

# A New Type of Signal Peptidase Cleavage Site Identified in an RNA Virus Polyprotein\*

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Pestiviruses, a group of enveloped positive strand RNA viruses belonging to the family *Flaviviridae*, express their genes via a polyprotein that is subsequently processed by proteases. The structural protein region contains typical signal peptidase cleavage sites. Only the site at the C terminus of the glycoprotein E<sup>rns</sup> is different because it does not contain a hydrophobic transmembrane region but an amphipathic helix functioning as the E<sup>rns</sup> membrane anchor. Despite the absence of a hydrophobic region, the site between the C terminus of E<sup>rns</sup> and E1, the protein located downstream in the polyprotein, is cleaved by signal peptidase, as demonstrated by mutagenesis and inhibitor studies. Thus, E<sup>rns</sup>E1 is processed at a novel type of signal peptidase cleavage site showing a different membrane topology. Prevention of glycosylation or introduction of mutations into the C-terminal region of E<sup>rns</sup> severely impairs processing, presumably by preventing proper membrane interaction or disturbing a conformation critical for the protein to be accepted as a substrate by signal peptidase.

Classical swine fever virus (CSFV)<sup>2</sup> belongs to the genus *Pestivirus*, which also includes the animal viruses bovine viral diarrhoea 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 in 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: NH<sub>2</sub>-N<sup>pro</sup>, C, E<sup>rns</sup>, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B-COOH. C, E<sup>rns</sup>, E1, and E2 are part of the virion (13, 14), with C forming the capsid, and E<sup>rns</sup>, E1, and E2 representing glycoproteins associated with the virus envelope. E<sup>rns</sup> and E2 elicit neutralizing antibody responses that can lead to protective immunity (15–18).

The pestivirus E<sup>rns</sup> protein is a highly unusual protein. It forms a disulfide-linked dimer of ~90 kDa, about half of which

is due to glycosylation (9, 13, 19). The protein displays homology to the RNases of the T<sub>2</sub>/S superfamily (20–22). In different test systems, it was shown that E<sup>rns</sup> indeed has RNase activity, a feature that is unique among viral glycoproteins (22–25). The protein is essential for virus growth (21), but the RNase activity is dispensable. Viruses, in which the RNase activity has been knocked out, are clinically attenuated (26, 27). A role for E<sup>rns</sup> in immune evasion has been proposed (28–31).

E<sup>rns</sup> can be found in virus-free cell culture fluid of infected cells and in the blood of infected animals (9, 29, 32). It lacks a hydrophobic region that could serve as a transmembrane anchor, and there is also no consensus sequence for glycosylphosphatidylinositol anchor addition. It is nevertheless bound to the virion and associated with intracellular membranes (9, 13, 14, 32, 33). E<sup>rns</sup> membrane binding is achieved by the C-terminal region forming an amphipathic helix that is inserted in plane into the membrane (32–34).

The topology of the E<sup>rns</sup> membrane anchor raises interesting questions concerning the protease that cleaves the polyprotein at the E<sup>rns</sup> C terminus. Processing of the region of the polyprotein encompassing the viral structural proteins and the first two nonstructural proteins p7 and NS2 is attributed to cellular proteases. The processing sites at the N termini of E<sup>rns</sup>, E2, p7, and NS2 comply with the demands for signal peptidase cleavage sites, namely a positively charged N-terminal region and a central hydrophobic region (h-region), followed by a more hydrophilic part (c-region) containing the cleavage site with small and uncharged residues at positions –3 and –1, as suggested by von Heijne (35, 36). The results of sequence and mutagenesis analyses clearly support the idea of SPase cleavage at these sites in the viral polyprotein (5, 6, 9).<sup>3</sup> In contrast, the site separating E<sup>rns</sup> and E1 does not comply with the above described features of a SPase cleavage site. The –3/–1 residues at the E<sup>rns</sup>/E1 cleavage site are in agreement with a sequence processed by SPase, but a hydrophobic region upstream of the cleavage site is missing. This fact raises the question of whether SPase cleaves this site as proposed in some reports or which other protease is responsible for processing the polyprotein here.

In the work presented here, we analyzed the cleavage at the E<sup>rns</sup> C terminus with different approaches, including mutagenesis and inhibitor studies. For the first time, we provide clear evidence that processing between E<sup>rns</sup> and E1 is executed by SPase, which reveals the existence of a new type of SPase cleavage site not described so far.

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<sup>2</sup> The abbreviations used are: CSFV, classical swine fever virus (formerly known as hog cholera virus); BFA, brefeldin A; BHK-21 cells, baby hamster kidney cells; mAb, monoclonal antibody; PNGase F, peptide:N-glycosidase F; SP, signal peptide; SPase, signal peptidase; SP-I, signal peptidase inhibitor; ER, endoplasmic reticulum; h-region, hydrophobic region; c-region, hydrophilic region containing the cleavage site.

<sup>3</sup> I. Bintintan and G. Meyers, unpublished results.

## EXPERIMENTAL PROCEDURES

**Cells and Viruses**—BHK-21 cells (kindly provided by T. Rüménapf) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids. The modified vaccinia virus strain Ankara containing the phage T7 RNA polymerase (MVA-T7) (37) was kindly provided by B. Moss (National Institutes of Health, Bethesda, MD).

**Construction of Recombinant Plasmids**—Restriction and subcloning were done according to standard procedures (38). Unless stated otherwise, all restriction and modifying enzymes were purchased from New England Biolabs (Frankfurt, Germany) and Fermentas GmbH (Sankt Leon-Roth, Germany). Plasmid pCITE 2a(+) was obtained from AGS (Heidelberg, Germany). It contains a T7 RNA polymerase promoter followed by a picornavirus internal ribosomal entry site. The synthetic DNA oligonucleotides were purchased from Metabion (München, Germany).

From the infectious cDNA clone of the CSFV strain Alfort/Tübingen (39), coding sequences were amplified with the pairs of primers NcoI (neu)/E1-3SXbaR, NcoI (neu)/E0-3SXbaI, E05SIII/E1-3SXbaR, and E05SIII/E0-3SXbaI and inserted into the NcoI/XbaI sites of pCITE 2a(+), yielding the constructs N<sup>pro</sup>-E1, N<sup>pro</sup>-E<sup>rns</sup>, SSeqE<sup>rns</sup>-E1, and SSeqE<sup>rns</sup>, respectively.

To obtain construct SSeqE1-E2, the cDNA of E1E2 was amplified from plasmid p578 (39). In addition, the coding sequence of the E<sup>rns</sup> signal peptide (SSeq) (nucleotides 1120–1173 of the full-length cDNA clone pA/CSFV (39)) was also incorporated in the SSeqE<sup>rns</sup>-E1 and SSeqE<sup>rns</sup> constructs. This sequence was amplified by PCR with the primers IB72, IB73, and E05SIII as follows. First, a PCR fragment was obtained from plasmid p578 with the primers IB72 and HPS38.2. This PCR product was then amplified with IB73 and HPS 38.2 and used for the generation of a further PCR fragment by amplification with E05SIII and E2 d(-). This PCR fragment was restricted with NcoI and XbaI and inserted into the NcoI/XbaI sites of pCITE 2a(+).

The construct mellSSeqE<sup>rns</sup>-E1 containing the signal peptide of mellitin (mellSSeq) was generated in a similar way as described above. The coding sequence of the mellitin signal peptide was obtained from Qiagen (Hilden, Germany) and amplified in three steps by PCR as described before. First, a PCR fragment was amplified from plasmid SSeqE<sup>rns</sup>-E1 with the primers IB131 and pCITErev. This PCR product was amplified with IB132 and pCITErev to obtain the second PCR fragment that contains part of the mellSSeq-coding sequence. The rest of the coding sequence was introduced by the third PCR with the second PCR fragment as template and the primers IB133 and E1-3SXbaR. The final product was cut with NcoI and XbaI and inserted into pCITE 2a(+), cut with NcoI/XbaI.

The constructs ppLSSeqE<sup>rns</sup>-E1 and ppLSSeqE<sup>rns</sup>-E1\* are also based on SSeqE<sup>rns</sup>-E1, with V5 tag and preprolactin signal peptide preceding E<sup>rns</sup>E1. The sequence coding for the preprolactin signal peptide coding sequence (ppLSSeq) was amplified from a preprolactin vector (kindly provided by Heiner Niemann) by PCR with the primers IB97 and IB98 for ppLSSeqE<sup>rns</sup>-E1 or

with IB97 and IB100 for ppLSSeqE<sup>rns</sup>-E1\*. In order to introduce the sequence coding for a V5 tag, the resulting PCR fragments were used as templates in a further PCR with primers IB94 and IB98 for ppLSSeqE<sup>rns</sup>-E1 or IB94 and IB100 for ppLSSeqE<sup>rns</sup>-E1\*. The resulting PCR products were cut with NcoI and EcoRI. The primers IB99 and E1-3SXbaR were used to amplify the E<sup>rns</sup>E1-coding fragment from the SSeqE<sup>rns</sup>-E1 plasmid. After restriction with EcoRI and XbaI, the resulting fragment was ligated together with the previously obtained fragment coding for V5-ppLSSeq and with pCITE 2a(+), cut with NcoI/XbaI.

QuikChange mutagenesis (Stratagene, Heidelberg, Germany) was employed according to the supplier's instructions to introduce substitutions, insertions or deletions. The constructs SSeqE<sup>rns</sup>-E1 and N<sup>pro</sup>-E<sup>rns</sup> served as templates for all mutagenesis approaches for characterization of the E<sup>rns</sup>/E1 cleavage site.

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 (40). Further details of the cloning procedures and the sequences of the primers used for cloning and mutagenesis are available on request.

**Transient Expression of Proteins**—BHK-21 cells were infected with vaccinia virus MVA-T7, subsequently transfected with the desired cDNA construct using SuperFect (Qiagen), and labeled with <sup>35</sup>S-amino acids as described earlier (32, 33). Where mentioned, tunicamycin (Roche Applied Science) or BFA (Calbiochem) was added to the medium during starving and labeling periods to a concentration of 10 µg/ml (tunicamycin) or 1 µg/ml (BFA).

**Preparation and Fractionation of Cell Extracts**—Crude extracts of transiently transfected cells were prepared under denaturing conditions as described before (32). Alternatively, transfected BHK-21 cells were harvested via fractionation essentially as described (32, 33, 41). Briefly, the tissue culture fluid of infected cells containing the secreted proteins was removed as the first fraction. The cells were harvested by scraping them into 1.5 ml of PBS and then passaged 10 times through a 27-gauge needle. Nuclei and cell debris obtained by low speed centrifugation (700 × g, 3 min) were collected in the pellet, and the supernatant of this centrifugation step was used to recover the membrane fraction by high speed centrifugation (107,000 × g; rotor TLA100.3; Beckmann TL100 centrifuge, for 25 min). The water-soluble proteins were found in the supernatant of this centrifugation step. All samples were resuspended in 1× radioimmune precipitation buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mg/liter bovine serum albumin, pH 7.6), denatured by the addition of 1% SDS, and heated to 95 °C before they were chilled on ice. After sonication, the samples were brought to a final concentration of 0.3% SDS by the addition of 1× radioimmune precipitation buffer and were used further in immunoprecipitation experiments.

**Immunoprecipitation and Quantification of E<sup>rns</sup>**—Immunoprecipitation of proteins was carried out as described (32) with 5 µl of rabbit E<sup>rns</sup> antiserum or 100 µl of mAb 24/16 (anti-E<sup>rns</sup>). After incubation of samples with *Staphylococcus aureus* (42), the bound immune complexes were eluted in sample buffer by

## Processing of Pestivirus E<sup>rns</sup>E1

heating to 95 °C. The *S. aureus* bacteria were removed by centrifugation, and the supernatants were analyzed by 10% SDS-PAGE. Where specified, the precipitated proteins were treated before electrophoresis with 1 μl of endoglycosidase H or PNGase F (New England Biolabs) for 1 h at 37 °C as suggested by the supplier.

The gels were exposed to PhosphorImaging plates and scanned with a Fujifilm Bas 1500 scanner (Raytest, Straubhardt, Germany). Signal intensities were determined using TINA 2.0 software (Raytest). The amount of E<sup>rns</sup> cleaved or uncleaved contained in all four steps of fractionation was taken as 100%.

The overwhelming amount of the viral glycoproteins analyzed here was found in the membrane fractions obtained after the low speed or high speed centrifugation. The soluble fraction contained only minimal amounts of viral proteins (data not shown). Therefore, only the results obtained for the tissue culture fluid and the membrane-containing fractions are shown in the present report.

**Cell-free Translation**—A coupled transcription/translation system (TNT<sup>®</sup> T7 coupled reticulocyte lysate system, Promega) supplemented with canine microsomal membranes (Promega) was used to study E<sup>rns</sup>E1 cleavage *in vitro*. The assays were performed as suggested by the supplier in a final volume of 12.5 μl. Proteins were labeled with [<sup>35</sup>S]methionine. In some cases, inhibitors were added at the final concentration indicated in the legend to Fig. 4. MeOSuc-Ala-Ala-Pro-Val chloromethyl ketone (signal peptidase inhibitor (SP-I)) was obtained from Sigma. The protease inhibitors *N*-[*N*-(3,5-difluorophenacyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (for γ-secretase), leupeptin (for serine/cysteine proteases), and 1,10-phenanthroline (for site-2 protease) were purchased from Sigma, and the signal peptide peptidase inhibitor 1,3-di-(*N*-carboxybenzoyl-*L*-leucyl-*L*-leucyl)amino acetone was from Calbiochem. The glycosylation acceptor peptide Ac-NYT-NH<sub>2</sub> was kindly provided by Mark O. Lively.

For *in vitro* translation of preprolactin, the RNA was generated by *in vitro* transcription using SP6 polymerase (New England Biolabs), basically as described before (43). The SP-I for the inhibition of the preprolactin cleavage was used at a concentration of 2.5 mM. For all of the other assays, SP-I was added to a final concentration of 1.5 mM.

Translation was carried out at 30 °C for 1 h or for 1 h 30 min. Thereafter, the samples were transferred on ice and provided with antibodies for immunoprecipitation. Where indicated, Proteinase K was added after completion of translation to destroy proteins that had not been translocated. For control purposes, Triton X-100 was added in some cases. The protease protection assays were conducted as described (32).

## RESULTS

**E<sup>rns</sup>E1 Is Cleaved in the ER**—The protease processing the E<sup>rns</sup>E1 site in the pestivirus polyprotein is not known, mainly because the amino acid sequence at the cleavage site does not fit into the schemes of the possible processing enzymes. As a first step toward identification of the responsible protease, we wanted to determine where in the cell the process-

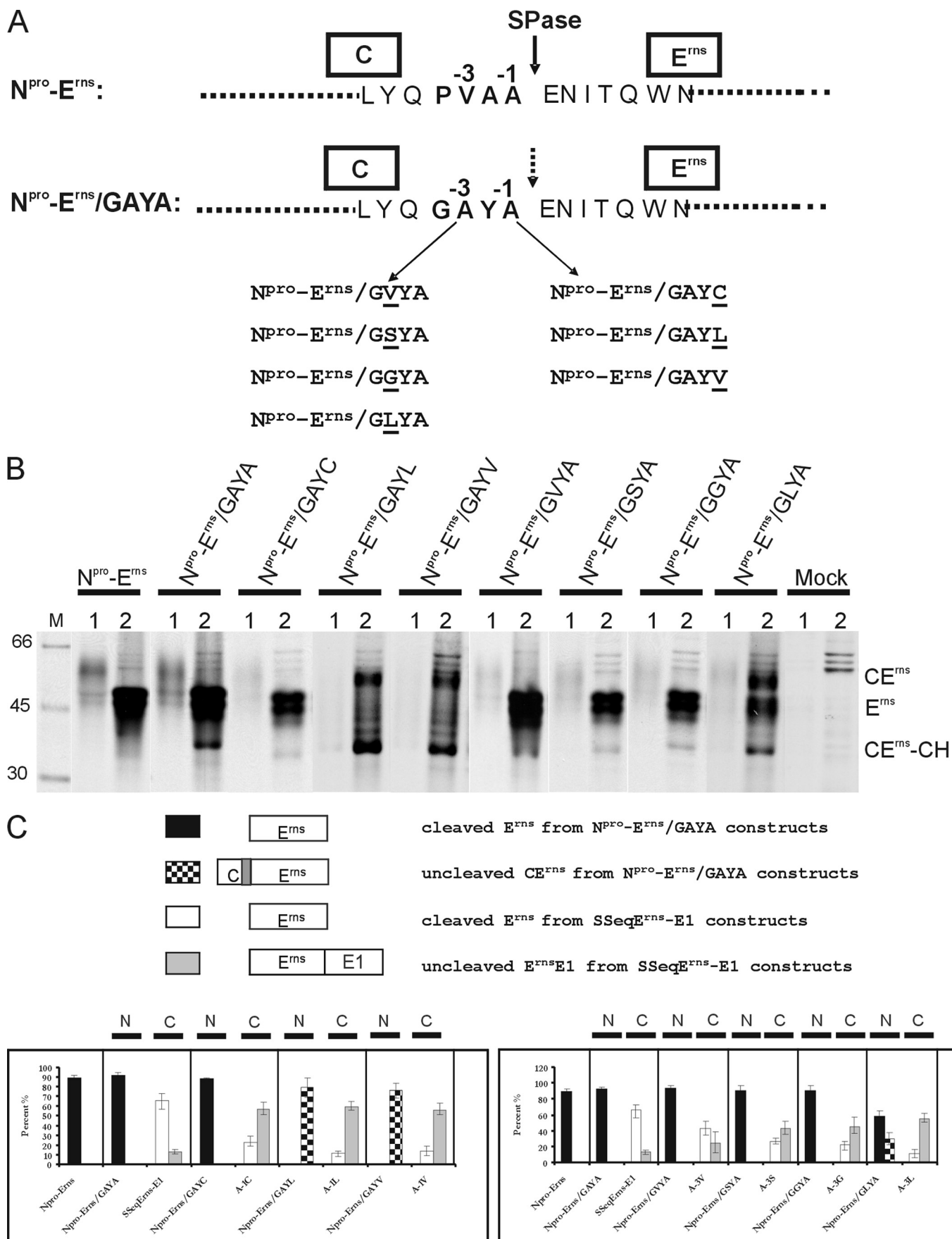
ing occurs. E<sup>rns</sup> is preceded by a signal peptide responsible for translocation of this protein and the following E1 into the ER. Processing of this fusion protein could occur either in the ER or in another compartment of the secretory pathway through which the protein is transported on its way out of the cell. Brefeldin A (BFA) can be used to prevent protein transport from the ER to the Golgi, resulting in accumulation of translocated proteins in the ER (44–48). Cells transiently expressing the region of the pestivirus polyprotein encompassing N<sup>pro</sup>/C/E<sup>rns</sup>/E1 (construct N<sup>pro</sup>-E1; Fig. 1A) were treated with BFA. Immunoprecipitation with an antiserum against E<sup>rns</sup> showed that, in addition to the E<sup>rns</sup>E1 precursor, also the processed E<sup>rns</sup> protein was present (Fig. 1B, lane 1). Due to the altered glycosylation in consequence of the BFA treatment, the bands migrated slightly differently than those in the control without BFA (Fig. 1B, lanes 1 and 2). To support this hypothesis, the precipitated proteins were treated with endoglycosidase H to remove the non-complex carbohydrates from the proteins. After this treatment, the products obtained in the presence of BFA were not distinguishable from the control (Fig. 1B, lanes 3 and 4). Cleavage of E<sup>rns</sup>E1 in the presence of BFA indicates that the processing occurs in the ER because BFA blocks ER to Golgi transport (44, 46–48). However, because BFA treatment also leads to redistribution of Golgi proteins into the ER (45), the involvement of Golgi components cannot be excluded by this assay. The results obtained with the endoglycosidase H assay localize E<sup>rns</sup>E1 processing to the ER or *cis*-Golgi compartment.

Processing of the E<sup>rns</sup>E1 precursor can also be observed after *in vitro* translation (Fig. 1C). Cleavage of the fusion protein is dependent on the presence of microsomal membranes (Fig. 1C, lanes 1 and 4, respectively), indicating that translocation of the protein into the membranous compartment is essential, which in consequence indicates that a protease within a membranous compartment cleaves the protein. In fact, E<sup>rns</sup>E1 is translocated in this assay into ER-derived vesicles before processing. This point is proven by a protease protection approach showing that the unglycosylated E<sup>rns</sup>E1 precursor (E<sup>rns</sup>E1-CH in Fig. 1C) was destroyed by Proteinase K, whereas the bands representing glycosylated E<sup>rns</sup>E1 and fully processed E<sup>rns</sup> were protected unless the membranes were destroyed with Triton X-100 (compare lanes 1, 2, and 3 in Fig. 1C). Because transport of proteins to vesicles originating from other membrane compartments is not known for microsomes, it can be concluded that processing of the E<sup>rns</sup>E1 precursor takes place in the ER.

**E<sup>rns</sup>E1 Cleavage Can Be Blocked by Mutations at the Cleavage Site**—Experimental support for the hypothesis that a protein is cleaved by SPase can be obtained by mutagenesis analysis for the –1- and –3-positions of the cleavage site. As a general rule, these positions have to be occupied by small and uncharged amino acids in an SPase cleavage site. The presence of large and/or charged residues at –1 or –3 blocks SPase processing (35).

The –1- and –3-positions of the E<sup>rns</sup>/E1 cleavage site are occupied by alanine (C-terminal sequence of E<sup>rns</sup>, “GAYA”). A variety of mutants were established in which the alanine residues were replaced by other amino acids (Fig. 2A). The effects of these mutations were analyzed in transient expression stud-





**TABLE 1**  
**Amino acids at positions –1 and –3 in signal peptides cleaved by SPase and in the E<sup>rn</sup>s/E1 cleavage site**

The upper part of the table presents the residues found in standard SPase cleavage sites with the incidence decreasing from left to right (35, 60, 70). Below, the residues tested in the context of the E<sup>rn</sup>s/E1 cleavage site are listed with the cleavage efficiency decreasing from left to right. The intensity of the decrease is indicated as follows: >, decrease; >>, strong decrease; >>>, very strong decrease of processing efficiency.

Positions	Residues
<b>Signal peptides</b>	
Position –1	Ala, Gly, Cys, Ser, Thr
Position –3	Ala, Val, Ser, Cys, Thr
<b>Site-directed mutagenesis at the E<sup>rn</sup>s/E1 site</b>	
Position –1	Ala >>> Gly >>> Cys > Ser > Leu > Val > Arg
Position –3	Ala > Val >>> Thr > Ser >>> Gly > Leu > Phe > Arg

tion of cleavage efficiency. This finding was not in agreement with the described requirements for a SPase cleavage site.

For control purposes, we wanted to test the same changes in the context of a typical SPase cleavage site and therefore introduced the mutations at the C-terminal end of the signal peptide preceding E<sup>rn</sup>s in the viral polyprotein (Fig. 3). We first changed the C terminus of the wild type signal peptide, which ends with the residues PVAA, into GAYA (construct N<sup>pro</sup>-E<sup>rn</sup>s/GAYA) to display the sequence found at the E<sup>rn</sup>s/E1 site (Fig. 3A). Afterward, several exemplary exchanges of the above described mutagenesis scheme were introduced into the latter sequence. In transient expression studies, results were obtained that would be expected for a typical SPase cleavage site with Leu at –1 or –3 and Val at –1 blocking processing but Val, Gly, or Ser at –3 and Cys at –1 having basically no influence on processing efficiency (Fig. 3, B and C). A direct comparison of the effects of different mutations introduced at the N-terminal or C-terminal cleavage sites of E<sup>rn</sup>s makes these differences obvious (Fig. 3C and Table 1).

Taken together, the results of the mutagenesis studies as summarized in Table 1 cannot answer the question of whether the E<sup>rn</sup>s/E1 site is cleaved by SPase, but it has to be stressed that all changes known to impair SP cleavage also block the E<sup>rn</sup>sE1 processing. The finding that also exchanges expected to be neutral in a typical SPase cleavage site reduce the processing efficiency severely might indicate that additional requirements have to be fulfilled to allow SPase activity in this special case.

**The Influence of Protease Inhibitors on E<sup>rn</sup>sE1 Cleavage**—Experiments with specific inhibitors can help to identify the protease active on a given substrate. Therefore, construct SSeqE<sup>rn</sup>s-E1 was translated in a cell-free system in the presence of different known protease inhibitors. Among the huge number of different substances blocking proteases, we selected inhibitors specific for intramembrane-cleaving proteases or signal peptidase to cover the likely candidates that could be

responsible for E<sup>rn</sup>sE1 cleavage. Cell-free translation of construct SSeqE<sup>rn</sup>s-E1 and immunoprecipitation with anti-E<sup>rn</sup>s monoclonal antibody 24/16 (mAb 24/16) resulted in detection of an unglycosylated precursor product of ~43 kDa (Fig. 4A, lane 7). In the presence of microsomal membranes and absence of inhibitor, two more bands were detected that represent the glycosylated precursor E<sup>rn</sup>sE1 of about 66 kDa and the cleaved glycosylated E<sup>rn</sup>s protein of ~48 kDa (lane 6). The addition of inhibitors against signal peptide peptidase,  $\gamma$ -secretase, site-2 protease-metalloprotease or of leupeptin as a serine/cysteine protease inhibitor did slightly influence the ratio between the different products but did not principally change the outcome (Fig. 4A, lanes 2–5). In contrast, the SP-I MeOSuc-Ala-Ala-Pro-Val chloromethyl ketone (49–52) was able to prevent processing so that the band representing processed E<sup>rn</sup>s was missing (Fig. 4A, lane 1).

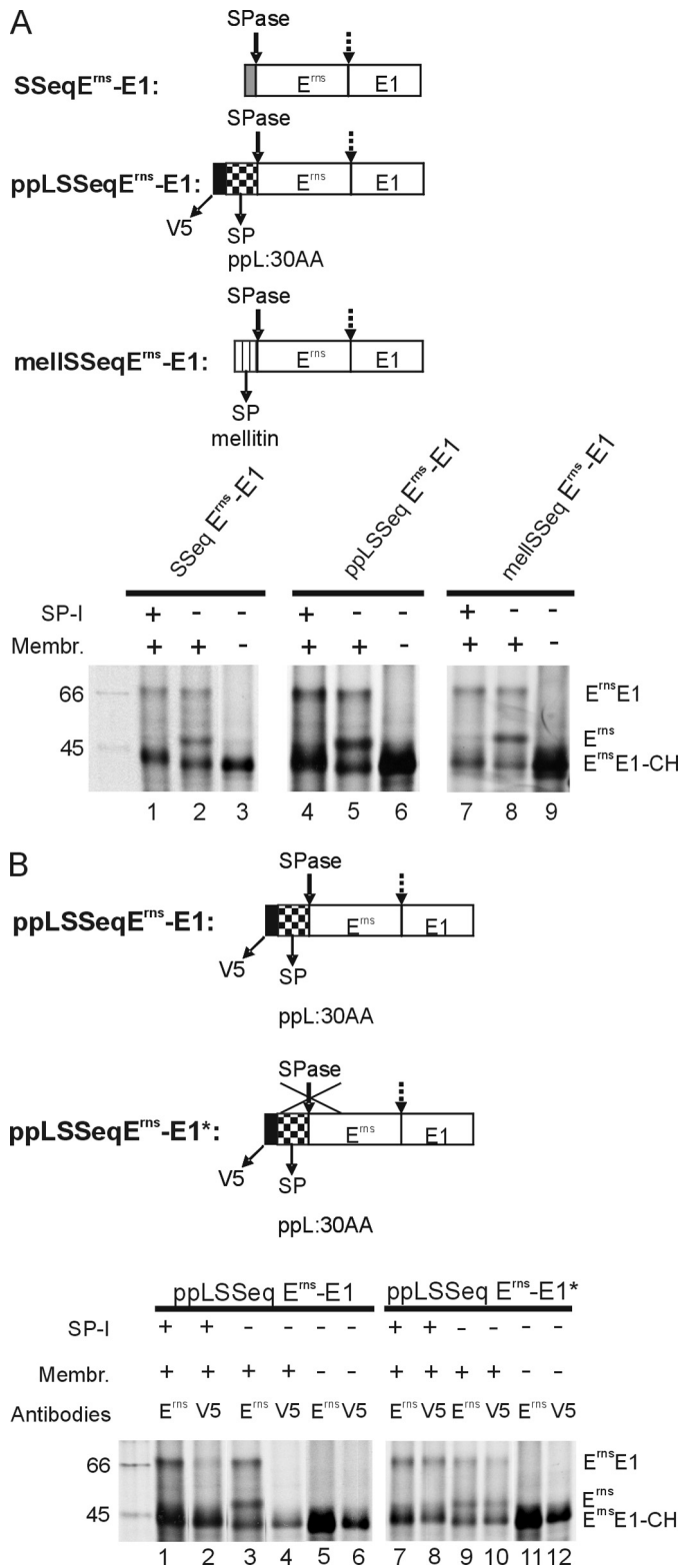
As controls for the SPase inhibition by SP-I, its influence on processing of two proteins containing typical SPase cleavage sites was tested. SSeqE1-E2 encodes a typical signal peptide, followed by CSFV E1 and E2. For this construct, the detection of the unprocessed glycosylated E1E2 precursor of about 70 kDa (E1E2 in Fig. 4B) after immunoprecipitation with an anti-E2 monoclonal antibody proves blocking of SPase processing. This result was obtained when SSeqE1-E2 was translated in the presence of SP-I (Fig. 4B, lane 1). However, the precursor was absent, when translation was done in the presence of membranes but in the absence of inhibitor (Fig. 4B, lane 2). Because free glycosylated E2 has about the same size as unglycosylated E1E2, the product of the processing is not readily detected but becomes visible after removal of N-linked carbohydrates with PNGase F (Fig. 4B, lane 3).

As a second control, construct ppl86AA was used, which encodes the N-terminal 86 amino acids of preprolactin. SPase processing leads to release of the corresponding prolactin fragment. Again, this fragment was almost not detected in the presence of SP-I (Fig. 4C). Taken together, the inhibitor studies strongly support the conclusion that processing of E<sup>rn</sup>sE1 is executed by SPase.

**The Type of Signal Peptide and Its Removal Are Not Crucial for E<sup>rn</sup>sE1 Processing**—The internal signal peptide responsible for translocation of E<sup>rn</sup>s into the ER might have special features important for E<sup>rn</sup>sE1 processing. More importantly, processing of a protein at more than one site can occur in a hierarchic order, where one cleavage has to precede another processing step. This principle is usually seen with SPase and signal peptide peptidase cleavages of signal peptides with signal peptide peptidase activity being dependent on the completion of the SPase cleavage (53–55). Similarly, hierarchical cleavage patterns are quite common for processing of viral polyproteins. Therefore,

**FIGURE 3. Processing efficiency of constructs with mutations at positions –1 and –3 in a standard signal peptide cleavage site.** A, the original sequence context in the internal signal peptide located between capsid protein C and E<sup>rn</sup>s in the CSFV polyprotein (construct N<sup>pro</sup>-E<sup>rn</sup>s, upper part) and the sequence of the mutant N<sup>pro</sup>-E<sup>rn</sup>s/GAYA with the C-terminal part of the signal peptide replaced by the GAYA motif found at the E<sup>rn</sup>s C terminus. Below, the names of the different constructs with the indicated mutations at –3 or –1 of N<sup>pro</sup>-E<sup>rn</sup>s/GAYA are given. B, the results obtained after transient expression, immunoprecipitation, and SDS-PAGE of the mutated proteins. C, diagrams showing the processing efficiency determined for the different mutants presented in B in comparison with the results obtained for the equivalent mutations in the context of the E<sup>rn</sup>s C terminus demonstrated in Fig. 2. On top, an explanation of the different types of bars is given. Below, the results for mutations affecting positions –1 (left diagram) or –3 (right diagram) are shown. Each section of the diagrams presents one type of mutation with its effect on processing of the standard SPase cleavage site at the N terminus of E<sup>rn</sup>s (N) or on processing at the E<sup>rn</sup>s C terminus (C). See also the legend to Fig. 2.





**FIGURE 5. Importance of the type of signal peptide and of signal peptide cleavage on E<sup>rns</sup>E1 processing.** *A*, the upper part shows schemes of the analyzed constructs in which E<sup>rns</sup>E1 was combined with different signal peptides. Below, SDS-PAGE with proteins precipitated with mAb 24/16 after *in vitro* translation of the constructs indicated above the gels. Translation was done in the presence or absence of microsomal membranes or SP-I, as indicated. *B*, top, constructs ppLSSeqE<sup>rns</sup>-E1 and variant ppLSSeqE<sup>rns</sup>-E1\* with the cleavage site SP/E<sup>rns</sup> blocked by mutation, are shown. Below, SDS-PAGE with products of *in vitro* translation precipitated with mAb 24/16 against E<sup>rns</sup> or an anti-V5 mAb, as specified above the gel. See also the legend to Fig. 1.

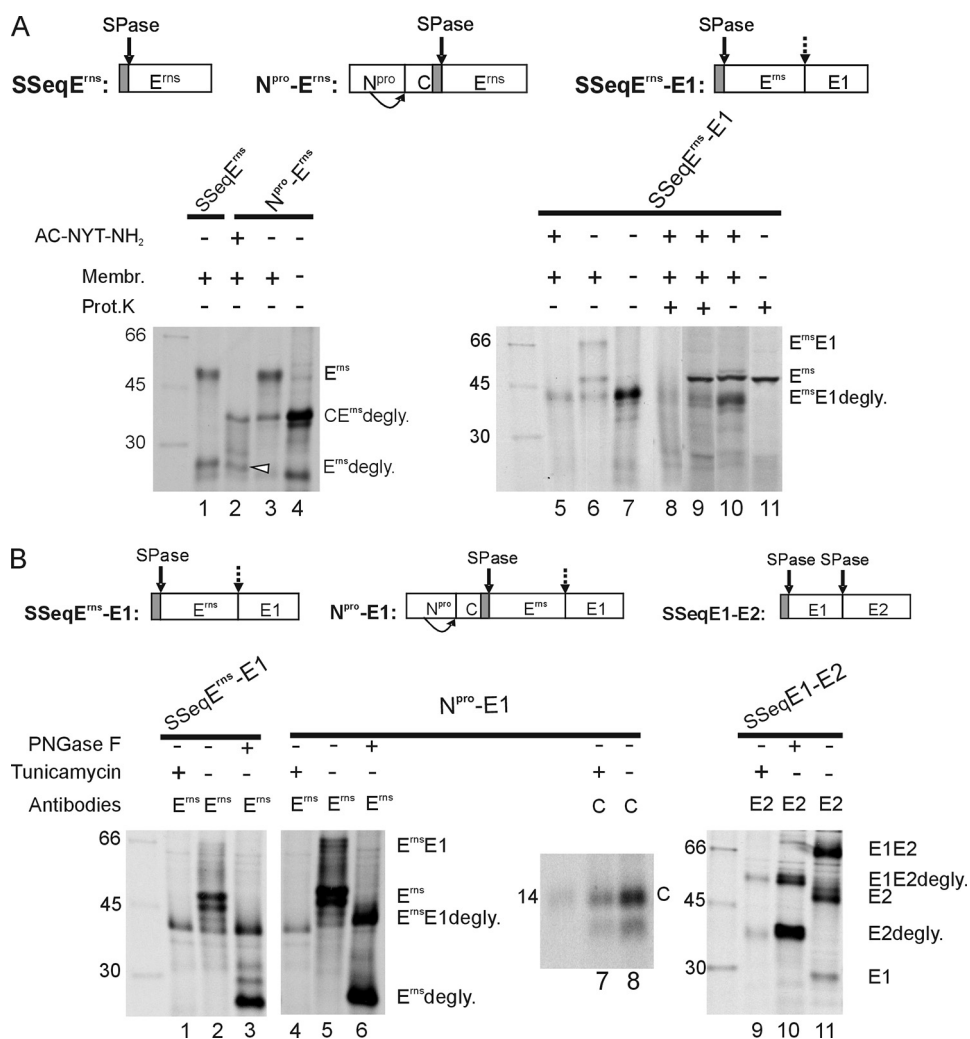
analyzed whether prevention of *N*-glycosylation has an impact on E<sup>rns</sup>E1 processing. Constructs N<sup>pro</sup>-E<sup>rns</sup> and SSeqE<sup>rns</sup>-E1 were translated *in vitro* in the presence or absence of Ac-NYT-NH<sub>2</sub>, a tripeptide substrate of glycosyltransferase that can competitively block glycosylation of other substrates (Fig. 6A) (48, 56). To provide a size marker for unglycosylated and glycosylated E<sup>rns</sup>, construct SSeqE<sup>rns</sup> was translated without the addition of the inhibitor (Fig. 6A, lane 1). After translation, E<sup>rns</sup> or fusion proteins containing E<sup>rns</sup> were precipitated with mAb 24/16. For N<sup>pro</sup>-E<sup>rns</sup>, processed E<sup>rns</sup> was clearly detected as an unglycosylated protein band of ~26 kDa in the presence of the competitive inhibitor (marked with a white arrowhead in Fig. 6A, lane 2). This finding proves that, despite the inhibition of glycosylation, SPase processing of the internal signal peptide connecting capsid protein C and E<sup>rns</sup> occurs. In contrast, cleaved unglycosylated E<sup>rns</sup> was not detected after translation of SSeqE<sup>rns</sup>-E1 in the presence of the inhibitor (Fig. 6, lane 5). To prove that the inhibitor did not prevent translocation of the proteins, proteinase protection assays were conducted. Unglycosylated E<sup>rns</sup>E1 was precipitated with the E<sup>rns</sup>-specific antibody when SSeqE<sup>rns</sup>-E1 was translated in the presence of membranes and treated with Proteinase K (Fig. 6A, lane 8). Because this band was somewhat hidden in a smear, a further control was conducted without immunoprecipitation. In this experiment, the unprocessed E<sup>rns</sup>E1 precursor was detected very clearly, regardless of whether the sample was treated with Proteinase K or not (Fig. 6A, lanes 9 and 10, respectively) but was not present when proteinase treatment was done with a sample translated in the absence of membranes (Fig. 6A, lane 11).

As a second approach, different CSFV expression constructs were transiently expressed in BHK-21 cells in the presence of the *N*-glycosylation inhibitor tunicamycin. In addition to SSeqE<sup>rns</sup>-E1, constructs N<sup>pro</sup>-E1 and SSeqE1-E2 were used, which contain the polyprotein region covering N<sup>pro</sup>/C/SP/E<sup>rns</sup>/E1 or SP/E1/E2, respectively (Fig. 6B). Proteins expressed from the transiently transfected cDNA constructs were labeled with radioactive amino acids *in situ* and subsequently precipitated with mAb 24/16 for E<sup>rns</sup>, A18 for E2, or rabbit antiserum D1\* for C. Before SDS-PAGE, part of the precipitates were treated with PNGase F to remove the *N*-linked carbohydrates.

Fully processed E<sup>rns</sup> without carbohydrates has a size of about 26 kDa. A corresponding band was detected for SSeqE<sup>rns</sup>-E1 or N<sup>pro</sup>-E1 when the constructs were expressed in the absence of tunicamycin, and the precipitated proteins were treated with PNGase F (Fig. 6B, lanes 3 and 6). In contrast, only the 43 kDa band of nonglycosylated E<sup>rns</sup>E1 was detected when expression was done in the presence of tunicamycin (Fig. 6B, lanes 1 and 4). Thus, processing of the E<sup>rns</sup>/E1 site was inhibited by tunicamycin treatment. However, this effect was specific for this individual processing site because the cleavage at the typical SPase sites at the N terminus of E<sup>rns</sup> or at the E1/E2 border occurred also when glycosylation was prevented. This fact could be concluded from the detection of the C protein (Fig. 6B, lane 7) or processed deglycosylated E2 (Fig. 6B, lane 9) when expression was done under tunicamycin treatment. Tunicamycin is a widely used substance that inhibits *N*-glycosylation but not translocation or SPase activity. Accordingly, processing of the C/E<sup>rns</sup> and the E1/E2 sites was observed, whereas process-



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**FIGURE 6. E<sup>rns</sup>E1 processing under prevention of glycosylation.** *A*, results obtained after *in vitro* translation. *Top*, the constructs used in the studies are presented schematically. *Below*, SDS-PAGE gels are shown with products precipitated with mAb 24/16 after *in vitro* translation of the indicated products in the presence or absence of the *N*-glycosylation acceptor (competitive inhibitor) AC-NYT-NH<sub>2</sub> (lanes 1–8). Lanes 8–11 show the results of Proteinase K protection assays, with lanes 9–11 containing products of *in vitro* translation without immunoprecipitation. The presence or absence of Proteinase K is indicated at the *top* of the gel. Lane 11 shows a control established by translation in the absence of membranes and inhibitor followed by Proteinase K treatment. Please note that in lanes 9–11 a strong unspecific band migrating a bit faster than E<sup>rns</sup>. The *white arrow* marks unglycosylated E<sup>rns</sup> cleaved by SPase from the N<sup>pro</sup>/C/E<sup>rns</sup> precursor. *B*, results of transient expression assays conducted with the constructs shown on *top*. The gels show the proteins precipitated with the given antibodies (E<sup>rns</sup>, mAb 24/16 against E<sup>rns</sup>; C, rabbit serum against C protein; E2, mAb A18 against E2) from the extracts of cells transfected with the indicated constructs. The transfected cells were treated with tunicamycin as indicated *above* the gels. Some of the precipitated proteins were deglycosylated with PNGase F (lanes 3, 6, and 10). See also the legends to Figs. 1 and 2. Please note that E1 is visible in lane 11 due to coprecipitation with E2 because of formation of a disulfide-linked heterodimer in infected cells (13). *Prot. K*, Proteinase K.

ing of the E<sup>rns</sup>E1 precursor was blocked in the presence of the drug. Taken together, the results show that processing of E<sup>rns</sup>E1 can only occur when the protein is glycosylated, a finding that strongly supports the conclusion that the overall conformation of the protein is especially important for processing of E<sup>rns</sup>E1.

**The Integrity of the E<sup>rns</sup> Membrane Anchor Is Crucial for E<sup>rns</sup>E1 Processing**—The prevention of *N*-glycosylation certainly has a major effect on the structure of a protein containing nine potential sites for *N*-glycosylation. To investigate whether also less invasive alterations of the E<sup>rns</sup> structure can influence E<sup>rns</sup>E1 processing, further mutants were tested. The C-terminal region of E<sup>rns</sup> is of special interest because it represents the membrane anchor of the protein that interacts with the lipid

bilayer via an amphipathic helix, thereby possibly presenting the cleavage site to SPase. Three different deletion mutants were established on the basis of SSeqE<sup>rns</sup>-E1. Each mutant protein contained a deletion of 14 amino acids of the E<sup>rns</sup> C-terminal region located upstream (E<sup>rns</sup>-Del 158–171), at the N-terminal border (E<sup>rns</sup>-Del 172–185), or in the center (E<sup>rns</sup>-Del 200–213) of the region mapped as membrane anchor (Fig. 7A). After transient expression of the latter two constructs, a severe reduction of E<sup>rns</sup>E1 processing was observed in comparison with the wild type construct. For E<sup>rns</sup>-Del 172–185, processing seemed to be completely blocked because no E<sup>rns</sup> could be detected. In contrast, construct E<sup>rns</sup>-Del 158–171 showed about wild type processing efficiency (Fig. 7B). Similar results were obtained for two further mutants with 14 amino acid deletions located further upstream of position 158 (data not shown).

A more subtle effect on the arrangement of the E<sup>rns</sup> amphipathic helix can be achieved by insertion of single alanine residues into the helix, resulting in a ~100° twist around the helix axis. This alteration provokes a significant disturbance of the amphipathic character of the helix without affecting the overall helical structure. Insertion of alanine at position 191 of the E<sup>rns</sup> sequence was shown before to reduce the efficiency of membrane binding from about 87% (wild type) to ~30% (32). When this mutation was introduced into SSeqE<sup>rns</sup>-E1, the transient expression analysis revealed a severe reduction of E<sup>rns</sup>E1 processing (Fig. 7B, construct E<sup>rns</sup>-Ala<sup>191</sup>). The amphipathic character of the helix can in part be restored when 3 or 4 alanine residues are inserted. Indeed, a higher processing efficiency was observed for construct E<sup>rns</sup>-3×Ala<sup>191</sup> compared with E<sup>rns</sup>-Ala<sup>191</sup>. However, the restoring effect was rather low, which fits with the results obtained in the analysis of membrane association of equivalent mutants at position 191 (32).

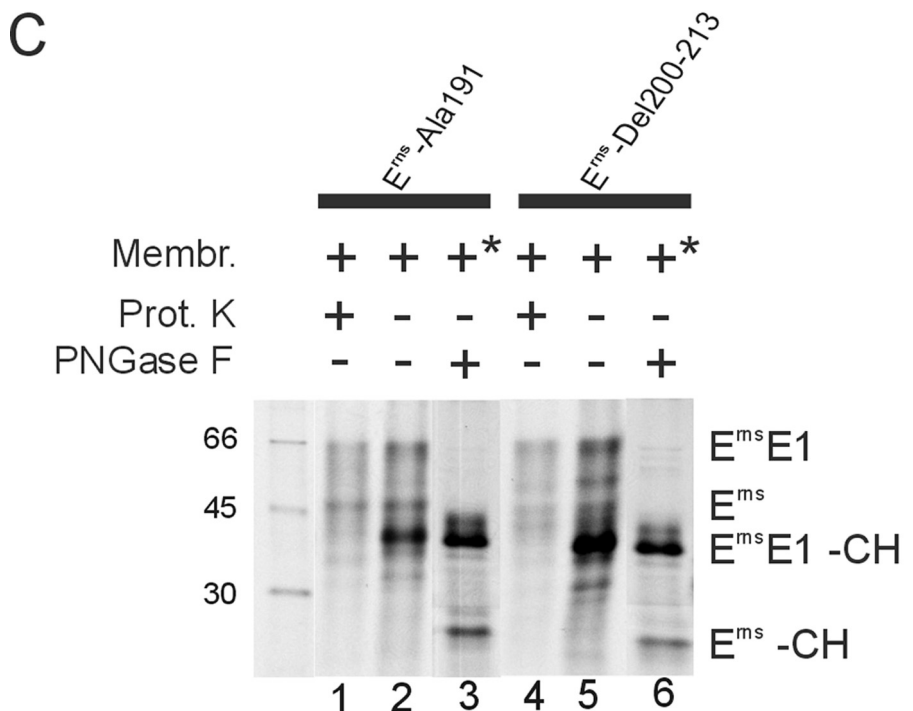
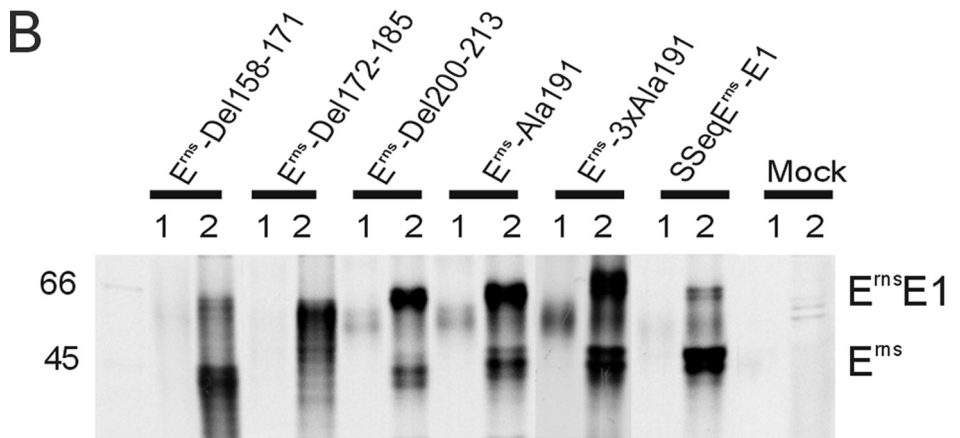
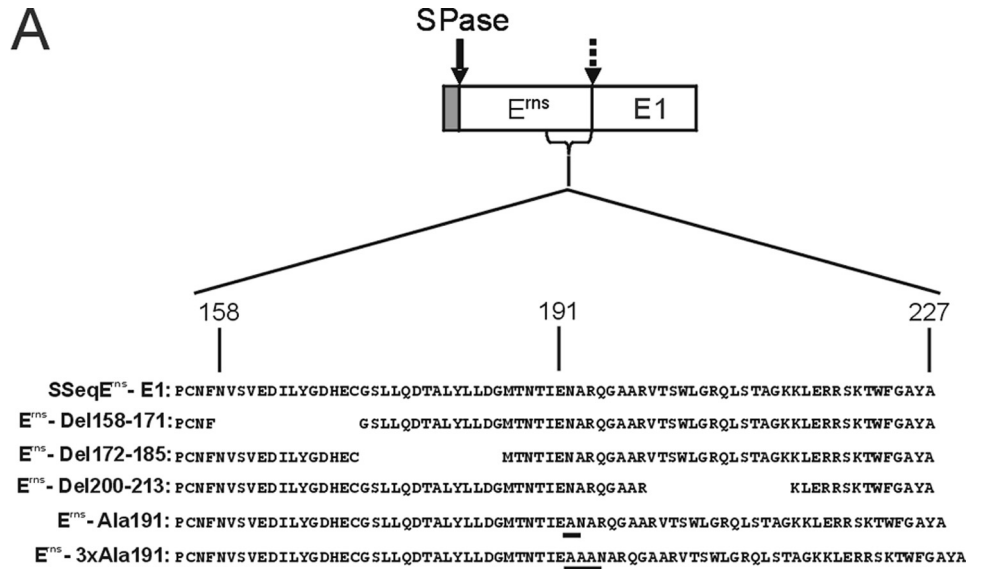
Secretion of the cleaved E<sup>rns</sup> of mutants E<sup>rns</sup>-Del 200–213, E<sup>rns</sup>-3×Ala<sup>191</sup>, and E<sup>rns</sup>-Ala<sup>191</sup> was more efficient than for the wild type or the E<sup>rns</sup>-Del 158–171 protein but less impressive than after expression of the mutated E<sup>rns</sup> alone (32), due to the overall much lower amount of cleaved E<sup>rns</sup> compared with

the yield after expression of E<sup>rns</sup> alone. As expected, the E<sup>rns</sup>E1 fusion protein is not secreted at all because the transmembrane region at the C terminus of E1 ensures efficient membrane binding of the fusion protein. To prove that the introduced mutations do not impair protein translocation, proteinase protection assays were conducted for constructs E<sup>rns</sup>-Ala<sup>191</sup> and E<sup>rns</sup>-Del 200–213. These assays showed that both the glycosylated E<sup>rns</sup>E1 precursor and the fully processed E<sup>rns</sup> protein were protected from the Proteinase K, whereas the unglycosylated precursor (E<sup>rns</sup>E1-CH) was destroyed (Fig. 7C, lanes 1 and 2 or lanes 4 and 5). The fact that the formerly mentioned bands represent glycosylated proteins and thus must have been translocated was also proven by deglycosylation with PNGase F of transiently expressed proteins precipitated from transfected BHK-21 cells (Fig. 7C, lanes 3 and 6). Moreover, the PNGase F-treated samples demonstrate again the poor processing of the two mutant constructs because the E<sup>rns</sup>-CH band is much less prominent than the precursor band E<sup>rns</sup>E1-CH.

Importantly, all of the different mutations with alterations affecting membrane interaction of the E<sup>rns</sup> C-terminal helix showed significantly reduced processing of the E<sup>rns</sup>E1 precursor, whereas changes outside of the helix had no significant effect.

**DISCUSSION**

Posttranslational modification of viral envelope proteins is usually done by the host cellular system that is normally responsible for trimming and maturation of cellular (surface) proteins. The possible modifications include removal of signal peptides by SPase and signal peptide peptidase cleavage, protein glycosylation, acylation, phosphorylation, and post-ER maturation cleavage by, for example, Golgi proteases. These processes are essential to obtain functional viral envelope proteins.



## Processing of Pestivirus E<sup>rns</sup>E1

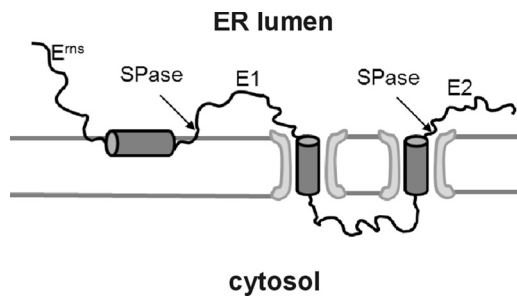
Positive strand RNA viruses express their proteins via polyproteins that are subsequently processed into the mature products. The enveloped members of this virus group employ host systems also for cleavage of the polyprotein region giving rise to the viral structural proteins. To enable such a processing scheme, the structural polyprotein adopts a multimembrane-spanning topology, in which individual proteins are separated by transmembrane regions, often organized in a head to tail arrangement of stop transfer sequence and signal peptide. These structures are then cleaved at typical SPase cleavage sites in the signal peptide moiety. Upon completion of the processing process, the transmembrane regions form the membrane anchor of the N-terminal cleavage product. The same concept is also found in pestiviruses with SPase cleavage occurring at typical SPase cleavage sites at the N termini of glycoproteins E<sup>rns</sup> and E2 and the non-structural proteins p7 and NS2. The only site that does not conform to SPase cleavage site requirements is the one between E<sup>rns</sup> and E1. This site has long been known to be special because processing of E<sup>rns</sup>E1 is delayed, so that E<sup>rns</sup>E1 can always be detected as a precursor in infected cells (9). It could be hypothesized that the E<sup>rns</sup>E1 fusion protein has a biological function in the virus life cycle, but the fact that viruses with an artificial deletion of the E<sup>rns</sup>-coding sequence can be efficiently complemented with E<sup>rns</sup> in *trans* argues against this hypothesis (21, 57, 58). On the other hand, it has recently been shown that processing of E<sup>rns</sup>E1 is crucial for pestivirus viability (59).

Because the E<sup>rns</sup>/E1 processing site lacks a hydrophobic h-region, which is regarded as a crucial part of a SPase cleavage site, it was speculated whether the delayed processing at this site was due to processing in a downstream compartment of the secretory pathway (9, 59) or resulted from inefficient SPase cleavage in consequence of the unusual structure. As reported here, the E<sup>rns</sup>E1 precursor is cleaved in the ER. Moreover, the data show that this site is cleaved by SPase despite its unusual features. This conclusion is based on the facts that processing can be blocked by a SPase inhibitor that was used before in a variety of analyses (49–52) and by typical exchanges of the amino acids at the –1- or –3-positions of the cleavage site. However, the mutagenesis analyses indicated again that the E<sup>rns</sup>/E1 cleavage site is unusual. Introduction of cysteine at position –1 considerably impaired E<sup>rns</sup>E1 processing, whereas the same exchange in a regular SPase cleavage site had basically no effect. Similarly, a glycine at position –3 in the context of the E<sup>rns</sup>/E1 site had almost the same inhibitory effect as a leucine in –3, whereas glycine at –3 was neutral when tested in the context of a typical SPase cleavage site. These results make it obvious that sequence requirements exceeding those known for a typical SPase cleavage site have to be fulfilled in order to allow processing of E<sup>rns</sup>E1. This hypothesis is supported by the results obtained

with mutants with alterations introduced rather far upstream of the E<sup>rns</sup>/E1 cleavage site. Two deletion mutants and a variant with a single alanine insertion 36 residues upstream of the cleavage site showed considerably reduced or even no processing of the precursor. In part, these results can simply be explained by the fact that mutations disturbing the C-terminal amphipathic helix lead to reduced membrane binding of the E<sup>rns</sup> C terminus, thereby impairing also the contact of the cleavage site with SPase. This is true for the alanine insertion at position 191 (32) and certainly has to be taken into account for the two deletions introduced into the C-terminal region. However, the argument of reduced membrane binding can hardly explain the results obtained with the point mutations at positions –3 and –1. Moreover, it also has to be questioned why the inhibition of protein glycosylation was able to prevent E<sup>rns</sup>E1 processing because this manipulation does not directly affect the C terminus of the protein. Membrane binding of a unglycosylated artificial fusion protein containing the amphipathic helix of E<sup>rns</sup> has been demonstrated (33). It therefore seems justified to speculate that, at least in the latter cases, structural constraints prevent proper interaction between enzyme and substrate, so that it cannot be cleaved.

When signal peptides, the regular substrates of SPase, were compared, a high degree of variation was detected that concerned both the length and sequences of the N-terminal region, h-region, and c-regions (35, 60). Thus, one could conclude that SPase is highly flexible with regard to its substrate as long as the general scheme and the –1, –3 rule are obeyed. It even can be questioned whether most of the typical features of a signal peptide are actually important for its acceptance as a substrate by SPase or whether these features are only necessary for the initiation of translocation. In an oversimplified view, one could regard only the c-region as the sequence interacting with the protease, with the –1, –3 residues being responsible for substrate recognition. However, such relaxed conditions would result in a huge number of SPase cleavage sites and thus contradict the fact that in reality SPase shows a high degree of substrate specificity (60, 61). It is therefore obvious that, in addition to the c-region, further parameters are necessary to define whether a certain sequence represents an SPase substrate. One of these parameters could be the context of substrate and protease with the translocon. However, it has been shown that purified SPase can execute a specific posttranslational cleavage of a substrate *in vitro* (62–66). In such assay mixtures, regular interaction of substrate and SPase with the translocon is highly unlikely. Similarly, the substrate investigated here is most likely not associated like a standard signal peptide with the Sec61 complex. The E<sup>rns</sup>/E1 cleavage site has to be inserted into the membrane after complete translocation of E<sup>rns</sup> and E1 because a fully glycosylated E<sup>rns</sup>E1 precursor can

**FIGURE 7. Effect of short deletions or alanine insertions in the E<sup>rns</sup> C-terminal region on E<sup>rns</sup>E1 processing.** A, construct SSeqE<sup>rns</sup>-E1 is shown with the C-terminal region of E<sup>rns</sup> presented as an enlargement with the sequence given in one-letter code (residue number in the mature E<sup>rns</sup> given at the top). The deletions and the positions of the inserted alanine residues (underlined) are demonstrated with the names of the corresponding expression constructs specified on the left. In analogy to the bovine viral diarrhea virus E<sup>rns</sup>, the amphipathic helix should start around position 180, as defined by an alanine insertion scanning approach (32). B, SDS-PAGE with the proteins precipitated with mAb 24/16 from tissue culture fluid (lanes 1) and cell extracts (lanes 2) of cells transfected with the indicated constructs. C, controls that were either done with Proteinase K protection assays using products of *in vitro* translation (lanes 1, 2, 4, and 5) or proteins precipitated from transfected cells (similar to B) that were deglycosylated with PNGase F. \*, the membranes were not added as in the *in vitro* translation assays but were present because of expression in cells. See also the legends to Figs. 1 and 2.



**FIGURE 8. Schematic presentation of the membrane topology and processing scheme of the pestivirus polyprotein region encompassing E<sup>rns</sup>, E1, and the N-terminal part of E2 (not drawn to scale).** Transmembrane regions and the E<sup>rns</sup> C-terminal amphipathic helix are shown as cylinders, whereas the rest of the proteins are indicated by black lines. The two gray horizontal lines represent the ER- or cytosol-facing surfaces of the ER membrane. The translocon is indicated as a light gray structure. SPase cleavage sites are marked by arrows and SPase. The signal sequence upstream of E<sup>rns</sup> responsible for E<sup>rns</sup> translocation is not shown. Note that the transmembrane region upstream of the E2 protein is a regular signal sequence with a typical transmembrane topology that stands in marked contrast to the in plane configuration of the E<sup>rns</sup> amphipathic helix preceding the E<sup>rns</sup>/E1 SPase cleavage site.

be detected within infected cells. Both glycosylation and generation of the E1 C terminus by SPase cleavage of the E1/E2 site can only be executed after completion of E1 translocation. The E<sup>rns</sup>E1 precursor is subsequently processed, as shown in pulse-chase experiments (9). The membrane interaction of the E<sup>rns</sup> amphipathic helix results in an in plane configuration of the E<sup>rns</sup>/E1 cleavage site region instead of the regular transmembrane situation with the signal peptide still interacting with the translocon. The different topology of the E<sup>rns</sup>E1 site is demonstrated in Fig. 8, where the processing of the pestivirus polyprotein region containing E<sup>rns</sup>, E1, and the N-terminal part of E2 is illustrated. The C terminus of E1 contains two transmembrane regions, a stop transfer sequence and a signal sequence responsible for translocation of E2. The signal sequence is processed by SPase in a typical transmembrane topology, whereas the E<sup>rns</sup>/E1 site is preceded by the in plane amphipathic helix.

It is known for signal peptides that the position of the cleavage site relative to the membrane surface is important for substrate recognition by SPase because artificial lengthening of the h-region of a signal peptide prevents SP cleavage (67). Moreover, it has been reported that the typical  $\alpha$ -helical conformation of the h-region must not extend into the c-terminal part of the c-region to ensure that the  $-1, -3$  residues are located in a region of extended conformation (66, 68, 69). Thus, a parameter for substrate recognition should be the correct presentation of a suitable cleavage site in a given "cleavage space" close to the membrane surface. For E<sup>rns</sup>E1, this hypothesis means that the amphipathic helix has to be inserted into the membrane in such a way that the cleavage site is presented and orientated correctly in this cleavage space. This requirement can explain why alteration of the ectodomain structure by prevention of glycosylation, changes affecting the amphipathic helix itself, or even subtle influences like point mutations can affect cleavage efficiency of this substrate because all of these alterations can disturb the conformation of the complex composed of the C-terminal amphipathic helix and the membrane. Even a subtle change of the conformation of this complex can affect the positioning of the cleavage site with respect to the enzyme.

These ideas indicate that a variety of demands have to be fulfilled to achieve the cleavage of a substrate like the E<sup>rns</sup>E1 precursor. To our present knowledge, the E<sup>rns</sup>/E1 site represents the first SPase cleavage site in which the membrane-traversing h-region is missing and is replaced by an amphipathic helix embedded in plane into the membrane. However, in the past, putative virus-specific biological features often turned out to be also used by the cells themselves, representing alternatives in the standard repertoire of cell biology that are designed to serve special demands.

Pestivirus E<sup>rns</sup> represents a very fascinating viral protein. It is an essential component of the virus particle, engaged in the infection process, but it is also involved in repression of the host's immune response to a pestivirus infection (26–31). The immune repressive function of E<sup>rns</sup> is connected with its enzymatic activity, namely its ability to hydrolyze RNA. So far, the target of the RNase is not known. Different theories have been put forward, and in all of them the fact that E<sup>rns</sup> is secreted from the infected cell plays a central role. Massive loss of the protein from the cell harboring replicating virus would be deleterious because it would interfere with the formation of infectious virus progeny. Thus, an equilibrium between retention and secretion has to be established that retains sufficient amounts of the protein within the infected cell. It is tempting to speculate that the unusual way by which this protein is bound to the membrane is one of the features playing a role in the establishment of this equilibrium. This unusual membrane anchor has imposed another problem on the virus, namely to assure processing of the unusual structure at the E<sup>rns</sup>/E1 border. As shown in this report, evolution has led to the point that this site is cleaved by SPase despite the absence of the hydrophobic h-region and, more importantly, a totally different membrane topology of the substrate. It can therefore be hypothesized that the interaction of this substrate with the enzyme is more sensitive to mutations and structural changes than the standard substrate. More information on the structure of the E<sup>rns</sup> C terminus in the context of a membrane environment is urgently needed to fully understand this interesting substrate/enzyme interaction. Most likely, the results of these future analyses will also shed some light onto the still quite unclear basis of SPase cleavage specificity in general and will probably provide yet further interesting open questions about the fascinating E<sup>rns</sup> protein.

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