The E2 Signal Sequence of Rubella Virus Remains Part of the Capsid Protein and Confers Membrane Association In Vitro

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Received 16 May 1990/Accepted 14 August 1990

The capsid (C) protein of rubella virus is translated from a 24S subgenomic mRNA as the first part of a polyprotein containing all three structural proteins of the virus. It is separated from the following protein (E2) by signal peptidase, which cleaves after the E2 signal sequence. We raised an antipeptide antiserum directed against the signal sequence and used the antiserum to show that this sequence is still a part of the C protein in the mature virion. Furthermore, we also showed that, when the C protein is synthesized by in vitro transcription and translation, the resultant protein is membrane associated. This association is not seen with a variant C protein which lacks the signal sequence, and a normally soluble protein (dihydrofolate reductase) becomes membrane associated when the signal sequence is placed at its carboxy terminus.

Rubella virus (RV) is the sole member of the *Rubivirus* genus in the *Togaviridae* family. Three major polypeptides have been identified from purified virus particles: C (33 kilodaltons [kDa], E2 (42 to 47 kDa) and E1 (58 kDa) (7, 43, 56). C proteins are complexed with the genomic 40S RNA, forming the inner nucleocapsid structure, which is surrounded by a lipid bilayer envelope containing the E2 and E1 proteins. The viral structural proteins are translated as a polyprotein from a 24S subgenomic mRNA in the order NH₂-C-E2-E1-COOH (9, 17, 18, 42, 44, 48, 51).

The assembly pathway of the RV virion is largely unknown. The replication strategy and the virion structure of RV resemble those of the alphaviruses, with the structural proteins of the alphaviruses being similarly translated as a polyprotein from a 26S subgenomic mRNA in the order NH₂-C-p62-E1-COOH. The assembly model suggested for the alphaviruses is that the nucleocapsid and the envelope are formed separately (for a review, see reference 20). The C protein is autoproteolytically cleaved from the nascent polyprotein precursor (2, 37) and remains in the cytoplasm, where nucleocapsids are formed. The release of the C protein reveals the amino-terminal signal sequence of p62, which initiates the translocation of membrane proteins into the endoplasmic reticulum (22, 26, 36, 55, 58; H. Garoff, D. Huylebroeck, A. Robinson, U. Tillman, and P. Liljeström, J. Cell Biol., in press; P. Liljeström and H. Garoff, unpublished results). These are transported to the plasma membrane, where final virus maturation (budding) occurs through binding of the nucleocapsid to the cytoplasmic protein domains of the membrane proteins (21, 50; K. Metsikkö and H. Garoff, unpublished results). In RV-infected cells, however, no free cytoplasmic nucleocapsids like those seen in alphavirus-infected cells have been identified and, therefore, the possibility exists that the core formation occurs simultaneously with the budding process (39 and references therein). Also, in contrast to the C protein of alphaviruses, the RV C protein is apparently not an autoprotease (9, 44). The cleavage between C and E2 proteins seems to be mediated by signal peptidase, which cleaves after the E2 signal sequence (9, 27). The E2 signal sequence would thus remain

attached to the carboxy terminus of the newly synthesized RV C protein. This signal sequence consists of a 21-residue stretch of uncharged, mainly nonpolar amino acids and is therefore long enough to function as a membrane-anchoring sequence (1, 13, 14, 53). If the C protein were a membrane-anchored protein, this would have important implications for the possible linkage of nucleocapsid formation and virus budding. Two central questions in the assembly of RV nucleocapsids are therefore whether the E2 signal sequence remains attached to the C protein throughout the process of virus maturation and if so, whether it makes the C protein membrane associated.

In the present work, we have studied these two important questions. We found that the E2 signal sequence is indeed part of the mature RV C protein. Furthermore, the E2 signal sequence was shown to confer membrane association on the in vitro-synthesized C protein; in this respect, therefore, the RV C protein differs significantly from its alphavirus counterpart.

MATERIALS AND METHODS

Cells and virus. B-Vero cells were grown in minimum essential medium supplemented with 10% fetal calf serum–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.3. The Therien strain of RV was used in all studies. Both the RV and B-Vero cells were generously provided by R. Pettersson, The Ludwig Institute for Cancer Research, Stockholm, Sweden. All tissue culture media were from GIBCO, and the tissue culture plastics were from Nunc.

cDNA cloning. The full-length cDNA clone for the RV structural region, assembled from pKTH351 (48) and pKTH2522 (containing the combined inserts of pKTH345 and pKTH349 [51]) (all three plasmids were the kind gift of R. Pettersson), produced only a prematurely terminated product in in vitro transcription-translation analysis, indicating an erroneous sequence in the region coding for the C protein. The cDNA for the capsid region was therefore recloned. Confluent B-Vero monolayers were infected with 5 PFU of RV per cell in minimum essential medium supplemented with 0.2% bovine serum albumin–10 mM HEPES. After 2 h at 37°C, the virus was removed and the cells were further incubated at 37°C in the same medium. At 10 h

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postinfection, dactinomycin (Sigma Chemical Co.) was added to a concentration of 0.4 $\mu\text{g/ml}$. At 24 h postinfection, the cytoplasmic RNA was extracted by a guanidine-hydrochloride method (35). Poly(A)⁺ RNA was purified by oligo(dT)-cellulose (type 3; Collaborative Research Inc.) column chromatography. Double-stranded cDNA was synthesized by priming poly(A) RNA with oligo(dT) (23, 25), and the cDNA molecules were size fractionated on a Sephacryl S-300 (Pharmacia) column. The double-stranded cDNA molecules were blunt ended with T4 DNA polymerase (Promega), and *EcoRI* linkers (Promega) were ligated onto the ends. After digestion with *EcoRI*, excess linkers were removed by Sephacryl S-300 column chromatography. The pooled cDNA molecules were cloned into *EcoRI*-cut and dephosphorylated pGEM2 (Promega) and transformed into *Escherichia coli* DH5 α cells. Clones positive for RV C protein cDNA were identified by colony hybridization (52), using a [³²P]dCTP-labeled (16) 124-base pair (bp) *KpnI*-*HpaII* fragment from the 5' end of the C gene as a probe. The cDNA from one of the positive clones (pcDNA17) was subcloned into M13mp18 as a 1,600-bp *EcoRI* fragment and sequenced by the dideoxy method, using Sequenase (U.S. Biochemical Corp.), as outlined in the protocol of the manufacturer. The sequence analysis revealed three single nucleotide differences compared with one of the previously published sequences of the Therien strain (48): (i) an A-to-G change at position -8 (numbering the A of the first AUG of the coding sequence as 1), (ii) a T-to-C change at position 23, and (iii) a G-to-C change at position 59. None of the three differences changed the amino acid sequence. The first two differences correspond to the nucleotides reported by Frey and Marr (17) for these positions in the Therien strain.

Plasmid constructs and in vitro mutagenesis. The full-length RV structural cDNA was assembled in pGEM2 under the SP6 polymerase promoter; bp -65 to 735 were from pcDNA17, bp 736 to 3196 were from the pKTH plasmids. In the resulting plasmid, pRVS4, the RV cDNA insert is flanked by *EcoRI* and *HindIII* sites from the pGEM2 multiple cloning site, and the *HindIII* site is preceded by an additional *SphI* site introduced by the subcloning procedures.

Site-directed mutagenesis was performed by using Sequenase as described previously (47), except that *E. coli* DH5 α F'IQ (GIBCO-BRL) was used instead of JM101. The mutant oligonucleotides were made at the Center for Biotechnology, Huddinge, Sweden, with an Applied Biosystems 380 B DNA synthesizer.

(i) **Plasmid pCE2.** pCE2 contains the coding region for RV C and E2 proteins (CE2 polypeptide) under the T7 promoter in pGEM3Zf+ (Promega). pCE2 was made by joining the 1,876-bp *EcoRI*-*PstI* fragment from pRVS4 and the 3,200-bp *PstI*-*EcoRI* fragment from pE2 (all the restriction enzymes used were from Promega or Boehringer Mannheim). pE2 in turn was constructed by cloning the 874-bp *StuI*-*PstI* fragment from pRVS4 into M13mp18. The 5' and 3' ends were altered by site-directed mutagenesis to create an artificial signal sequence (34) or the final nine residues of E2 followed by two in-frame stop codons, respectively. The mutating oligonucleotides were 5'-CCC GGC GCG CGC GGT TGG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CTT CCG TCT TCC CAT GGT ACC GAG CTC GAA-3' (5' end) and 5'-GCC AGT GCC AAG CTT TCA TTA GCC ATA GGC GGG GGG GTT GTA CCC CTG CAG GAC GAC CGC-3'. The correct double mutant was subcloned as an *EcoRI*-*HindIII* fragment into pGEM3Zf+ to give pE2.

(ii) **Plasmid pDE2.** pDE2 contains the coding region for the

mouse dihydrofolate reductase (dhfr)-E2 fusion protein (DE2 protein) under the T7 promoter in pGEM3Zf+. The construct includes the carboxy-terminal region of the C gene encoding the E2 signal sequence. Signal peptidase is thus expected to cleave the DE2 precursor to D_{ss} (dhfr with the E2 signal sequence) and E2 proteins. pDE2 was constructed by first replacing the 814-bp *EcoRI*-*TaqI* fragment of pRVS4 (bp -65 to 749) by the 550-bp *EcoRI*-*AccI* fragment from pGEM2-dhfr (57) resulting in pdhfr-r. The *TaqI* and *AccI* sites used were blunt ended with Klenow fragment (Promega) and mung bean nuclease (Pharmacia), respectively. pDE2 was then constructed by joining the 972-bp *EcoRI*-*PstI* fragment from pdhfr-r and the 3,200-bp *PstI*-*EcoRI* fragment from pE2.

(iii) **Plasmid pC.** pC contains the coding sequence for the C protein followed by a translational stop codon immediately downstream from the putative signal peptidase cleavage site between C and E2. The coding sequence is under the SP6 promoter in pGEM2. The plasmid was constructed by first subcloning the 1,600-bp *EcoRI* fragment from pcDNA17 into M13mp18 and mutating it with the oligonucleotide 5'-GGCT GGAGCTAGGCGCGC-3'. Correct mutants were identified by single-track dideoxy sequencing and were resequenced from the stop codon up to the *SphI* site. The 800-bp *SphI*-*PstI* fragment was then used to replace the corresponding section of pcDNA17 to create pC.

(iv) **Plasmid pC_{ss}.** pC_{ss} is identical to pC except that the C protein encoded (C_{ss}) lacks the E2 signal sequence. A translational stop codon was introduced three amino acids upstream from the E2 signal sequence by site-directed mutagenesis, which was done as described above except that the mutating oligonucleotide was 5'-CGGATGCGTCAAG GATGG-3'. The 800-bp mutated *SphI*-*PstI* fragment was again used to replace the corresponding section of pcDNA17 to give pC_{ss}.

(v) **Plasmid pGEM-SFV d-4.** pGEM-SFV d-4 contains the coding sequence for the Semliki Forest virus (SFV) C protein and a p62 protein variant lacking the last 15 amino acids of the cytoplasmic domain in pGEM1. The construction of the plasmid is described elsewhere (H. Garoff et al., in press).

In vitro transcription. For RNA synthesis, 5 μg of plasmid DNA was incubated at 37°C with 20 U of SP6 or T7 RNA polymerase in a final volume of 50 μl and in the presence of 40 mM HEPES-KOH (pH 7.4)-6 mM magnesium acetate-2 mM spermidine-hydrochloride (Sigma)-100 μg of bovine serum albumin (Sigma) per ml-0.5 mM m⁷G(5')ppp(5')G (Pharmacia)-5 mM dithiothreitol (DTT; Sigma)-0.5 mM each of ATP, CTP, and UTP (Pharmacia)-50 U of RNasin (Promega). After 5 min of incubation, 1 μl of 25 mM GTP (Pharmacia) was added and the incubation was continued for 55 min. The RNA was separated into aliquots and stored at -70°C. pCE2 and pDE2 were linearized with *HindIII* and transcribed with T7 RNA polymerase (Promega). pC, pC_{ss} and pGEM-SFV d-4 were used unlinearized and transcribed with SP6 RNA polymerase (Promega).

In vitro translation. The 15- μl in vitro translation assay was made up of 1.5 μl of transcription reaction, 6 μl of rabbit reticulocyte lysate, 2 μl of H₂O and 5.5 μl of a translation mixture containing 20 μCi of [³⁵S]methionine (SJ 1515; Amersham), 100 μM potassium acetate (pH 7.4), 40 μM spermidine-hydrochloride, 50 μM each of 19 amino acids (no methionine; Sigma), 10 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate (Sigma), and 1.7 μg of creatine phosphokinase (Sigma) per ml. Where indicated, 2 μl of salt-washed dog pancreas microsomal membranes was substi-

tuted for the H_2O . The reticulocyte lysate and the microsomal membranes were prepared as described previously (30 and 31, respectively). The translation mixture was incubated at 30°C for 60 min. Where appropriate, N-linked glycosylation was inhibited by including in the reaction an acceptor peptide (*N*-benzoyl-Asn-Leu-Thr-*N*-methylamide) for N-linked glycosylation at a final concentration of 200 μ M (49).

Posttranslational analysis. (i) Protease assay. The 15- μ l translation mixture was adjusted to 10 mM $CaCl_2$. One microliter of proteinase K (GIBCO-BRL; 0.5 mg/ml) was added, and digestion was carried out at 0°C for 30 min in the presence or absence of 1% Triton X-100. The digestion was stopped by adding phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 1 mg/ml, and the incubation was continued on ice for an additional 5 min. The samples were processed immediately for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

(ii) Sedimentation in physiological salt (PS). After 60 min at 30°C, puromycin (Sigma) was added to the translation mixture to a final concentration of 1 mM and the incubation was continued for 10 min. Five microliters of carrier membranes (dog pancreas microsomal membranes) was added to the translation mixture, and the concentrations of EDTA and potassium acetate were adjusted to 25 mM and 150 mM, respectively, in a final volume of 110 μ l. After 20 min of incubation on ice, the membranes were pelleted through a 50- μ l sucrose cushion for 5 min at 25 lb/in² in a Beckman Airfuge, using an 18° A-100 rotor. The sucrose cushion contained 0.25 M sucrose, 50 mM triethanolamine, 150 mM potassium acetate, 25 mM EDTA, and 1 mM DTT (24). The supernatant fraction (sucrose cushion included) was trichloroacetic acid (TCA) precipitated by adding an equal volume of 20% TCA. The TCA-precipitated proteins and the pelleted membranes were taken up in SDS-PAGE sample buffer preheated to 95°C and processed for SDS-PAGE.

(iii) HS extraction. The high-salt (HS) extraction was carried out similarly to the sedimentation in PS, except that 800 mM KCl was substituted for potassium acetate in the sample and in the buffer for the sucrose cushion.

(iv) Carbonate extraction (high pH). For carbonate extraction, 100 μ l of 50 mM Na_2CO_3 (pH 11.1) was added to 10 μ l of translation mixture and 5 μ l of carrier membranes, and the extraction was carried out as described above. The sucrose cushion contained 0.2 M sucrose, 30 mM HEPES (pH 11.5), 150 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM DTT (24).

(v) Immunoprecipitation of in vitro-made C proteins. The in vitro-made proteins were solubilized in 1% SDS, diluted into Triton X-100 buffer (2.5% Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4], 20 μ g of phenylmethylsulfonyl fluoride per ml), as described previously (3), and mixed with 10 μ l of anti-E2 signal sequence antiserum (see below) or the corresponding preimmune serum. The immunoprecipitation was carried out as described below.

Preparation of antipeptide antibodies against the E2 signal sequence. Peptide NH_2 -KATVAVGTTARA-COOH (made at the Center for Biotechnology with an Applied Biosystems 430 A peptide synthesizer), corresponding to the carboxy terminus of RV E2 signal sequence, was coupled to the hemocyanin carrier via the extra amino-terminal lysine in the following reactions: 2 mg of hemocyanin (Sigma) in 0.6 ml of 10 mM K_2PO_4 (pH 6.0)–0.15 M NaCl (K-PBS) was mixed with the linker, 1 mg of benzoquinone (Sigma) in 0.1 ml of ethanol, and the mixture was incubated at room temperature

in the dark for 30 min. Free benzoquinone was removed by gel filtration through a 10-ml Sephadex G-50 (Pharmacia) column equilibrated with K-PBS. The peptide was reacted with the hemocyanin-benzoquinone complex by mixing 400 μ g of peptide (in 80 μ l of H_2O) with half of the pooled first pink peak from the gel filtration column and adding 1/10 volume of 1 M sodium carbonate buffer, pH 9.0. The mixture was incubated at room temperature overnight, and the reaction was stopped by adding 1/10 volume of 1 M lysine, pH 7.4, and incubating the mixture for 4 h at room temperature. The mixture was then dialyzed against K-PBS at 4°C overnight. The peptide coupled to hemocyanin (125 μ g) was mixed with an equal volume of complete Freund adjuvant (Sigma) and injected into rabbits subcutaneously. Four booster injections at 2-week intervals were given in incomplete Freund adjuvant. Rabbits were bled before the first immunization (preimmune sera) and 10 days after the last injection.

Labeling of viral proteins in infected cells and immunoprecipitation. Confluent B-Vero monolayers grown on 3.5-cm dishes were infected with 5 PFU per cell as described above. At 24 h postinfection, the medium was replaced by methionine-free minimum essential medium supplemented with 0.2% bovine serum albumin–10 mM HEPES and incubated for 30 min at 37°C. Cells were labeled with 100 μ Ci of [³⁵S]methionine per dish for 15 min in the methionine-free medium. Monolayers were washed twice with ice-cold PBS (10 mM $NaPO_4$ [pH 7.4], 150 mM NaCl) and solubilized in 300 μ l of Nonidet P-40 (NP-40) buffer (1% NP-40, 50 mM Tris-hydrochloride [pH 7.4], 150 mM NaCl, 2 mM EDTA, 20 μ g of phenylmethylsulfonyl fluoride per ml). To immunoprecipitate RV C proteins, 10 μ l of anti-E2 signal sequence antiserum (or the corresponding preimmune serum) was added to 100 μ l of lysate and incubated on ice for 30 min. After the addition of 40 μ l of 1:1 (vol/vol) slurry of protein A-Sepharose (Pharmacia), the incubation was continued overnight at 4°C. The immunoprecipitations were washed and processed for SDS-PAGE, as described previously (55). Five microliters of rabbit antiserum against RV (anti-RV, a generous gift from C. Oker-Blom, Labsystems Oy, Helsinki, Finland) was used similarly to immunoprecipitate all the structural proteins of RV.

Preparation of radioactively labeled virus. Confluent B-Vero monolayers were infected with 5 PFU per cell as described above. After 24 h, the medium was replaced by methionine-free medium containing 50 μ Ci of [³⁵S]methionine per ml. The incubation was continued at 37°C for 12 h, and the virus-containing culture medium was collected. The medium was cleared by spinning at 400 $\times$ g for 5 min in an Heraeus Minifuge T centrifuge, and the virus was pelleted in a JA 18.1 rotor at 17,000 rpm for 1 h (10°C) in a Beckman J2-21 centrifuge. The virus was solubilized with NP-40 buffer and immunoprecipitated with anti-E2 signal sequence antiserum and anti-RV antiserum as described above.

SDS-PAGE. Samples for SDS-PAGE were solubilized in SDS-PAGE sample buffer (0.1 M Tris-hydrochloride [pH 6.8], 20% glycerol, 5 mM EDTA, 0.02% bromphenol blue, 4% SDS, 50 μ M DTT) by heating at 95°C for 5 min. The samples were alkylated with an excess of iodoacetamide and spun for 2 min in an Eppendorf microcentrifuge at full speed before being applied to the gel. SDS-PAGE was performed in 10% gels (32). Gels were fixed in 30% methanol–20% acetic acid for 30 min at room temperature, rinsed in H_2O for 30 min, and then treated for fluorography with 1 M sodium salicylate (8). The gels were dried and exposed to Kodak XAR-5 film or Fuji X-ray film at –70°C. The quantification of

types of the proteins translated *in vitro* from these coding sequences were analyzed (Fig. 3). In the absence of dog pancreas microsomal membranes, CE2 gave a major product with an apparent molecular mass of 70 kilodaltons (kDa) (Fig. 3, lane 1), in agreement with the molecular mass for the CE2 polypeptide predicted from the coding sequence (67 kDa). Other lower-molecular-mass products were also present, probably resulting from internal initiation or premature termination events, which are known to occur in this system when higher-molecular-mass products are synthesized (57). When microsomal membranes were included in the translation reaction, a fraction of the precursor was processed into polypeptide chains with apparent molecular masses that correspond to those of the C and E2 proteins of RV (lane 2). The addition of the N-linked glycosylation inhibitor decreased the molecular mass of E2 from 42 to 30 kDa, whereas that of the C protein remained unchanged (lane 3). The unglycosylated E2 protein migrated slightly faster than the C protein, and its molecular mass agreed well with that reported for E2 synthesized in the presence of tunicamycin (43). The correct topology of the proteins relative to the microsomal membranes was shown by proteinase K digestion. E2 is a transmembrane protein with only a short cytoplasmic domain and most of the protein was, as expected, protected from exogenously added proteinase K,

Plasmids coding for wild-type and mutant C proteins.

Figure 1 shows the amino acid sequence around the C-E2 junction in the RV structural polyprotein. The stretch of hydrophobic and noncharged amino acids (underlined in the figure) preceding the amino terminus of E2 acts as the signal sequence for E2 (27). We constructed several variants of the RV structural cDNA to address the following two questions: (i) whether this signal sequence is retained on the C protein or cleaved off after the synthesis of the protein or at a later stage in virus maturation, and (ii) if retained, whether the signal sequence could provide a membrane anchor for the C protein. The coding sequences of these constructs, pCE2, pDE2, pC, and pC_{-ss}, are schematically presented in Fig. 2. pCE2 encodes the CE2 protein, which is a precursor for RV C and E2 proteins. pDE2 encodes a protein fusion of dhfr to the last 50 amino acids of the C protein, including the E2 signal sequence. DE2 should thus be a precursor for D_{ss} (dhfr plus the E2 signal sequence region) and E2. pC encodes a C protein, and pC_{-ss} encodes a variant which lacks the E2 signal sequence.

The cleavage, glycosylation, and translocation pheno-

FIG. 3. In vitro translation of CE2, DE2, C, and C_{ss} . CE2 (lanes 1 to 5), DE2 (lanes 6 to 10), C (lanes 11 to 14), and C_{ss} (lanes 15 to 18) were translated in the absence (–) or presence (+) of microsomal membranes (RM). Where indicated, an acceptor peptide for N-linked glycosylation (ACC) was included in the translation reaction to inhibit the N-linked glycosylation. Proteinase K treatment of the translation reactions in the absence (–) or presence (+) of Triton X-100 is indicated by P and TX-100, respectively. The positions of the CE2 precursor, the DE2 precursor, E2, the unglycosylated E2 (E2'), C, and the D_{ss} protein are indicated.

implying that it was sequestered in the membrane vesicles (lane 4). The slight shift in the molecular mass of E2 seen in lane 4 is most likely due to the digestion of its cytoplasmic tail. Complete digestion of E2 was achieved when the integrity of the membrane vesicles was disrupted by Triton X-100 (lane 5). The C protein was completely susceptible to proteinase K both in the presence and absence of Triton X-100. From these data, we concluded that C and E2 were synthesized and correctly processed in our in vitro system.

A similar analysis of the proteins produced from the DE2 mRNA is shown in Fig. 3, lanes 6 to 10. In the absence of microsomal membranes, a major band of 62 kDa, the molecular mass of which corresponds to that calculated for the DE2 precursor, was seen (lane 6). As in the case of CE2 translation, several other bands of lower molecular masses were also present. As expected, in the presence of microsomal membranes a fraction of the precursor was processed into polypeptide chains with apparent molecular masses corresponding to those calculated for D_{ss} and E2 (lane 7). Addition of the N-glycosylation inhibitor peptide did not affect the size of D_{ss} but caused the disappearance of the E2 band (lane 8). Unfortunately, the unglycosylated form of the E2 could not be identified, since one of the unspecific bands produced by this construct comigrated with the band corresponding to the unglycosylated E2 (compare to lane 3). The E2 protein was correctly inserted into the membrane (since it was largely resistant to exogenously added protease unless Triton X-100 was included in the digestion reaction) (lanes 9 and 10), thereby showing that the E2 signal sequence in the DE2 polyprotein is functional in initiating translocation of E2. D_{ss} was completely susceptible to the protease in both the absence and presence of Triton X-100.

Figure 3, lanes 11 to 18, shows the analysis of the proteins encoded by pC and pC_{ss} . The mobility of neither protein was affected by the absence or presence of microsomal membranes in the translation (lanes 11, 12, 15, and 16), and both C protein variants were completely susceptible to proteinase K, indicating that they were not translocated into vesicles (lanes 13, 14, 17, and 18). The C_{ss} protein migrated slightly faster than the C protein made from pC, with the

difference in the apparent molecular masses of the two proteins being in agreement with the absence of 26 amino acids in the C_{ss} variant. The lower yields of C and C_{ss} in lanes 12 and 16 were due to an inhibitory effect of the microsomal membranes on protein synthesis (45).

The E2 signal sequence is part of the mature C protein. Our first objective was to determine whether the E2 signal sequence remains attached to the newly made and virion C proteins. To address this question, we prepared an antiserum directed against the last 10 amino acids in the E2 signal sequence (see Materials and Methods). C proteins made from the pCE2 and pC constructs, as well as C proteins from RV-infected cells and from extracellular virus particles, could be immunoprecipitated with this antiserum but not with the corresponding preimmune serum (Fig. 4A). The D_{ss} protein could also be immunoprecipitated with the antiserum (data not shown). On the other hand, C_{ss} was not immunoprecipitable with the antiserum (lanes 5 and 6), confirming its specificity. In order to be certain that all the virion C protein contained the signal sequence, and not just a small subfraction, we performed quantitative immunoprecipitation on the solubilized virus. This was made difficult by the low affinity of the anti-peptide antiserum; nevertheless, five rounds of immunoprecipitation with the anti-peptide antiserum collected 94% of the C protein. The remaining 6%, which was precipitated by a polyclonal anti-RV antiserum, was indistinguishable by SDS-PAGE from the C protein precipitated by the first antiserum. It could therefore be concluded that the E2 signal sequence remains attached to both the newly synthesized and the virion C protein.

This interpretation of the immunoprecipitation results was further confirmed by comparing the molecular masses of C proteins made from the pCE2, pC, and pC_{ss} constructs. The C protein from pCE2 was found to be identical to the one made from pC and clearly different from C_{ss} (Fig. 4B, lanes 1 to 3), again indicating that the E2 signal sequence remains attached to the newly synthesized C protein. Interestingly, the C protein could be resolved into several closely migrating bands. The in vitro-made C proteins from pCE2 and pC consisted predominantly of three bands, and that from pC_{ss} consisted of two, whereas the C proteins from the virion or from infected-cell lysates were seen as a major faster-migrating form and a minor slower-migrating form. A close comparison of the in vivo- and in vitro-made C proteins suggested that the two bands from the virion and the intracellular C proteins corresponded to the two faster-migrating bands of the C proteins made from pC or pCE2. By using a high-resolution gradient gel, it could be shown that all the forms of C_{ss} migrated differently from those of the wild type C (data not shown). The nature of this heterogeneity in the C protein is unknown. Heterogeneity could result from usage of the two in-frame AUG codons at the 5' end of the C gene for the initiation of protein synthesis (9, 17, 48). However, deletion of the first AUG in the capsid-coding sequence again produced three forms (albeit slightly faster migrating) of the C protein (data not shown). This confirmed the results of Clarke et al. (10), who also found that the first AUG is used as the sole initiation site in vitro. Alternatively, the heterogeneity could result from differential proteolytic processing at the carboxy terminus of the C protein. Processing at the carboxy terminus appears to be an unlikely explanation, since the C proteins expressed from pC, pC_{ss} , and pC_{+30} (a variant containing the 30 amino-terminal residues of E2 after the C protein sequence) in the absence of membranes, and thus containing different carboxy termini, all showed microheterogeneity in size. The fact that the

FIG. 4. (A) Immunoprecipitation of C proteins with anti-E2 signal sequence antiserum. The C proteins from pCE2 (lanes 1 and 2), pC (lanes 3 and 4), pC_{ss} (lanes 5 and 6), RV-infected cells (lanes 7 and 8), and RV virions (lanes 9 and 10) were immunoprecipitated with the anti-E2 signal sequence antiserum (odd numbers) or with the corresponding preimmune serum (even numbers). (B) Comparison of in vitro- and in vivo-made C proteins. The C proteins made from pC (lane 1), pC_{ss} (lane 2), and pCE2 (lane 3), RV structural proteins immunoprecipitated from infected cells (lane 4), and extracellular virus particles (lane 5) were analyzed by SDS-PAGE. The positions of the CE2 precursor, E1, E2, and C are indicated. The band migrating slightly slower than the intracellular E2 [E2(c)] in lane 4 is a host contaminant protein, since the protein is not recognized by monoclonal antibodies directed against the E2 or E1 proteins and is found in mock-infected cells.

antiserum against the E2 signal sequence immunoprecipitated all three forms of the C proteins made from pCE2 or pC (Fig. 4A, lanes 1 and 3) also suggests that the carboxy termini of these forms are homogenous. Therefore, we believe that the heterogeneity does not result from sequence differences at the ends. There appears to be some form of as yet unidentified posttranslational modification occurring which is specific to the C protein sequence, a conclusion supported by the fact that the D_{ss} protein migrated as a single band (Fig. 3, lane 7).

The E2 signal sequence confers membrane association on the in vitro-made C protein. The E2 signal sequence is inserted into the membrane when it directs the translocation of the ectodomain of E2 into the endoplasmic reticulum. It is possible, therefore, that the E2 signal sequence remains membrane associated after the signal peptidase cleavage, which would mean that the C protein is anchored in the membrane. The possible membrane integration of the wild-type C protein, the C_{ss} and D_{ss} proteins, was tested by a membrane copelleting assay by using three different buffer conditions: PS, HS, and high pH carbonate. The sedimentation at PS concentration leaves both the peripheral and integral membrane proteins membrane associated. On the other hand, treatment of membranes with carbonate (pH ≥ 11) transforms the vesicles into membrane sheets and is known to release most proteins that are not integrated into the lipid bilayer of the membrane (19). HS extraction is another common method used to assess insertion of the protein into lipid bilayer (6, 38). HS extraction is expected to compete out the ionic interactions that bind proteins to membranes directly or indirectly (through other membrane proteins). The E2 protein of RV was used as a control for an integral membrane protein and wild-type dhfr was used as a control for a soluble protein. The E2 protein was found almost entirely in the pellet fraction under all conditions (Fig. 5A), while almost all (97%) of the dhfr was found in the soluble fraction (Fig. 5D, lanes 10 to 13).

Figure 5A shows the copelleting assay for the C protein

made from the CE2 polypeptide; the quantification for this and for the other constructs is shown in Table 1. At the PS concentration, about 70% of the recovered C protein was found in the membrane pellet (Fig. 5A, lanes 2 and 3). The 30% soluble component was apparently not due to an artificial release of the protein from membranes caused by the experimental procedures used, since when pelleted vesicles were extracted and pelleted for a second time under the same conditions, a negligible amount of the C protein was found in the supernatant (data not shown). The copelleting material was reduced to 43 to 49% after HS or carbonate extractions (lanes 4 to 7). We noticed that the CE2 precursor also showed detectable membrane association; this precursor has three large hydrophobic domains, and, even if the precursor were not translocated, these hydrophobic segments might mediate membrane association that is not completely disrupted by the procedures used.

About 30% of C_{ss} cosedimented with the membranes under PS conditions (Fig. 5B, lane 2), whereas most of the protein was found in the soluble fraction after HS and carbonate extractions (91 and 89%, respectively [lanes 4 to 7]). Much more of the C protein made from pC was membrane associated under all conditions: 56% at the PS concentration and 35 and 38% after the HS and carbonate extractions, respectively (Fig. 5C). The fastest-migrating form of these C proteins was consistently found preferentially in the pellet fraction. The presence of the carboxy-terminal E2 signal sequence also changed the properties of dhfr. As mentioned above, the wild-type protein was always found in the soluble fraction; in contrast, 60% of D_{ss} was membrane associated under PS conditions, and the amount of this copelleting material remained essentially unchanged after the high-pH extraction and was reduced only slightly after the HS extraction (Fig. 5D, lanes 3 to 8).

The C protein of SFV. As the C protein of SFV lacks a carboxy-terminal hydrophobic segment but has otherwise similar overall features to the RV C protein (e.g., size and distribution of charged amino acids), it was of interest to see

FIG. 5. Membrane copelleting assay. The products of *in vitro* transcription-translation of various constructs were treated with PS buffer (lanes PS), HS buffer (lanes HS), or high pH buffer (lanes C); and the proteins were separated into a membrane-bound fraction (lanes P) and a soluble fraction (lanes S) by centrifugation. The corresponding unfractionated samples (lanes T) are also shown. (A) pCE2 (the HS fractionation is from a separate experiment); (B) pC_{ss}; (C) pC; (D) pDE2 (lanes 2 to 8) and pdhfr (lanes 9 to 13). Lane 1 shows total translation of DE2 in the absence of microsomal membranes.

whether it behaved differently from the RV counterpart in our tests for membrane integration. The SFV C protein was expressed from pGEM-SFV d-4, which encodes the C and p62 d-4 proteins (H. Garoff et al., in press), the latter being a p62 protein variant with a deletion in its cytoplasmic domain. In the absence of microsomal membranes, C protein and unglycosylated p62 d-4 were made (Fig. 6, lane 1), whereas in the presence of membranes, a higher-molecular-mass species representing the glycosylated form of p62 d-4 appeared (lane 2). Surprisingly, under PS conditions, 60% of the SFV C protein copelleted with the membranes (lanes 3 and 4). However, this membrane association was disrupted by the HS extraction, since only 6% of the recovered C protein remained in the pellet fraction after this treatment (lanes 5 and 6). The high pH extraction was not as effective as the HS extraction, but most (73%) of the SFV C protein was also found in the soluble fraction after the high pH treatment. The glycosylated form of the p62 d-4 protein was found in the pellet fraction under all conditions, as expected

of an integral membrane protein. The identity of the fuzzy band between the unglycosylated p62 d-4 and C protein bands in the soluble fractions is unknown, but it could be a dimer form of the C protein. Alternatively, it could be a degradation product of the untranslocated p62 d-4 precursor caused by TCA precipitation, since it is not found in the original *in vitro* translation (lane 2).

DISCUSSION

The processing of the RV structural polyprotein by signal peptidase (9, 27) raised the possibility that, in contrast to the

TABLE 1. Quantification of the membrane copelleting assay

Protein	% [Mean (±SD)] recovered ^a		
	PS	HS	C
C from CE2	70 (4.6)	43 (6.1)	49 (9.6)
C _{ss}	30 (11.0)	9 (4.5)	11 (4.0)
C	56 (4.2)	35 (ND)	38 (14.4)
D _{ss}	60 (16.8)	51 (ND)	63 (9.1)
SFV C	60 (ND)	6 (ND)	27 (ND)

^a The percentage of the recovered material in the pellet fraction is shown. Recovery was usually 70 to 90% compared with that of the unfractionated sample. The numbers are mean values from 2 to 4 experiments. ND, Not determined (quantifications only from two experiments).

FIG. 6. *In vitro* translation and membrane copelleting assay of SFV C and p62 d-4 proteins. *In vitro* transcription-translation and copelleting were performed as described in the legend to Fig. 5. Lane 1 shows the total translation product in the absence of microsomal membranes. All other lanes are as described in the legend to Fig. 5. The positions of the glycosylated p62 d-4 (p62*), the unglycosylated p62 d-4 (p62), and the C protein are indicated.

alphaviruses, the hydrophobic signal sequence responsible for initiating the translocation of the RV E2 envelope protein could remain part of the preceding capsid protein. By using an antiserum directed against the last 10 amino acids of the E2 signal sequence, we showed that this signal indeed remains attached to both the newly synthesized (in vitro-made) C protein and that in the native virus. This conclusion was further supported by comparing the molecular masses of the in vitro-made wild-type C protein with artificially created variants containing defined carboxy termini. In this respect, RV is therefore quite different from the otherwise structurally related alphaviruses, in which the C protein autoproteolytically cleaves itself from the polyprotein and the signal sequence of the following membrane protein (p62) remains part of that protein (2, 37; H. Garoff et al., in press). West Nile virus (family *Flaviviridae*), which uses a similar structural polyprotein to that of the togaviruses, has a core protein that, when newly synthesized, also contains a hydrophobic stretch (the signal sequence for the following envelope protein) at the carboxy terminus (40). There is some evidence that the newly synthesized core protein of this virus is also membrane associated (40); however, in this case stringent extraction conditions (HS or high pH) were not used in the membrane pelleting assay. Our experiments with SFV C protein suggest that considerable care must be used in evaluating such experiments (see below). In the case of the West Nile virus, the hydrophobic carboxy terminus is removed, probably by a viral enzyme, prior to or during virus assembly (40). In contrast, it is clear from our results that the RV C protein retains its hydrophobic anchor even in the mature virion.

According to the loop and helical hairpin models for translocation initiation (15, 29, 54), the signal sequence inserts into the lipid bilayer in a helical configuration with the amino terminus of the signal on the cytoplasmic side of the membrane. Such a mode of insertion has also been postulated to result in the "amino terminus in" orientation of the integral membrane proteins with combined signal-anchor sequences such as the asialoglycoprotein receptor (28, 46), the invariant chain of class II histocompatibility antigen (33), and the transferrin receptor (57). Therefore, it is reasonable to assume that the long hydrophobic E2 signal sequence of RV also confers a transmembrane configuration on the carboxy terminus of the C protein during the synthesis of RV structural proteins. We have, in our assays, obtained results that support this close association of the RV C protein with the membrane. By using high pH or HS washes of the microsomal membranes in *in vitro* translations, a clear correlation between the presence of the hydrophobic E2 signal sequence on the protein and membrane association was seen: 43 to 49% of the wild-type C protein and 51 to 63% of the D_{ss} protein remained membrane associated after HS or high pH extractions, whereas the soluble counterparts (C_{-ss} and wild-type dhfr) were almost entirely removed from the membranes under similar conditions. Between 35 and 38% of the C protein made from pC was also found to be stably associated with membranes. This membrane association must arise from posttranslational, and therefore probably signal recognition particle-independent, insertion into the membranes, since the hydrophobic E2 signal sequence domain of the C protein would not appear until synthesis of the protein was complete and the polypeptide chain detached from the ribosome. The mode of insertion in this case may be similar to that of cytochrome *b₅*, which also contains a carboxy-terminal membrane anchor and inserts into mem-

branes posttranslationally and independently of the signal recognition particle (4, 6, 41).

The association of the RV C protein with membranes was less efficient than that of the RV E2 protein or other integral membrane proteins in similar assays (12, 19). This can possibly be explained by the structural differences between the E2 signal sequence and normal membrane anchors. Membrane proteins which span the lipid bilayer once have, in general, a 20-or-more-residues-long peptide which traverses the bilayer and divides the protein into luminal and cytoplasmic domains (53). Typically, the anchor peptide consists of uncharged, mostly hydrophobic amino acids. Furthermore, the anchor region is usually flanked by charged residues, especially on its cytoplasmic side. A comparison of the RV E2 signal sequence with these consensus features shows several nonideal properties. The uncharged region is less hydrophobic than is normal for anchor sequences and includes a number of residues (three glycines, a proline, and a glutamine) normally excluded from transmembrane domains (5, 53). In addition, the fact that the RV C protein lacks a luminal domain could also contribute to the destabilization of the membrane association of the protein. Although the RV C protein was found to bind less efficiently to the membranes than the RV E2 protein after HS or high pH extractions, our results are still compatible with a transmembrane location for the E2 signal sequence. Previously, it has been convincingly shown for the SFV p62 protein that the acquisition of a transmembrane configuration during protein synthesis is much less dependent on the anchor consensus features than is stable membrane association (11, 12). The functional significance of the observed membrane association of the RV C protein is unknown, but it could provide the basis for a membrane-associated assembly pathway for the nucleocapsid.

Our studies on the membrane association of the SFV C protein also raise a note of caution concerning the use of cosedimentation assays at PS concentrations: the SFV C protein, even though it lacks hydrophobic segments of significant length, showed considerable membrane association under such conditions. The C protein of SFV is rich in positively charged residues, and the observed membrane association could be due to unspecific ionic interactions of these residues with the negatively charged surface of microsomal membranes. More stringent conditions of HS or high pH are necessary to assess true membrane association in the case of these highly charged proteins. We also noticed that about 30% of the in vitro-made C protein and D_{ss} protein behaved as soluble proteins even at PS concentrations. At present, it is unclear if this soluble fraction is an artifact of the *in vitro* translation system or truly reflects the situation *in vivo*. Work is currently in progress to examine the membrane association of the RV C protein in infected cells.

ACKNOWLEDGMENTS

We thank R. Pettersson for the generous gifts of the RV strain, the B-Vero cell line, and the plasmids; C. Oker-Blom for the generous gift of antibodies; A. Peters for the oligonucleotide and peptide synthesis; A. Robinson for providing the reticulocyte lysate and the microsomal membranes; M. Ekström for cell culture; and P. Liljeström for critical reading of the manuscript.

This work was supported by the Swedish Medical Research Council (B88-12X-08272-01A), the Swedish National Board for Technical Development (87-02750P), and the Swedish Natural Science Research Council (B-BV 9353-301). M.S. was the recipient of a short-term fellowship from the Finnish Academy.

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