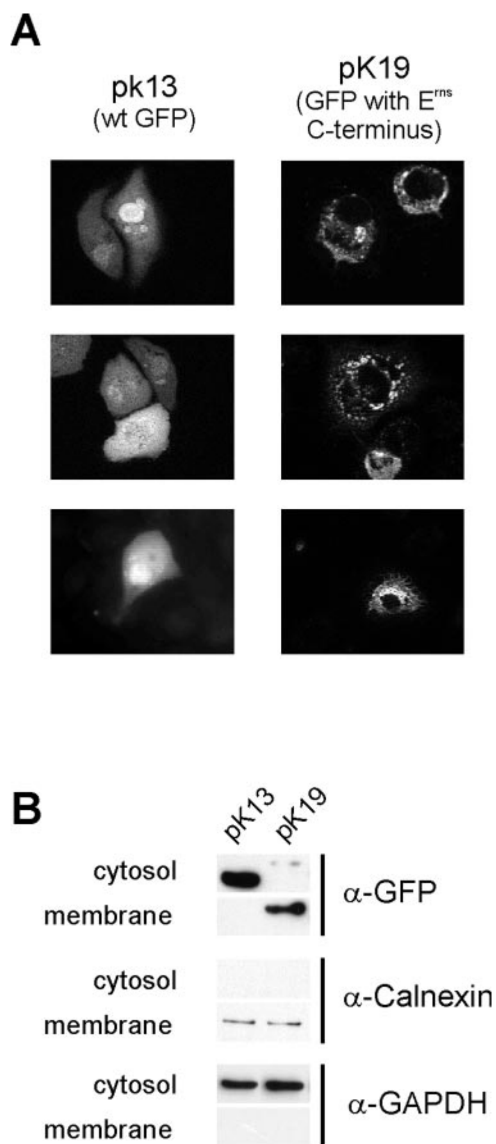


FIG. 3. Transient expression of constructs pK13 and pK19, which encode GFP or GFP fused at its carboxy terminus with the 56 C-terminal amino acids of E^{rns}. (A) Fluorescence microscopic analysis. (B) Western blot with a GFP-specific antiserum (1:1,000 in PBS-Tween) of protein extracts obtained after fractionation of the cells. The fractionation was done by ultracentrifugation as described above. "Membrane" represents fraction 3, and "cytosol" represents fraction 4. Western blots with antisera against the cellular proteins calnexin (1: 2,000 in PBS-Tween) and GAPDH (1:1,000 in PBS-Tween) served as controls for the effective fractionation (see the legends to Fig. 1 and 2).

of the BVDV sequence, but residual membrane binding was observed for all constructs except pK78 and the control, pK13.

In a second set of experiments, fractionation was done with Triton X-114 without prior separation of cellular components via ultracentrifugation. This type of fractionation leads to separation of membranes and nonmembranous components (mainly cytosol) (3, 53). Membrane proteins are predominantly found in the membrane but bleed more or less into the aqueous fraction, whereas water-soluble proteins are found exclusively in the aqueous phase (Fig. 4B, control with antiserum α-Aldolase). Thus, detection of a protein in the membrane fraction results from true membrane binding and does not represent contamination. Importantly, the detergent-based separation is more stringent than the method based on centrifugation, so that loosely membrane-bound proteins are found entirely in the soluble fraction. Comparison of the results of Triton X-114-based fractionation (Fig. 4B) with those obtained after centrifugation (Fig. 4A) shows that the products of several constructs found in the membrane pellet after ultracentrifugation are bound to the membrane so weakly that they no longer show up in the detergent phase.

Selected GFP fusion constructs with truncated E^{rns} C-terminal sequences were also analyzed by immunofluorescence (Fig. 4C). Consecutive truncations of the C or the N terminus of the E^{rns} moiety resulted in less condensation of the signal and increased staining of the cytoplasm and the nucleus. Taken together, both N-terminal and C-terminal truncation of the E^{rns} C-terminal sequence seem to impair membrane binding ability consecutively. Similar data were obtained in transient-expression studies in BVDV-susceptible porcine kidney (PK15) cells without the use of vaccinia virus with both Triton-X114 extraction and alkaline-carbonate-based extraction with sodium carbonate, pH 11.5 (not shown). The latter method uses disruption of membranes by incubation at alkaline pH, which results in conversion of membrane vesicles into open membrane sheets that can be collected by ultracentrifugation, whereas peripheral membrane proteins and soluble proteins from within the vesicles are found in the supernatant (40). Since similar results were obtained with different cell lines and fractionation methods, an important influence of the cell type, the expression system, or the fractionation procedure can be excluded.

The truncation experiments showed that a core sequence composed of amino acids 202 to 212 has to be present in order to observe detergent-resistant membrane binding. This result could indicate that this sequence alone would be able to confer membrane anchoring. We therefore established a further set of constructs that contained only the coding sequence of the core region or derivatives thereof fused to the 3′ end of the GFP gene (Table 3). Expression of these constructs and analysis of the localization of the products revealed that even after the less stringent centrifugation-based fractionation, none of the resulting proteins showed considerable membrane binding (Fig. 5A). Only traces of the product of construct pB6 were found in the membrane fraction at all, and this construct contains a total of five extra amino acids in addition to the core region. In immunofluorescence analyses, all the constructs gave results resembling those of wt GFP, for example, pB6 and pB7 in Fig. 5B. Thus, the core region is obviously necessary but not sufficient for anchoring a protein to a lipid bilayer. Interestingly, both amino-terminal and carboxy-terminal extensions of the core sequence are able to restore membrane binding ability, as can be concluded from the truncation experiments shown above.

To verify the results obtained with the GFP fusion constructs, some of the experiments described above were repeated with E^{rns} constructs. Triton X-114 extraction of cells transiently expressing wt E^{rns}, E^{rns}/dCT, or E^{rns}/TM showed that the C-terminally truncated protein was not present in the

TABLE 2. C-terminal sequences of E^{rns} proteins encoded by expression constructs[a]

Name	BVDV sequence added to GFP C terminus
	*180 *190 *200 *210 *220
pK13	
pK19	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTWFGAYA
pK27	LTTWLGRQLGILGKKLENKSKTWFGAYA
pK46	TTWLGRQLGILGKKLENKSKTWFGAYA
pK48	TWLGRQLGILGKKLENKSKTWFGAYA
pK50	WLGRQLGILGKKLENKSKTWFGAYA
pK37	LGRQLGILGKKLENKSKTWFGAYA
pK34	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTW
pK32	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSK
pK57	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLEN
pK66	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILG
pK76	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGI
pK81	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLG
pK78	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGR

[a] Asterisks mark amino acid positions.

detergent fraction, whereas E^{rns}/TM was predominantly found in the detergent fraction and the wt protein was distributed between both phases (Fig. 6A).

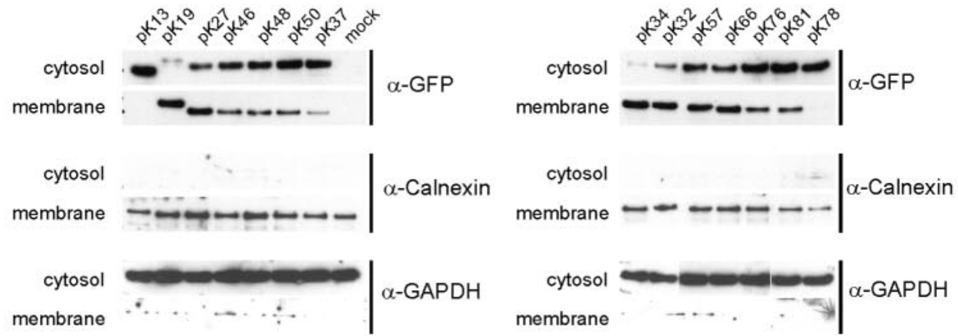
C-terminal sequences with truncations were also tested in the context of the original protein (Table 4). Consecutive C-terminal truncations resulted in the expected increase of E^{rns} secretion and considerable decrease of protein amounts recovered from the membrane fractions (fractions 2 and 3) (Fig. 6B). Similarly, internal deletions within the core region of the membrane anchor (Table 4) led to detection of smaller amounts of protein in the membrane fractions and higher yields in fractions 1 and 4 (Fig. 6B). Thus, it can be concluded that membrane binding of either the cytoplasmic reporter protein or the endoplasmic reticulum (ER)-translocated authentic glycoprotein was influenced in basically the same way by the tested mutations.

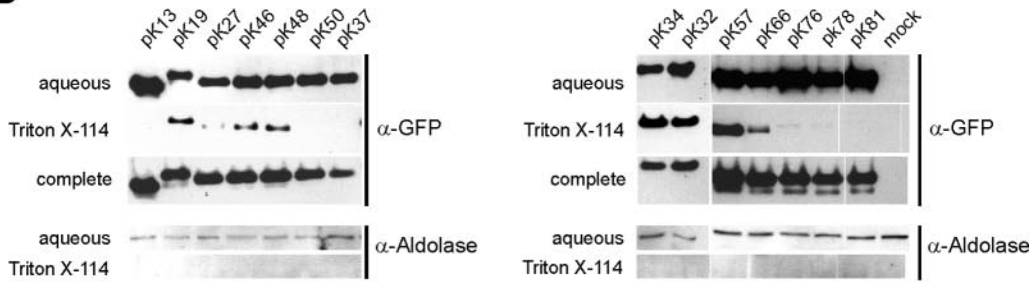
DISCUSSION

The pestivirus E^{rns} protein is a highly glycosylated structural protein that is found in the pestivirus polyprotein between the viral capsid protein and the two other glycoproteins, E1 and E2 (reviewed in references 20 and 34). Early studies of the processing of the structural-protein region of the polyprotein revealed that the cleavage between E^{rns} and E1 is delayed in comparison to the other cleavage events (45). This difference in processing kinetics corresponded to the results of the sequence analysis. The cleavage sites C/E^{rns}, E1/E2, E2/P7, and P7/NS2 all represent typical signalase cleavage sites composed of a hydrophobic stretch of amino acids fulfilling the requirements of a signal/transmembrane sequence, followed by a von Heijne processing site (55). In contrast, the E^{rns}/E1 site lacks a hydrophobic stretch of residues. Since E^{rns} terminates with A-Y/H-A, which meets part of the prerequisites for a signalase cleavage site, it might nevertheless be cleaved by a signalase, and the delay in processing at this site could then be explained by the absence of the hydrophobic part of the cleavage signal. Alternatively, a yet unknown protease might be involved. Importantly, the absence of the hydrophobic sequence implies that E^{rns} does not contain a C-terminal transmembrane sequence that could serve as a membrane anchor.

Even though considerable amounts of the E^{rns} synthesized in an infected cell are secreted into the supernatant, most of the translated protein is retained until it is attached by a so far unknown mechanism to the budding viral particle and enters the secretory pathway as part of the virion (45). Similarly, 84% of the E^{rns} protein expressed after transfection of cDNA constructs is found within the cell at about 16 h posttransfection. These features of a protein translocated to the ER are generally connected with binding to a lipid membrane. The absence of a classical transmembrane/stop transfer sequence at the carboxy terminus of E^{rns} raises the question of whether, and how, the protein is anchored to the membrane. Protein sequencing showed that the amino-terminal signal sequence is cleaved off and therefore cannot serve as a transmembrane region of mature E^{rns} (45). According to computer predictions, the E^{rns} sequence does not contain a signal for the addition of a glycophosphatidylinositol anchor (11, 12, 45). Nevertheless, E^{rns} is a membrane-bound protein, as shown here for the first time. We provide evidence that the carboxy-terminal amino acids are involved in membrane anchoring. We cannot, of course, exclude a contribution of other regions of the protein to membrane association, but the experiments with GFP fu-

FIG. 4. Results of the fractionation of cells transiently expressing cDNA constructs coding for GFP or GFP with a set of carboxy-terminal extensions representing different parts of the C terminus of the E^{rns} protein. The features of the different constructs are given in Table 2. Fractionation was done either by centrifugation (A) or by extraction with Triton X-114 (B). (A) See the legend to Fig. 3 for further details. (B) A Western blot with an antiserum directed against the cellular cytoplasmic protein aldolase (1:500 in PBS-Tween) served as a control for the purity of the membrane fraction. In addition to the fractionated samples, aliquots of nonfractionated protein extracts (complete) were loaded on the gel. (C) Fluorescence microscopic analysis of selected GFP variants containing the C- or N-terminally truncated E^{rns} membrane anchor.


A
pK13 pK19 pK27 pK46 pK48 pK50 pK37 mock
pK34 pK32 pK57 pK66 pK76 pK81 pK78
cytosol
membrane
α-GFP
α-Calnexin
α-GAPDH


B
pK13 pK19 pK27 pK46 pK48 pK50 pK37
pK34 pK32 pK57 pK66 pK76 pK78 pK81 mock
aqueous
Triton X-114
complete
α-GFP
α-Aldolase

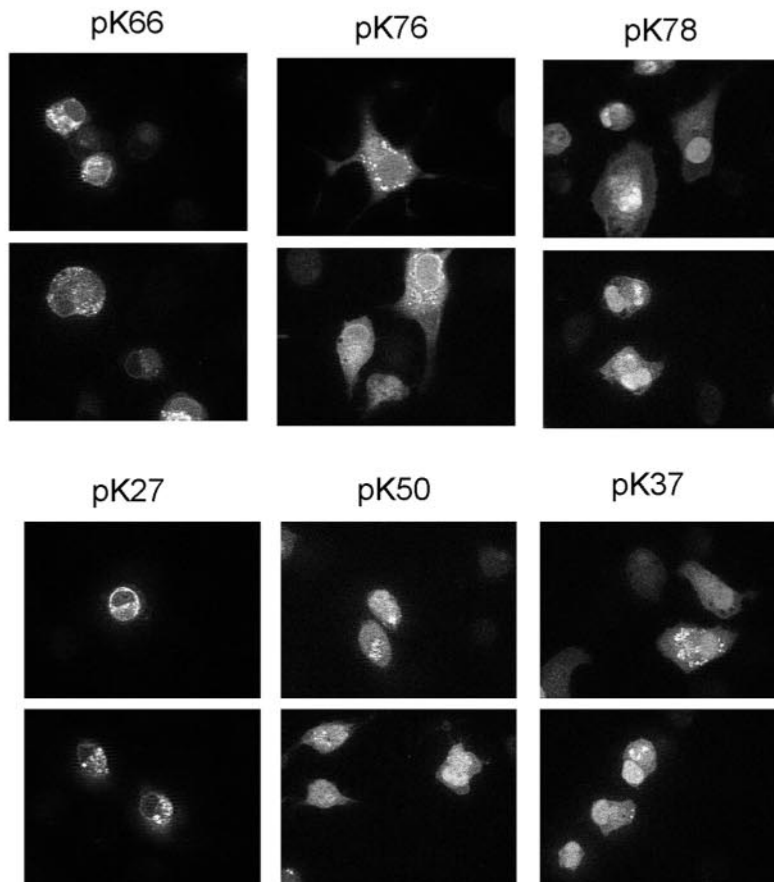

C
pK66
pK76
pK78
pK27
pK50
pK37

TABLE 3. C-terminal sequences of E^{rns} proteins encoded by expression constructs[a]

Name	BVDV sequence added to GFP C terminus
	*180 *190 *200 *210 *220
pK19	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTWFGAYA
pB6	KLTTWLGRQLGILGKK
pB10	TTWLGRQLGILG
pB8	TTWLGRQLG
pB9	WLGRQLGILG
pB7	WLGRQLG

[a] Asterisks mark amino acid positions.

sions clearly show that the 56 carboxy-terminal amino acids alone have the potential to hook a protein to lipid membranes. First experiments with the respective CSFV sequence showed that it functions equivalently (data not shown), so it seems justified to regard the carboxy terminus of the pestivirus E^{rns} protein as its membrane anchor.

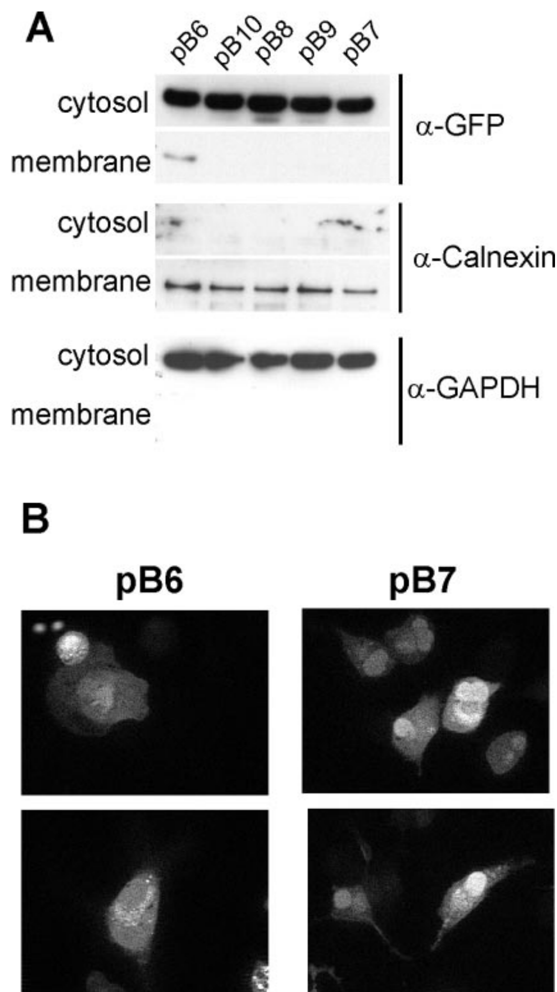


FIG. 5. Analysis of the membrane binding activities of GFP constructs containing the determined core region of the E^{rns} membrane anchor. (A) Western blot analysis after cell fractionation centrifugation as in Fig. 3. (B) Fluorescence microscopic analysis of two selected GFP variants. The features of the different constructs are given in Table 3. See also the text and the legends to Fig. 1 to 3.

Our truncation experiments with GFP constructs showed that shortened E^{rns} C-terminal sequences can also serve as membrane anchors, but with more or less reduced efficiency. Importantly, the experiments revealed that a core region composed of amino acids 202 to 212 of E^{rns} is crucial for membrane binding. However, the core region alone is not sufficient but has to be flanked by upstream or downstream sequences. This result indicates that membrane binding is presumably not achieved through a signal for addition of some kind of a hydrophobic anchor. Rather, the sequence itself interacts directly with membranes, and truncation beyond a minimal length abolishes this ability or lowers the binding force below the level detectable in our assays. This conclusion holds true for both the cytoplasmic GFP fusion proteins that bind to the cytoplasmic face of membranes posttranslationally and the original E^{rns} protein bound to membranes inside a so-far-unknown intracellular compartment. Compared to the GFP constructs, the original E^{rns} protein seems to be much more dependent on the integrity of the C-terminal sequence, since the truncations have a considerably stronger effect on membrane binding of the latter protein.

Besides its function as a membrane anchor, the E^{rns} carboxy terminus is known to exhibit two further interesting features. The most carboxy-terminal region of the protein contains a glycosaminoglycan binding site (25, 53). The basic motif KKL ENKSK (amino acids 213 to 220) was shown to be responsible for the binding. Since the addition of purified E^{rns} with mutations of K214 and K218 was no longer able to block BVDV infection of cells, the hypothesis was put forward that interaction of this sequence with acidic groups on the surfaces of the target cells is important for virus infection. Electrostatic interaction of the basic residues present in this motif and the surrounding sequence with negatively charged phospholipids could also confer membrane binding of E^{rns}. However, the negligible effect of the high-salt extraction of pelleted membranes on E^{rns} membrane association shows that this interaction is at least not the major binding force.

The carboxy-terminal sequence of E^{rns} was found to be able to translocate E^{rns} or other proteins from the outside through the plasma membrane into a target cell (33). Similar translocation activities were also found for other (homologous) sequences, but the mechanism underlying the translocation process is still not well understood. The core sequence necessary for translocation was narrowed down in truncation experiments to residues 194 to 218 for optimal function, but peptides with amino termini downstream of 194 or caboxy termini upstream of position 218 were still internalized. The truncation

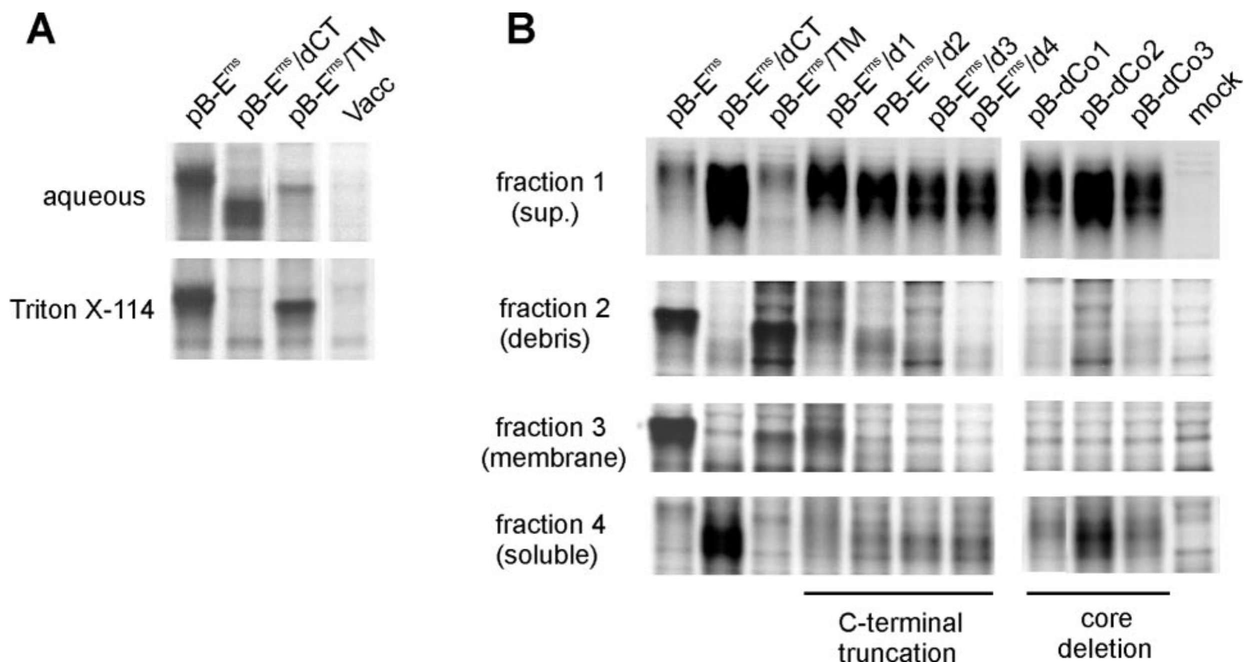


FIG. 6. (A) Triton X-114 extraction of whole cells transiently expressing wt E^rns, E^rns/dCT, and E^rns/TM. E^rns was detected in the separated phases by immunoprecipitation and PAGE (see the legend to Fig. 4). (B) Determination of the influence of C-terminal truncation and deletions within the core region of the E^rns membrane anchor on membrane binding of E^rns. Cell fractionation was done by centrifugation. The deletions present in the proteins derived from the different constructs are indicated in Table 4. See also the text and the legends to Fig. 1 to 3.

experiments conducted for identification of the translocation activity (33) and the anchor function of the E^rns C terminus, respectively, did not yield fully congruent results, but the core sequence for membrane association also represented the central part of the region that provides optimal translocation activity. This finding is perhaps not surprising, since both features rely on the interaction of the peptide with a membrane. However, there are obvious fundamental differences between the functions of an "anchor" and a "transporter." The situation is even more puzzling, since E^rns seems to be translocated unidirectionally from the outside into the cell, while anchoring of newly synthesized E^rns has to occur in an "outside" orientation, so the differences cannot be explained by a simple preference for an "in" or "out" configuration. Attempts to explain this discrepancy on the basis of the presently available data would be mere speculation.

The data presented clearly show that the C-terminal sequence of E^rns interacts with membranes and is able to tightly associate a protein with a membrane. A highly interesting question is which mechanism leads to membrane binding. As already discussed above, the available data indicate that membrane binding is an intrinsic activity of the sequence itself and apparently is not based on addition of a hydrophobic moiety to this sequence. The E^rns C terminus does not represent a typical transmembrane region, and the results of the extraction assays with Triton X-114, as well the solubilization experiments with pH 11.5 or 6 M urea, show that E^rns is less tightly bound to the membrane than a typical integral membrane protein anchored by a transmembrane helix. On the other hand, the resistance to high-salt extraction and the rather small effect of the treatment with 4 M urea argue against the possibility that E^rns represents a peripheral membrane protein. Langedijk proposed that the C-terminal sequence of E^rns could fold into an amphipathic helix, which could be responsible for membrane interaction (33). This helix would be characterized by a basic surface opposite to the hydrophobic face. Membrane binding of such a

TABLE 4. C-terminal sequences of E^rns proteins encoded by expression constructs[a]

Name	BVDV E^rns with C-terminal or internal deletions at C terminus
	*180 *190 *200 *210 *220
Wt	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTWFGAYA
pB-E^rns/d1	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGILGKKLENKSKTW
pB-E^rns/d2	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLGI
pB-E^rns/d3	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGRQLG
pB-E^rns/d4	TSVIQDTAHYLVDGMTNSLESARQGTAKLTTWLGR
pB-dCo1	TSVIQDTAHYLVDGMTNSLESARQGTAKLTT ILGKKLENKSKTWFGAYA
pB-dCo2	TSVIQDTAHYLVDGMTNSLESARQGTA KKLENKSKTWFGAYA
pB-dCo3	TSVIQDTAHYLVDGMTNSLESARQGTA ILGKKLENKSKTWFGAYA

[a] Asterisks mark amino acid positions.

helix could be driven both by hydrophobic interaction between the apolar face of the helix and the fatty acid chains within the lipid bilayer and by electrostatic forces between the positively charged surface of the helix and the negatively charged phosphate groups of phospholipids. Sequence comparison showed that the character of most of the residues relevant for the amphipathic nature is conserved among pestiviruses so that several hydrophobic residues are found on the one face whereas a net sum of five positively charged residues are located on the opposite side. In addition, two tryptophan residues are present at positions that would allow the ring systems to localize to the interphase, which is preferred by this amino acid. Further experiments are needed to verify the relevance of the putative helix and individual amino acids for the membrane binding of the sequence.

Membrane association by in-plane amphipathic α-helices has been described, or at least proposed, for several other proteins, e.g., prostaglandin H synthase, squalene cyclase, hepatitis C virus NS5A protein, Semliki Forest virus NSP1, and poliovirus or hepatitis A virus 2C protein (5, 10, 29, 32, 39, 41–43, 49, 52, 60). All these proteins share specific features with a larger group of membrane proteins called tail-anchored proteins (TAPs). Typically, TAPs represent proteins that are bound to membranes directly via a short terminal anchor sequence without involvement of a signal sequence (4, 30, 56). This feature implies that TAPs are located at the inner faces of cellular membranes with the bulk of the protein exposed to the cytoplasm. More often than amphipathic helices, standard hydrophobic transmembrane sequences serve as a tail anchor, and in most cases, the anchor sequence is located at the C terminus of the protein, implying posttranslational membrane binding (1). As indicated by the results of our GFP experiments, the E^{rns} C terminus is able to function as such a tail anchor, since its presence is sufficient to confer membrane association on a GFP without a signal sequence. However, it has to be kept in mind that the usual function of this sequence is to provide a membrane anchor to a protein that is translocated into the ER. To our knowledge, E^{rns} is the first viral glycoprotein described as being bound to the membrane by this unusual mechanism. A highly interesting question for future work is therefore why E^{rns} contains this atypical membrane anchor and not a standard hydrophobic transmembrane region. When considering this question, the different functions of E^{rns} have to be regarded. Even though evidence is increasing that the E2 protein represents the (major) receptor binding protein of the virus (35), E^{rns} is also essential for infection and accordingly is targeted by virus-neutralizing antibodies (19, 20, 24, 57). Besides its role as a structural protein, E^{rns} displays further activities. It is the only known viral structural glycoprotein that exhibits RNase activity (15, 18, 47, 61). The enzymatic function of the protein is not essential for virus replication, but abrogation of the RNase activity leads to considerable virus attenuation (36, 37). Different hypotheses have been put forward to explain this activity by interference with the host or host cellular immune response. The secretion of the protein from the infected cell plays an important role in these considerations. It might well be that the immunomodulatory effect of E^{rns} is dependent on its secretion and perhaps on its ability to be efficiently internalized by a target cell. Detection of the protein in the supernatant of transiently transfected cells shows that secretion of E^{rns} represents an intrinsic property of the polypeptide that is not dependent on virus replication or the presence of other viral proteins. The atypical membrane anchor could be necessary to meet the different requirements, namely, establishment of a certain equilibrium between secretion and retention, attachment of the protein to the virion, and translocation of the secreted protein into the so far unknown target cell of the RNase. Further elucidation of the mechanism of E^{rns} membrane anchoring not only may be interesting from a biochemical point of view but will also represent an important contribution to understanding the pathogenesis of pestivirus-induced diseases.

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REFERENCES

1. **Abell, B. M., M. R. Pool, O. Schlenker, I. Sinning, and S. High.** 2004. Signal recognition particle mediates post-translational targeting in eukaryotes. EMBO J. **23:**2755–2764.
2. **Bolin, S. R., V. Moennig, N. E. Kelso Gourley, and J. Ridpath.** 1988. Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. Arch. Virol. **99:**117–123.
3. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256:**1604–1607.
4. **Borgese, N., S. Colombo, and E. Pedrazzini.** 2003. The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. J. Cell Biol. **161:**1013–1019.
5. **Brass, V., E. Bieck, R. Montserret, B. Wolk, J. A. Hellings, H. E. Blum, F. Penin, and D. Moradpour.** 2002. An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. J. Biol. Chem. **277:**8130–8139.
6. **Collett, M. S., R. Larson, S. Belzer, and E. Retzel.** 1988. Proteins encoded by bovine viral diarrhea virus: the genome organization of a pestivirus. Virology **165:**200–208.
7. **Collett, M. S., M. A. Wiskerchen, E. Welniak, and S. K. Belzer.** 1991. Bovine viral diarrhea virus genomic organization. Arch. Virol. Suppl. **3:**19–27.
8. **Devereux, J., P. Haeberli, and O. A. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Donis, R. O., W. Corapi, and E. J. Dubovi.** 1988. Neutralizing monoclonal antibodies to bovine viral diarrhoea virus bind to the 56K to 58K glycoprotein. J. Gen. Virol. **69:**77–86.
10. **Echeverri, A., R. Banerjee, and A. Dasgupta.** 1998. Amino-terminal region of poliovirus 2C protein is sufficient for membrane binding. Virus Res. **54:**217–223.
11. **Eisenhaber, B., P. Bork, and F. Eisenhaber.** 1999. Prediction of potential GPI-modification sites in proprotein sequences. J. Mol. Biol. **292:**741–758.
12. **Eisenhaber, B., P. Bork, Y. Yuan, G. Loffler, and F. Eisenhaber.** 2000. Automated annotation of GPI anchor sites: case study *C. elegans*. Trends Biochem. Sci. **25:**340–341.
13. **Elbers, K., N. Tautz, P. Becher, T. Rümenapf, and H.-J. Thiel.** 1996. Processing in the pestivirus E2-NS2 region: identification of the nonstructural proteins p7 and E2p7. J. Virol. **70:**4131–4135.
14. **Harada, T., N. Tautz, and H. J. Thiel.** 2000. E2-p7 region of the bovine viral diarrhea virus polyprotein: processing and functional studies. J. Virol. **74:**9498–9506.
15. **Hausmann, Y., G. Roman-Sosa, H. J. Thiel, and T. Rumenapf.** 2004. Classical swine fever virus glycoprotein E^{rns} is an endoribonuclease with an unusual base specificity. J. Virol. **78:**5507–5512.
16. **Heinz, F. X., M. S. Collett, R. H. Purcell, E. A. Gould, C. R. Howard, M. Houghton, J. M. Moormann, C. M. Rice, and H.-J. Thiel.** 2000. Family Flaviviridae, p. 859–878. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
17. **Horiuchi, H., K. Yanai, M. Takagi, K. Yano, E. Wakabayashi, A. Sanda, S. Mine, K. Ohgi, and M. Irie.** 1988. Primary structure of a base non-specific ribonuclease from *Rhizopus niveus*. J. Biochem. **103:**408–418.

18. **Hulst, M. M., G. Himes, E. Newbigin, and R. J. M. Moormann.** 1994. Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology **200:**558–565.
19. **Hulst, M. M., and R. J. Moormann.** 1997. Inhibition of pestivirus infection in cell culture by envelope proteins E(rns) and E2 of classical swine fever virus: E(rns) and E2 interact with different receptors. J. Gen. Virol. **78:**2779–2787.
20. **Hulst, M. M., and R. J. Moormann.** 2001. Erns protein of pestiviruses. Methods Enzymol. **342:**431–440.
21. **Hulst, M. M., H. G. van Gennip, and R. J. Moormann.** 2000. Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E^{rns}. J. Virol. **74:**9553–9561.
22. **Hulst, M. M., H. G. van Gennip, A. C. Vlot, E. Schooten, A. J. de Smit, and R. J. Moormann.** 2001. Interaction of classical swine fever virus with membrane-associated heparan sulfate: role for virus replication in vivo and virulence. J. Virol. **75:**9585–9595.
23. **Hulst, M. M., D. F. Westra, G. Wensvoort, and R. J. Moormann.** 1993. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. J. Virol. **67:**5435–5442.
24. **Iqbal, M., H. Flick-Smith, and J. W. McCauley.** 2000. Interactions of bovine viral diarrhoea virus glycoprotein E(rns) with cell surface glycosaminoglycans. J. Gen. Virol. **81:**451–459.
25. **Iqbal, M., and J. W. McCauley.** 2002. Identification of the glycosaminoglycan-binding site on the glycoprotein E(rns) of bovine viral diarrhoea virus by site-directed mutagenesis. J. Gen. Virol. **83:**2153–2159.
26. **Iqbal, M., E. Poole, S. Goodbourn, and J. W. McCauley.** 2004. Role for bovine viral diarrhea virus E^{rns} glycoprotein in the control of activation of beta interferon by double-stranded RNA. J. Virol. **78:**136–145.
27. **Kessler, S. W.** 1981. Use of protein A bearing staphylococci for the immunoprecipitation and isolation of antigens from cells. Methods Enzymol. **73:** 442–459.
28. **König, M., T. Lengsfeld, T. Pauly, R. Stark, and H.-J. Thiel.** 1995. Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. J. Virol. **69:**6479–6486.
29. **Kusov, Y. Y., C. Probst, M. Jecht, P. D. Jost, and V. Gauss-Muller.** 1998. Membrane association and RNA binding of recombinant hepatitis A virus protein 2C. Arch. Virol. **143:**931–944.
30. **Kutay, U., E. Hartmann, and T. A. Rapoport.** 1993. A class of membrane proteins with a C-terminal anchor. Trends Cell Biol. **3:**72–75.
31. **Lackner, T., A. Muller, A. Pankraz, P. Becher, H. J. Thiel, A. E. Gorbalenya, and N. Tautz.** 2004. Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an RNA virus. J. Virol. **78:**10765–10775.
32. **Lampio, A., I. Kilpelainen, S. Pesonen, K. Karhi, P. Auvinen, P. Somerharju, and L. Kaariainen.** 2000. Membrane binding mechanism of an RNA virus-capping enzyme. J. Biol. Chem. **275:**37853–37859.
33. **Langedijk, J. P.** 2002. Translocation activity of C-terminal domain of pestivirus Erns and ribotoxin L3 loop. J. Biol. Chem. **277:**5308–5314.
34. **Lindenbach, B. D., and C. M. Rice.** 2001. Flaviviridae: the viruses and their replication, p. 991–1042. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.
35. **Maurer, K., T. Krey, V. Moennig, H. J. Thiel, and T. Rumenapf.** 2004. CD46 is a cellular receptor for bovine viral diarrhea virus. J. Virol. **78:**1792–1799.
36. **Meyer, C., M. Von Freyburg, K. Elbers, and G. Meyers.** 2002. Recovery of virulent and RNase-negative attenuated type 2 bovine viral diarrhea viruses from infectious cDNA clones. J. Virol. **76:**8494–8503.
37. **Meyers, G., A. Saalmüller, and M. Büttner.** 1999. Mutations abrogating the RNase activity in glycoprotein E^{rns} of the pestivirus classical swine fever virus lead to virus attenuation. J. Virol. **73:**10224–10235.
38. **Meyers, G., N. Tautz, P. Becher, H.-J. Thiel, and B. Kümmerer.** 1996. Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. J. Virol. **70:**8606–8613.
39. **Nina, M., S. Berneche, and B. Roux.** 2000. Anchoring of a monotopic membrane protein: the binding of prostaglandin H2 synthase-1 to the surface of a phospholipid bilayer. Eur. Biophys. J. **29:**439–454.
40. **Okamoto, T., R. B. S. P. E. Schwab, and M. P. Lisanti.** 1999. Analysis of the association of proteins with membranes, p. 5.4.1–5.4.17. *In* J. S. Bonifacino, M. Dasso, J. Lippincott-Schwartz, J. B. Harford, and K. M. Yamada (ed.), Current protocols in cell biology. John Wiley & Sons, Inc., New York, N.Y.
41. **Paul, A. V., A. Molla, and E. Wimmer.** 1994. Studies of a putative amphipathic helix in the N-terminus of poliovirus protein 2C. Virology **199:**188–199.
42. **Penin, F., V. Brass, N. Appel, S. Ramboarina, R. Montserret, D. Ficheux, H. E. Blum, R. Bartenschlager, and D. Moradpour.** 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J. Biol. Chem. **279:**40835–40843.
43. **Picot, D., P. J. Loll, and R. M. Garavito.** 1994. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. Nature **367:**243–249.
44. **Rümenapf, T., R. Stark, G. Meyers, and H.-J. Thiel.** 1991. Structural proteins of hog cholera virus expressed by vaccinia virus: further characterization and induction of protective immunity. J. Virol. **65:**589–597.
45. **Rümenapf, T., G. Unger, J. H. Strauss, and H.-J. Thiel.** 1993. Processing of the envelope glycoproteins of pestiviruses. J. Virol. **67:**3288–3295.
46. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
47. **Schneider, R., G. Unger, R. Stark, E. Schneider-Scherzer, and H.-J. Thiel.** 1993. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science **261:**1169–1171.
48. **Shmulevitz, M., and R. Duncan.** 2000. A new class of fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped fusogenic reoviruses. EMBO J. **19:**902–912.
49. **Spencer, A. G., E. Thuresson, J. C. Otto, I. Song, T. Smith, D. L. DeWitt, R. M. Garavito, and W. L. Smith.** 1999. The membrane binding domains of prostaglandin endoperoxide H synthases 1 and 2. Peptide mapping and mutational analysis. J. Biol. Chem. **274:**32936–32942.
50. **Stark, R., G. Meyers, T. Rümenapf, and H.-J. Thiel.** 1993. Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. J. Virol. **67:**7088–7095.
51. **Tautz, N., K. Elbers, D. Stoll, G. Meyers, and H.-J. Thiel.** 1997. Serine protease of pestiviruses: determination of cleavage sites. J. Virol. **71:**5415–5422.
52. **Teterina, N. L., A. E. Gorbalenya, D. Egger, K. Bienz, and E. Ehrenfeld.** 1997. Poliovirus 2C protein determinants of membrane binding and rearrangements in mammalian cells. J. Virol. **71:**8962–8972.
53. **Thiel, H.-J., R. Stark, E. Weiland, T. Rümenapf, and G. Meyers.** 1991. Hog cholera virus: molecular composition of virions from a pestivirus. J. Virol. **65:**4705–4712.
54. **van Zijl, M., G. Wensvoort, E. de Kluyver, M. Hulst, H. van der Gulden, A. Gielkens, A. Berns, and R. Moormann.** 1991. Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. J. Virol. **65:**2761–2765.
55. **von Heijne, G., and Y. Gavel.** 1988. Topogenic signals in integral membrane proteins. Eur. J. Biochem. **174:**671–678.
56. **Wattenberg, B., and T. Lithgow.** 2001. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. Traffic **2:**66–71.
57. **Weiland, E., R. Ahl, R. Stark, F. Weiland, and H.-J. Thiel.** 1992. A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. J. Virol. **66:**3677–3682.
58. **Weiland, E., R. Stark, B. Haas, T. Rümenapf, G. Meyers, and H.-J. Thiel.** 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide linked heterodimer. J. Virol. **64:**3563–3569.
59. **Weiland, F., E. Weiland, G. Unger, A. Saalmuller, and H. J. Thiel.** 1999. Localization of pestiviral envelope proteins E(rns) and E2 at the cell surface and on isolated particles. J. Gen. Virol. **80:**1157–1165.
60. **Wendt, K. U., A. Lenhart, and G. E. Schulz.** 1999. The structure of the membrane protein squalene-hopene cyclase at 2.0 A resolution. J. Mol. Biol. **286:**175–187.
61. **Windisch, J. M., R. Schneider, R. Stark, E. Weiland, G. Meyers, and H.-J. Thiel.** 1996. RNase of classical swine fever virus: biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. J. Virol. **70:**352–358.
62. **Wiskerchen, M., S. K. Belzer, and M. S. Collett.** 1991. Pestivirus gene expression: the first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. J. Virol. **65:**4508–4514.
63. **Wyatt, L. S., B. Moss, and S. Rozenblatt.** 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology **210:**202–205.
64. **Xu, J., E. Mendez, P. R. Caron, C. Lin, M. A. Murcko, M. S. Collett, and C. M. Rice.** 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J. Virol. **71:**5312–5322.