Translocation and cleavage of rubella virus envelope glycoproteins: identification and role of the E2 signal sequence

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The structural proteins of rubella virus (RV) are translated as a large polyprotein precursor, p110, which is processed to produce the mature virion components, the 33K capsid protein (C) and the two envelope glycoproteins, E1 (58K) and E2 (42K to 47K). The precise processing mechanism has not been elucidated; however it must include at least two proteolytic cleavages to release the individual virion components from the polyprotein, and it must provide for their dichotomous intracellular distribution. The C protein remains in the cytoplasm where it participates in the formation of nucleocapsids, while the envelope glycoproteins enter the cellular secretory pathway and are N-glycosylated and cleaved. Sequence analysis of the 24S mRNA encoding the polyprotein precursor suggests that both E1 and E2 are preceded by signal peptides for translocation across the membrane of the rough endoplasmic reticulum. A recent study has provided direct evidence that the putative signal peptide preceding E1 can in fact mediate translocation of E1. In this study, we have used in vitro translation-translocation assays to examine further the processing of RV glycoproteins. We have shown that the putative signal sequence preceding E2 can mediate translocation of the E2 protein in the absence of an intact E1 signal peptide. The experiments also revealed that cleavage of the E2–E1 polyprotein requires (i) the E2 signal peptide, (ii) microsomal membranes and (iii) sequences beyond the proximal half of the E1 signal peptide. Together these results suggest that separation of the E2 signal sequence as well as the proteolytic cleavage of E1 from E2 is performed by the cellular enzyme, signal peptidase.

Rubella virus (RV) is the only member of the genus Rubivirus, within the family Togaviridae (Porterfield et al., 1978; Schlesinger & Schlesinger, 1986). The virion consists of three structural proteins: the capsid protein, C, and two envelope glycoproteins, E1 and E2 (Oker-Blom et al., 1983; Pettersson et al., 1985; Toivonen et al., 1983; Waxham & Wolinsky, 1983). The virion contains two or more forms of E2 (E2α and E2β) which differ in the structures of their oligosaccharide side chains (Oker-Blom et al., 1983). The genome of RV is a single-stranded 40S RNA of positive polarity, with a 5' cap and a 3' poly(A) tract (Oker-Blom et al., 1984). Transcription of the 3' one-third of the genomic RNA produces a 24S subgenomic mRNA, which is translated to produce a precursor polyprotein, p110 (Clarke et al., 1988; Kalkkinen et al., 1984), that contains the viral structural proteins in the sequence NH2--C-E2-E1--COOH (Oker-Blom, 1984). The three individual virion proteins are produced by processing of this primary translation product, but the precise processing mechanism has not been determined.

The C protein is thought to associate with viral RNA to form progeny nucleocapsids in the cytoplasm of the infected cell. Subsequently these nucleocapsids are enveloped and released, possibly by budding at internal membranes. This strategy of viral morphogenesis requires proteolytic cleavages, which produce the individual virion components, and a dichotomy in the intracellular distribution of the products. Presumably the cellular secretory pathway is used to accomplish the ultimate disposition of both E2 and E1.

Proteins enter the secretory pathway of mammalian cells by translocation across the membrane of the rough endoplasmic reticulum (RER) (Walter et al., 1984; Walter & Lingappa, 1986; Wickner & Lodish, 1985). The prototype translocation mechanism begins with a cytoplasmic interaction between the 'signal peptide' in a nascent polypeptide chain and a cellular ribonucleoprotein component called the signal recognition particle. The signal recognition particle–signal peptide–ribosome complex then binds to the signal recognition particle

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receptor, or docking protein, located in the membrane of the RER. This interaction is accompanied by the release of the signal recognition particle and cotranslational passage of the polypeptide chain across the membrane of the RER. The signal peptide is cleaved by signal peptidase, located in the lumen of the RER, creating a new amino terminus for the mature protein. In the case of integral membrane proteins, translocation is interrupted by the appearance of a hydrophobic transmembrane anchor, or ‘stop-transfer’ sequence, which mediates a stable association between the protein and the membrane. Deletion of signal peptides from proteins that normally enter the secretory pathway, including the E1 protein of RV, results in the expression of proteins that are unstable in the cytoplasm (Gething & Sambrook, 1982; Hobman et al., 1988; Jabbar & Nayak, 1987; Sekikiwa & Lai, 1983).

Typical signal peptides consist of approximately 20 amino acids, with a basic amino terminus, a central hydrophobic core of about 10 to 12 amino acids, and small, neutral amino acids (e.g. Gly, or especially Ala) located at positions $-1$ and $-3$ relative to the signal peptide cleavage site (Perlman & Halvarson, 1983; von Heijne, 1984). The amino-terminal amino acid sequences of both E2 and E1 have been determined (Kalkkinen et al., 1984), and identified within the amino acid sequence derived from the nucleotide sequence of the 24S mRNA (Clarke et al., 1987; Frey & Marr, 1988; Frey et al., 1986; Nakhasi et al., 1986; Takkinen et al., 1988; Vidgren et al., 1987). Examination of the regions upstream from the amino termini of E2 and E1 reveals that both are preceded by sequences that have the characteristics typical of signal peptides (see Fig. 2). A recent report has provided direct evidence that E1 is in fact preceded by a functional signal peptide (Hobman et al., 1988).

We have investigated further the mechanism of RV glycoprotein processing by evaluating the functions of the putative E2 signal peptide and the E1 signal peptide. By in vitro translation-translocation experiments in a mammalian cell-free system, we have shown that the putative signal peptide preceding E2 is both functional in the absence of the E1 signal peptide and important for efficient processing of the polyprotein. Furthermore, proteolytic cleavage of the E2/E1 polyprotein required not only the presence of the E2 signal peptide, but also microsomal membranes and sequences beyond the proximal half of the E1 signal peptide. This suggests that the proteolytic separation of E2 from E1 occurs concurrently with cleavage of the E1 signal peptide by the action of cellular signal peptidase.

Several plasmids were constructed for the in vitro transcription-translation experiments by using standard recombinant DNA procedures (Maniatis et al., 1982). The RV coding sequences containing the putative signal sequence of E2 were derived from the plasmid pAc701-RVE that has been described in detail previously (Oker-Blom et al., 1989). The constructs lacking the corresponding sequence were obtained from the plasmid pAc311-RVE (Fig. 1), that was constructed as follows. pKTH2522 is a plasmid containing the entire coding sequence for the RV envelope glycoproteins (Oker-Blom et al., 1989). This plasmid was digested with BssHIII, the comigrating 1064 or 1066 bp fragments were gel-purified, and the 5' protruding ends were repaired with the Klenow fragment of Escherichia coli DNA polymerase. The fragments were dephosphorylated with calf thymus alkaline phosphatase, ligated with phosphorylated BglII linkers and T4 DNA ligase (New England Biolabs) and digested with BglII and PstI. The plasmid T7SVSP (kindly supplied by Dr J. Strauss; see also Oker-Blom & Summers, 1989) was digested with XbaI and HindIII, the ends were filled in with DNA polymerase, and BglII linkers were inserted to generate T7SVSP-2. The BglII-PstI fragment of this plasmid was replaced with the BglII-PstI fragment described above, to produce pGEM-4RVSV, which contains the 1066 bp RV fragment. The PstI–EcoRI fragment of this plasmid was replaced with the PstI–EcoRI fragment from the plasmid pKTH2522, to generate pGEM-4RV. The unique EcoRI site in plasmid pGEM-4RV was converted to a BglII site by linker insertion to generate pGEM-4RV(BglII). Finally the gel-purified BglII fragment from pGEM-4RV(BglII) was inserted into the unique BamHI site of the baculovirus transfer plasmid pAc311 (Luckow & Summers, 1988) to generate the final recombinant transfer plasmid, pAc311-RVE. This plasmid encodes an RV E2–E1 polyprotein that lacks the E2 signal peptide and was used for the construction of pBS-E2(−) and pBS-E2/E1(−), respectively (Fig. 2).

All constructs were cloned into a pBluescript vector (Stratagene Cloning Systems) after it had been modified to contain a BglII site (pBSSK-Bgl) and expressed in vitro as described below. The general structures of the plasmids are presented in Fig. 2(a), whereas the sequences around the region of the E2 signal sequence are shown in Fig. 2(b). Briefly, the gel-purified EcoRV–PstI fragments from the plasmids pAc701-RVE and pAc311-RVE were inserted into the plasmid pBSSK-Bgl. The resulting constructs were designated pBS-E2(+) and pBS-E2(−) respectively (Fig. 2). The plasmid pBS-E2(+) encodes the RV E2 glycoprotein, preceded by 50 RV-encoded amino acids, including the putative signal peptide of 21 amino acids. pBS-E2(−) encodes a mature E2 glycoprotein preceded by only three RV-specific amino acids. Neither of these constructs encodes an intact E1 signal peptide or downstream E1-coding sequences. The PstI–BglII fragment from
pKTH2522 (Fig. 1) was then inserted into both pBS-E2(+), and pBS-E2(-), to produce pBS-E2/E1(+) and pBS-E2/E1(-), respectively (Fig. 2). The plasmids pBS-E2/E1(+) and pBS-E2/E1(-) are identical to pBS-E2(+) and pBS-E2(-) respectively, but they also contain the entire E1 signal peptide and the complete coding region of the E1 protein. Finally, a PstI–BamHI fragment from the plasmid T7SVSP, containing the coding region of 120 amino acid residues of the carboxyl terminal end of the Sindbis virus E1 glycoprotein, was inserted into the PstI–BglII site of pBS-E2(+), to create pBS-E2/SV(+). This plasmid is identical to pBS-E2/E1(+), except that the RV sequences downstream from the middle of the E1 signal peptide were replaced with sequences encoding the carboxyl terminal end of the Sindbis virus E1 glycoprotein. Thus the putative signal peptidase cleavage site between the E1 signal peptide and the E1 protein has been eliminated. The plasmids were linearized with BglII, except for pBS-E2/SV(+), which was linearized with NsiI, and each was transcribed in vitro using T7 RNA polymerase (Bethesda Research Laboratories), under the conditions recommended by the manufacturer. Two μl aliquots of the 100 μl transcription reaction were used directly for in

Fig. 1. Cloning strategy for the plasmid construct pAc311-RVE lacking the putative E2 signal sequence.
Translation of pBS-E2(+), produced a 34K protein in the absence of membranes and a 39K protein in the presence of membranes (Fig. 3a). In contrast, translation of pBS-E2(-) produced a 30K protein, whether or not the membranes were added. These data suggest that the putative E2 signal peptide is functional, as it mediated translocation and consequently N-glycosylation of E2 in the absence of an intact E1 signal sequence.

In the absence of membranes, the translation product of pBS-E2/E1(+) migrated as a single protein with an apparent $M_\text{r}$ of 84K, whereas in the presence of membranes, p84 was converted to two products of about 58K and 39K. These represent cleaved and N-glycosylated forms of E1 and E2, respectively. In contrast, translation of pBS-E2/E1(-) in the presence or absence of membranes produced only one protein: p84. This protein essentially comigrated with the p84 produced in the absence of membranes with pBS-E2/E1(+).

Together these data indicate that E2 is preceded by a functional signal peptide that is required for trans-

![Diagram of RV cDNA constructs](https://example.com/diagram.png)

**Fig. 2.** RV cDNA constructs used in this study. (a) General structures. The pBS-E2/E1(+) construct, encodes an RV E2-E1 polyprotein in which the putative signal peptide preceding E2 is intact, E2/E1(+), whereas pBS-E2/E1(-) encodes an RV E2-E1 polyprotein that lacks
location of the E2/E1 polyprotein into the cellular secretory pathway, and for proteolytic processing of the polyprotein.

The in vitro translation of the proteins synthesized from the constructs pBS-E2(+) and pBS-E2/E1(+) were further studied using endo-β-N-acetylglucosaminidase H (endo H) (Tarentino & Maley, 1974). The product synthesized in the presence of membranes and treated with endo H using pBS-E2(+) was smaller (29K) than that synthesized in the absence of membranes (34K), revealing that the E2 signal peptide was removed (Fig. 3b). Similarly, the E2 protein from pBS-E2/E1(+) underwent translocation, signal peptide cleavage and N-glycosylation.

The results of in vitro translation experiments using pBS-E2/SV(+) are shown in Fig. 3(b). The hybrid polyprotein synthesized from this construct was translocated and N-glycosylated in the presence of microsomal membranes, as shown by an increase in its size and by its sensitivity to endo H. Again, the product synthesized in the presence of membranes and treated with endo H was smaller (38K) than the product synthesized in the absence of membranes (42K), suggesting that the E2 signal peptide was cleaved. However the E2 products synthesized from both pBS-E2(+) and pBS-E2/E1(+) were smaller than the corresponding E2 product from the hybrid construct, in which the E1 signal peptide is truncated. This suggests that translocation alone does not ensure the proteolytic cleavage of E2 from E1 in the polyprotein precursor. A third component contained in the sequences downstream from the proximal one-half of the E1 signal peptide is necessary also. We speculate that this component is the signal peptidase cleavage site following the E1 signal peptide.

Although the general features of RV replication are quite well understood (Pettersson et al., 1985), the precise molecular mechanism of processing of the viral structural proteins remains to be determined. Elucidation of the nucleotide sequence of the 24S mRNA has revealed that both E2 and E1 are preceded by amino acid sequences with the characteristics typical of signal peptides (Clarke et al., 1987; Perlman & Halvarson, 1983; Takkinen et al., 1988; Vidgren et al., 1987; von Heijne, 1984). Hobman et al. (1988) have provided direct evidence that the putative signal peptide preceding E1 is functional. In this study, we have provided experimental

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**Fig. 3.** Radioimmunoprecipitation and SDS-PAGE analysis of in vitro translated RV proteins. (a) The plasmids pBS-E2(+), pBS-E2(−), pBS-E2/E1(+) and pBS-E2/E1(−) were transcribed and translated in the absence and presence of canine microsomal membranes (MM) as described in the text. The negative control was reticulocyte lysate with or without microsomal membranes, but without the addition of RNA. (b) Plasmids pBS-E2(+), pBS-E2/E1(+) and pBS-E2/SV(+) were used for translation as above, but after synthesis the protein products were treated with endo H. The 14C-labelled Mr markers (Bethesda Research Laboratories) are shown on the left.
evidence that the putative signal peptide preceding E2 is functional as it mediated translocation and glycosylation of the E2 protein. This is in complete agreement with a recently published study by Hobman & Gillam (1989). In addition we have provided evidence that the signal peptide preceding E2 is functional in the absence of an intact E1 signal peptide and that it is required for processing of the E2/E1 polyprotein in vitro. The polyprotein produced from constructs with or without the E2 signal peptide was not cleaved in the absence of microsomal membranes. Similarly, a hybrid polyprotein lacking RV sequences downstream from the proximal half of the E1 signal peptide was not cleaved. Previously it was suggested that host cell proteases are responsible for the proteolytic processing of the RV polyprotein, based upon the finding that cleavage did not occur unless the polyprotein was translocated across the membrane of the RER (Clarke et al., 1987). Consistent with this, our results suggest that there are three requirements for the proteolytic separation of E2 from E1: (i) a functional E2 signal peptide, (ii) microsomal membranes (RER) and (iii) sequences downstream from the proximal half of the E1 signal peptide. Based upon these requirements, we propose that cellular signal peptidase simultaneously accomplishes cleavage of the E2 signal peptide and the proteolytic separation of E2 from E1.

The general replication strategy of RV closely resembles that of the members of the Alphavirus genus (Pettersson et al., 1985). The cleavage of the capsid protein where alphaviruses are concerned is accomplished by an autoproteolytic activity that resides within the capsid protein itself (Schlesinger & Schlesinger, 1986). It has been suggested previously that the release of the RV capsid protein from the p110 polyprotein precursor in vitro requires microsomal membranes (Clarke et al., 1987). Therefore it can not be excluded that the E2 signal peptide functions as an internal translocation signal and that the proteolytic separation of the C protein from E2, like the separation of E2 from E1, could be accomplished by signal peptidase. Moreover the C protein would remain membrane-bound, as a result of the presence of the E2 signal peptide. This would maintain an association between the C protein and one or both of the envelope glycoproteins, which would ensure that they are transported together, as a complex, while still maintaining their dichotomous intracellular distribution. This association might be required for proper assembly of progeny virion particles. There is also a possibility that RV, like alphaviruses, contains a small (2.5K) intergenic peptide between E2 and E1 (Schlesinger & Schlesinger, 1986; Waxham & Wolinsky, 1983). This peptide could be produced by proteolytic cleavage at a second site located upstream of the E1 signal sequence. This cleavage might occur at a late stage of processing, perhaps in the Golgi apparatus, but not in vitro. Experiments designed to elucidate the transmembrane domains for E2 and E1 and to determine the carboxy-terminal sequences of C and E2 are obviously needed to test these speculations and to provide a more complete understanding of the pathway of RV glycoprotein biosynthesis and processing.

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References


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