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Assembly in vitro of a spanning membrane protein of the endoplasmic reticulum: The E1 glycoprotein of coronavirus mouse hepatitis virus A59

(cell-free protein synthesis/enveloped animal viruses)

PETER ROTTIER*, DOROTHEE BRANDENBURG*, JOHN ARMSTRONG†, BEN VAN DER ZEIJST*, AND GRAHAM WARREN†

*Institute of Virology, Veterinary Faculty, State University of Utrecht, 3508 TD Utrecht, The Netherlands; and †The European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, Federal Republic of Germany

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ABSTRACT **The E1 glycoprotein of coronavirus mouse hepatitis virus A59 was synthesized *in vitro* by translation of viral mRNA in the presence of dog pancreatic microsomes. Its disposition in the membrane was investigated by digestion with proteases and by selective NH_2-terminal labeling. The protein spans the membrane, but only small portions from the NH_2 and COOH terminus are exposed respectively in the lumenal and cytoplasmic domains; the bulk of the molecule is apparently buried in the membrane. The protein lacks a cleavable leader sequence and does not acquire its characteristic O-linked oligosaccharides in rough microsomes. It may enter the membrane at any stage during synthesis of the first 150 amino acid residues. These unusual features of the protein might help to explain why it is not transported to the cell surface *in vivo* but remains in intracellular membranes, causing the virus to bud there.**

Studies of membrane biogenesis at the molecular level have been greatly facilitated by the use of enveloped animal viruses as probes. The viral spike glycoproteins of Semliki Forest, Sindbis, vesicular stomatitis, and influenza viruses are all synthesized in the rough endoplasmic reticulum (rough ER) (1–4). They then pass through the stacks of flattened Golgi cisternae (5, 6) before reaching the plasma membrane (PM) where budding of the virus occurs. A similar pathway is inferred for the host cell proteins that span the PM.

Much less is known about the biogenesis of proteins of intracellular membranes, such as those of the ER and Golgi complex (7), but analogous studies to those noted above could be carried out with viruses whose site of budding is in these membranes. For example, bunyaviruses and coronaviruses have long been known to bud into intracellular membranes, but it is only recently that a few of them have been characterized sufficiently to allow a detailed biochemical study (8–11).

The most intensively studied coronavirus is mouse hepatitis virus (MHV). MHV buds into the ER and perhaps membranes in the Golgi region (12–14). The assembled virions then appear to follow the route taken by both secretory and PM proteins (5, 6, 15). Virions are found in the lumen of ER and the Golgi cisternae and in vacuoles that probably fuse with the PM, releasing virus from the cell (13, 16). The intracellular budding site of coronavirus appears to be determined by one of its two envelope glycoproteins, E1, which stays in internal membranes after its synthesis on membrane-bound ribosomes (13, 14). The other glycoprotein, E2, is also assembled in the rough ER; part of it is incorporated into the budding virions in which it is needed for subsequent infectivity but not for virus maturation and release (12, 17). Some passes to the cell surface where it fuses adjacent cells together, thereby spreading the infection. In some respects, E2 is similar to the spike glycoproteins of those viruses that bud at the PM. It appears to take the same route through the cell, passing through the Golgi complex, to be fatty-acylated, and to have normal N-linked oligosaccharides (18). In contrast, the E1 protein has neither fatty acid groups nor N-linked oligosaccharides; instead, it has O-linked oligosaccharides (12, 17, 18), which are probably acquired in the Golgi complex (14, 19) as the virions pass through the stacks of Golgi cisternae. This pattern of post-translational modification is unique among viral glycoproteins so far characterized.

The budding site of the coronaviruses suggested that they might serve as a useful model for the biogenesis of membranes on the intracellular transport pathway; in particular, the behavior of the E1 glycoprotein implicates it as a possible model intracellular transmembrane protein. As a first step in characterization, we report the assembly of this protein in microsomal membranes in cell-free extracts. The results show several striking differences between this glycoprotein and those that normally are transported to the cell surface.

MATERIALS AND METHODS

Coronavirus MHV-A59. This strain of MHV was grown in Sac^- cells and labeled with [^{35}S]methionine as described (17, 20). Iodination of the virus was carried out with IODO-GEN (21), and the final specific activity was 1.9 μCi/μg (1 Ci = 37 GBq). Iodination did not affect the mobility of the viral proteins on NaDodSO$_4$/polyacrylamide gels.

Poly(A)$^+$ RNA. Polyadenylylated RNA was prepared from infected cells as described (22) except that poly(U)-Sepharose (23) was used to select the poly(A)$^+$ RNA.

Dog Pancreatic Microsomes. Prepared as described by Blobel and Dobberstein (24) and treated with EDTA (2), they were provided by David Meyer (European Molecular Biology Laboratory).

Cell-Free Protein Synthesis. Reticulocyte lysates were prepared as described (25). Proteins were synthesized in reaction mixtures containing (per 25 μl) 10 μl of reticulocyte lysate, 2.5 μl (2.5 μg) of poly(A)$^+$ RNA (where specified), 2.5 μl (0.05 A_{280}) of dog pancreatic microsomes (where specified), and the following components (final concentrations in parentheses): Hepes (pH 7.2; 20 mM), potassium acetate (80 mM), magnesium acetate (0.65 mM), dithiothreitol (2 mM), creatine phosphate (8 mM), rabbit skeletal muscle creatine kinase (40 μg/ml), spermidine·HCl (0.5 mM; Serva), 19 ami-

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Abbreviations: ER, endoplasmic reticulum; PM, plasma membrane; MHV, mouse hepatitis virus.

no acids excluding methionine (25 μM each), and [^{35}S]methionine (1 mCi/ml; New England Nuclear at 1500 Ci/mmol or the Radiochemical Centre at 910 Ci/mmol). The mixture was normally incubated for 1 hr at 30°C.

Synchronized Protein Synthesis. Protein synthesis was modeled as described (26) except that 1.2 μM edeine (27) was used to inhibit initiation.

***N*-Formyl-[^{35}S]Methionyl-tRNA^{Met}.** This was prepared as described (28) with yeast tRNA, *Escherichia coli* synthetases and transformylase, and calcium leucovorin, which was provided by H. van Steeg and M. Kasperaitis (Department of Molecular Cell Biology, State University, Utrecht).

Immunoprecipitation. Translation products were incubated overnight at 4°C with mouse anti-MHV-A59 antiserum (20), mouse serum obtained prior to immunization ("preimmune" mouse serum), or monoclonal anti-E1 (provided by M. Koolen, Institute of Virology, State University, Utrecht) and then were treated for a further 5 hr at 4°C with 5 μg of affinity-purified rabbit anti-mouse IgG provided by Brian Burke (European Molecular Biology Laboratory). The immunocomplexes were isolated essentially as described (6).

Protease Digestions. Translation mixtures were diluted 1:5 with 50 mM Tris·HCl buffer, pH 7.4/100 mM NaCl containing proteinase K (Serva; 1.25 mg/ml) and incubated at 37°C for 15 min in the presence or absence of 0.05% saponin (Sigma). After being cooled on ice, they were treated for 10 min with excess phenylmethylsulfonyl fluoride to inhibit proteinase K activity. The samples were then treated with trichloracetic acid or extracted with Triton X-114 (29).

Gel Electrophoresis. Samples were prepared for NaDodSO$_4$/PAGE as described (figures 1 and 2 in ref. 6; other figures in ref. 22) and were fractionated on a 15% polyacrylamide gel with a 5% stacking gel as described by Maizel (30) or on a 15% polyacrylamide gel with a 4% stacking gel as described previously (22). The gels were fixed, treated with EN^{3}HANCE (New England Nuclear), dried, and exposed at −70°C to Kodak XR-5 film (6), or they were washed twice with dimethyl sulfoxide, treated with 20% (wt/wt) 2,5-diphenyloxazol (Merck) in dimethyl sulfoxide, incubated in water, dried, and exposed at −70°C to Fuji RX film.

RESULTS

E1 Assembled in Microsomal Membranes Is Neither Cleaved nor Glycosylated. Sac$^-$ cells were infected with coronavirus MHV-A59; 8–9 hr later, poly(A)$^+$ RNA was extracted. When translated in a reticulocyte lysate, many proteins were synthesized (compare lanes 1 and 2 with 3 and 4 in Fig. 1) and two [nucleocapsid (N) and E1] were tentatively identified as viral proteins, the rest presumably being derived from host cellular mRNAs. The identity of the E1 protein was confirmed by using specific antibodies. A M_r 23,500 polypeptide was specifically precipitated from the total translation mixture by antiserum to the whole virus (Fig. 1, lane 8) and a monoclonal anti-E1 antibody (Fig. 1, lane 10) but not by preimmune serum (Fig. 1, lane 9).

E1 synthesized in the absence of dog pancreatic microsomes (Fig. 1, lane 3) could be digested completely by protease (Fig. 1, lane 5). Synthesis in the presence of microsomes (Fig. 1, lane 4) yielded an E1 protein of the same molecular weight, but most of this was resistant to protease digestion (Fig. 1, lane 6), indicating that assembly into the membrane had occurred. The absence of any change in molecular weight after assembly into microsomes strongly suggests that the signal sequence is uncleaved, a suggestion confirmed by NH_2-terminal labeling of the protein (see below). The assembled E1 protein also comigrated with unglycosylated E1 from virions (Fig. 1, cf. lanes 10 and 11), suggesting that addition of O-linked oligosaccharides occurs after the completed protein has been transported from the rough ER.

E1 Spans the Lipid Bilayer. E1 was separated from soluble proteins, which included most of the background of nonviral proteins, by exploiting the phase separation properties of Triton X-114 (29). Because E1 behaves as an integral membrane protein (8), it was selectively extracted into the detergent phase of a Triton X-114 suspension at 30°C (Fig. 2, lane 2), leaving soluble proteins such as the nucleocapsid and host-cell proteins in the aqueous phase (Fig. 2, lane 3).

After protease treatment of translation mixtures containing microsomal membranes, the E1 form of M_r 23,500 was no longer visible, and the major product had a M_r of 22,000 (Fig. 2, cf. lanes 4 and 5; Fig. 3, cf. lanes 1 and 2). Thus, a fragment with an apparent M_r of 1500 had been removed from the equivalent of the cytoplasmic side of the ER membrane *in vivo*. Previous studies have shown that E1 is accessible to protease in intact virions (8). The different forms of E1, which are presumably O-glycosylated to different extents (17) on that part of the protein exposed on the virion surface, are all digested to a common fragment of M_r 21,000 (Fig. 3, cf. lanes 5 and 6). Because the virus buds into the ER

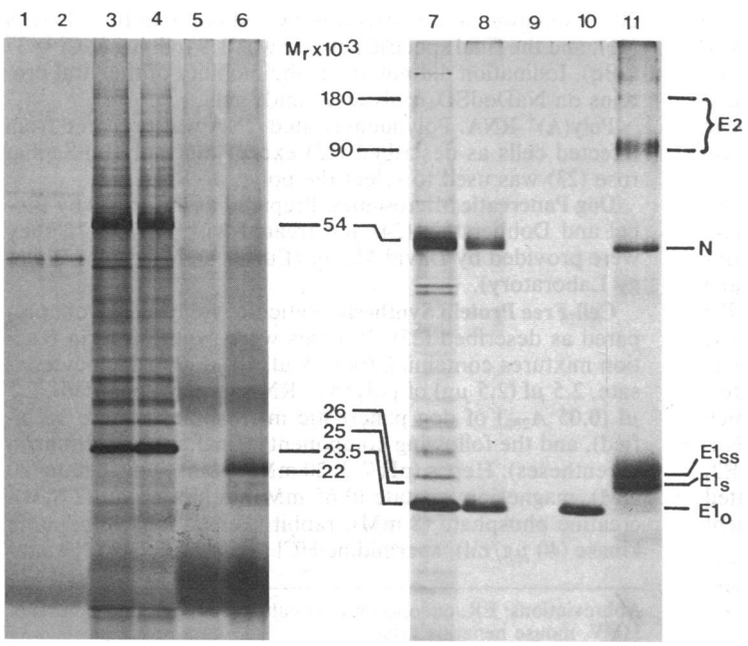

FIG. 1. Synthesis of the E1 protein in the presence and absence of microsomal membranes. Incubation in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of poly(A)$^+$ RNA from infected cells and in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of microsomal membranes. Aliquots (2 μl) were taken for direct analysis by NaDodSO$_4$/PAGE; 5-μl aliquots were treated with proteinase K and applied to lanes 5 (without membranes) and 6 (with membranes). The identity of the capsid (N) and E1 protein was confirmed by immunoprecipitation from 2.5-μl aliquots taken from proteins synthesized in the presence of microsomal membranes (lane 7) by using polyclonal anti-coronavirus antibodies (lane 8), nonimmune serum (lane 9), and monoclonal anti-E1 (lane 10). Lane 11 is iodinated virus. E1$_o$ is unglycosylated E1, whereas E1$_s$, E1$_{ss}$ have O-linked oligosaccharides. E2 is the other glycoprotein of coronavirus MHV-A59.

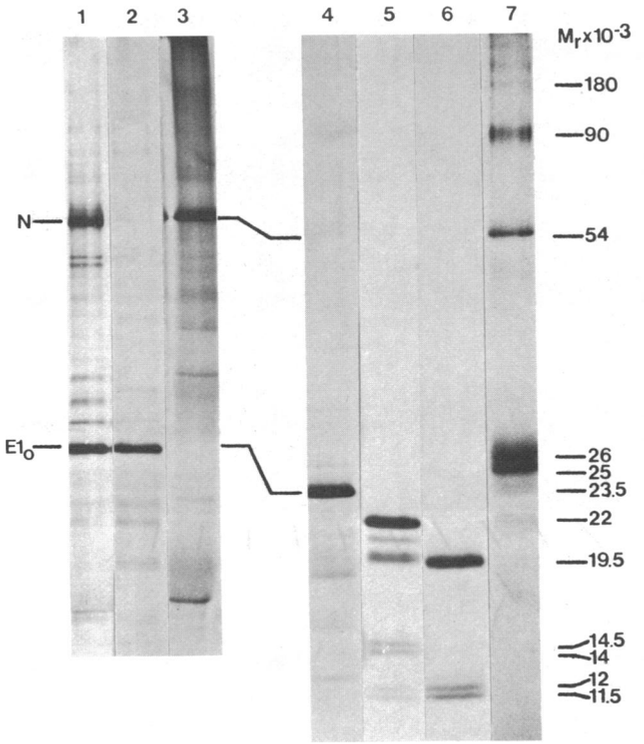

FIG. 2. Protease digestion of the E1 protein synthesized in the presence of microsomal membranes. Lanes: 1–3, purification of the E1 protein by Triton X-114 extraction; 4–6, protease digestion of E1 synthesized in the presence of microsomal membranes and purified by Triton X-114 extraction. After translation of poly(A)$^+$ RNA in the presence of microsomal membranes (lane 1), a 5-μl aliquot was extracted with Triton X-114 at 30°C, and the detergent phase containing purified E1 (lane 2) was separated by centrifugation from the aqueous phase (lane 3) containing, among other proteins, the viral capsid protein (N). Aliquots (2.5 μl) of the original incubation (lane 4) were treated with proteinase K in the absence (lane 5) or presence (lane 6) of 0.05% saponin. Lane 7 contains iodinated virus.

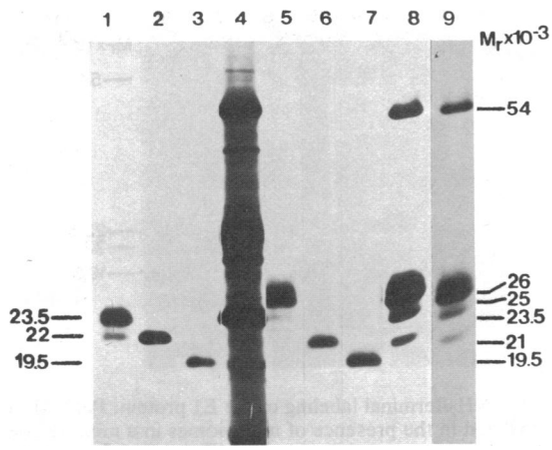

FIG. 3. Protease digestion of labeled virions and of the E1 protein synthesized in the presence of microsomal membranes. Lanes 1–4 are directly comparable to lanes 4–6 and 1 in Fig. 2. Lanes 5–8 show the protease digestion of [^{35}S]methionine-labeled virus. The original virus (30,000 cpm) (lane 8) was treated with proteinase K in the presence (lane 5) or absence (lane 6) of phenylmethylsulfonyl fluoride or in the presence of 0.05% saponin (lane 7). Phenylmethylsulfonyl fluoride was added before extraction with Triton X-114. Note that the M_r 19,500 E1 fragment is produced by digestion of both virions and microsomes in the presence of saponin (cf. lanes 3 and 7). Lane 9 is a shorter exposure of lane 8.

lumen, the virion outer surface is topologically equivalent to the lumenal side of the ER. Thus, E1 is accessible to protease from the cytoplasmic and lumenal sides of the ER membrane and, therefore, must span the bilayer.

This was demonstrated directly by permeabilizing the microsomal vesicles with the detergent saponin. When microsomal vesicles were treated with protease in the presence of 0.05% saponin, the E1 protein was quantitatively converted into a fragment with a M_r of 19,500 (Fig. 2, cf. lanes 4 and 6; Fig. 3, cf. lanes 1 and 3). Treatment of intact virions under the same conditions gave a fragment of exactly the same size (Fig. 3, cf. lanes 5 and 7), the size of which was that expected if E1 had been digested from both sides of the membrane. Thus, it would seem that saponin makes the membrane permeable to added protease but does not affect the protein otherwise. Complete disruption of the bilayer with Triton X-100 made the E1 protein completely sensitive to protease digestion (data not shown). An E1 fragment of the same mobility (M_r 19,500) was observed in some experiments in the absence of saponin (Fig. 2, lane 5; Fig. 4, lane 7). Because microsomal vesicles are known to be leaky to proteases to some extent (31), the fragment is probably derived by digestion of E1 from both sides of the bilayer. For analytical purposes, saponin treatment is advantageous in ensuring that all of the microsomal vesicles are permeable to the protease.

The E1 fragment that is resistant to protease digestion from both sides of the membrane is large enough to contain up to seven polypeptide segments spanning the bilayer, although its precise topology in the membrane remains to be elucidated. However, the presence of minor proteolytic fragments of E1 does suggest that one loop of this fragment is accessible to protease to a limited extent on the outside of microsomal vesicles. Depending on which part of the loop is cleaved, the minor fragment had a M_r of 14,000 or 14,500 (Fig. 2, lane 5). In the presence of saponin, this dropped to M_r 11,500 and 12,000 (Fig. 2, lane 6). The proposed topology that would result in these fragments is presented in Fig. 6 and will be discussed below.

The NH_2 Terminus of E1 Is on the Lumenal Side of the ER Membrane. Because E1 is not proteolytically cleaved during assembly in microsomal vesicles, it proved possible to label the NH_2 terminus selectively by using *N*-formyl-[^{35}S]methionyl-tRNA^{Met}. This label was present on the assembled E1 protein (Fig. 4, lane 3) and on the M_r 22,000 fragment generated by protease digestion (Fig. 4, lane 2), showing that the NH_2 terminus was not on the cytoplasmic side of the membrane. Digestion in the presence of saponin resulted in complete loss of the label (Fig. 4, lane 1), indicating that the NH_2 terminus is on the lumenal side of the ER membrane. The presence of each of the proteolytically derived forms of E1 was confirmed in parallel experiments with [^{35}S]methionine as the radiolabel (Fig. 4, lanes 5–8).

E1 Can Enter the Membrane at Late Stages of Synthesis. Protein synthesis was started in the cell-free system by the addition of poly(A)$^+$ RNA. After 2 min edeine was added to prevent further initiation (27). At different times samples were taken for direct analysis by NaDodSO$_4$/PAGE or were added to microsomal membranes and incubated at 30°C until 30 min after the addition of poly(A)$^+$ RNA to allow assembly to occur. These samples then were treated with protease to distinguish between soluble E1 and that assembled in microsomal membranes and finally were extracted with Triton X-114 and fractionated by NaDodSO$_4$/PAGE. Full-length E1 appeared after about 12 min of synthesis at 30°C (Fig. 5 *Upper*), corresponding to a synthetic rate of about 18 amino acids polymerized/min. This rate was not affected when synthesis was carried out in the presence of microsomal membranes (data not shown). If microsomal membranes were added at any time up to 8 min after the start of synthesis, assembly would still occur, as judged by resistance to proteolysis (Fig. 5 *Lower*). This corresponds to the synthesis

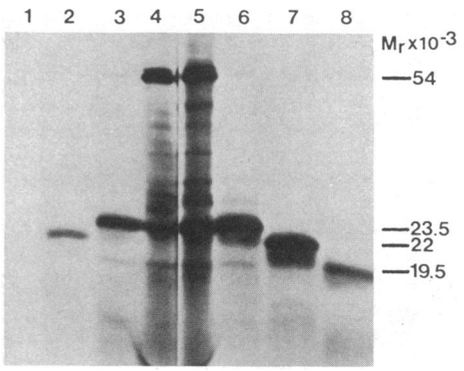

FIG. 4. NH_2-terminal labeling of the E1 protein. Poly(A)$^+$ RNA was translated in the presence of microsomes in a mixture containing 0.1 mM methionine and *N*-formyl-[^{35}S]methionyl-tRNA^{Met} instead of [^{35}S]methionine. After incubation for 1 hr at 30°C, a 6-μl aliquot of the reaction mixture was fractionated by $NaDodSO_4$/PAGE (lane 4), and a 20-μl aliquot was extracted with Triton X-114 to purify E1 (lane 3). A further two aliquots (20 μl each) were treated with proteinase K in the absence (lane 2) or presence (lane 1) of 0.05% saponin. For comparison purposes (lanes 5–8), a normal translation with [^{35}S]methionine was performed, and a 2-μl aliquot was fractionated by $NaDodSO_4$/PAGE (lane 5). Further 5-μl aliquots were extracted with Triton X-114 before (lane 6) or after treatment with proteinase K in the absence (lane 7) or presence of 0.05% saponin (lane 8).

of 65–70% of the E1 protein or 140–150 amino acids. If microsomal membranes were added at later times, however, E1 did not acquire resistance to proteolysis (Fig. 5 *Lower*).

DISCUSSION

We have studied the assembly into membranes *in vitro* of E1 glycoprotein from coronavirus MHV-A59 as a model for those integral membrane proteins that are not transported to the PM after their synthesis in the ER. Our results show that, in comparison to other PM and secretory proteins, this protein has several unusual features in addition to those previously known.

First, the bulk of the protein becomes resistant to proteolysis when it is inserted into the lipid bilayer (Figs. 1–3); apparently only short regions of M_r 2,500 and 1,500 from the NH_2 and COOH termini are exposed to the lumenal and cytoplasmic compartments, respectively (Fig. 6). The remainder is sufficiently large to span the membrane several times. This would be a novel feature for a viral glycoprotein, although several nonviral proteins are known that apparently cross the membrane more than once (32–35). Only bacteriorhodopsin is comparable, however, in appearing to lack substantial domains on either side of the membrane (36), but this protein is found in the unusual membrane of an archaebacterium.

The analysis of the protein's disposition in microsomal membranes was greatly facilitated by use of the detergent saponin. This reagent apparently permeabilizes the microsomes to allow entry of proteases without exposing the buried part of the membrane protein to digestion. Thus, the method should be of general use in investigating the topology of membrane proteins, particularly those that are not constituents of enveloped viruses and, therefore, lack the experimental advantage of being accessible in the virion in the "inverted" orientation.

Second, an NH_2-terminal leader peptide is not cleaved from the molecule after insertion into the membrane (Fig. 1). Other examples of spanning-membrane proteins without a cleavable signal sequence are rhodopsin (37) and band III protein (38). The same phenomenon has been found for a secreted protein, ovalbumin (39), and for an ER membrane

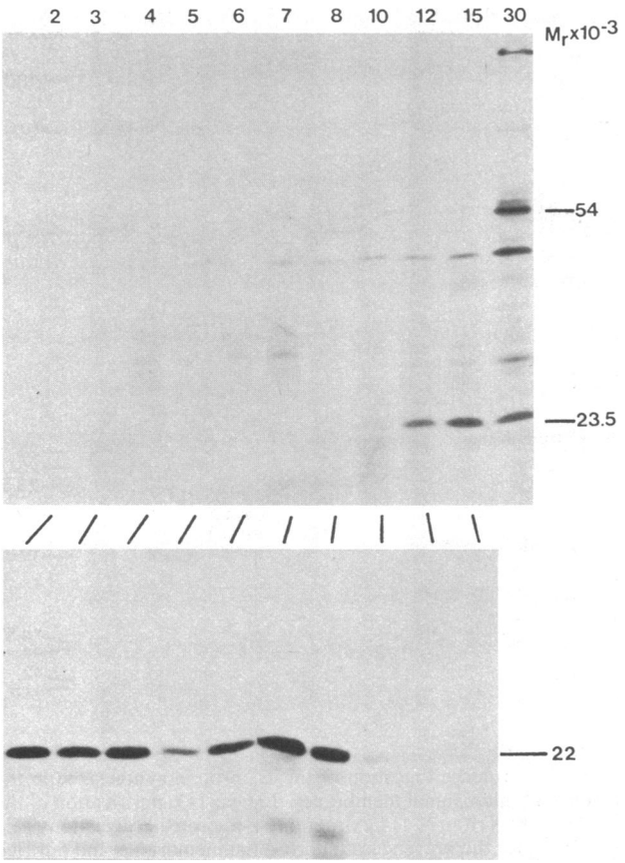

FIG. 5. Addition of microsomal membranes at different times after initiation of protein synthesis. Poly(A)$^+$ RNA was added at zero time to initiate synthesis at 25°C; 2 min later edeine was added to a final concentration of 1.2 μM to prevent further initiation, and the mixture was incubated further at 30°C. At the times shown in minutes (above the lanes), 1-μl aliquots were removed for direct analysis by $NaDodSO_4$/PAGE (*Upper*), and 10-μl aliquots were treated with microsomal membranes and incubated at 30°C for 30 min. These samples were then treated with proteinase K, extracted with Triton X-114, and fractionated by $NaDodSO_4$/PAGE (*Lower*).

protein, cytochrome P_{450} (40); in the latter case, however, it is not clear that the protein contains a domain on the lumenal side of the membrane.

Third, the molecule can penetrate the membrane even after a large portion of it, ≈140–150 amino acids, has been synthesized (Fig. 5); thus, the signal for membrane insertion in principle could be located anywhere within this region. A further comparison with ovalbumin and band III can be made. It has been proposed that the signal sequence for

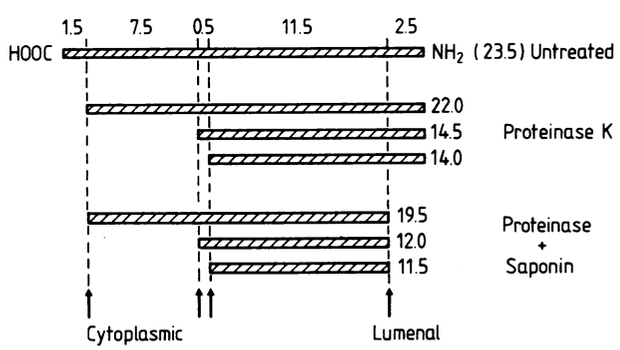

FIG. 6. Proteolytic cleavages of the E1 protein on each side of the microsomal membrane. Arrows indicate the observed sites of cleavage by proteinase K to give fragments with molecular weights shown $\times 10^{-3}$.

these proteins is internal rather than NH_2-terminal (38, 41), although the precise location for ovalbumin remains controversial (42). An indirect indication that the signal for E1 is in the NH_2-terminal region is provided by the experiment shown in Fig. 4. E1 translated *in vitro* with formylmethionine as its NH_2 terminus can enter the membrane, but apparently not with the same efficiency as the normal protein does (compare lanes 2 and 3 with lanes 7 and 6). Presumably, NH_2-terminal methionine normally is cleaved from the protein before insertion into the membrane. However, formylmethionine is resistant to cleavage (43) and, by interfering with an NH_2-terminal signal structure, might reduce the efficiency of penetration.

Fourth, the protein does not acquire its oligosaccharides, which are O-linked rather than N-linked, in the rough ER. This confirms the cell fractionation data obtained by Niemann *et al.* (14); it seems likely that these oligosaccharides are acquired as the completed virions pass through the Golgi complex (14, 19).

Finally, it appears that only a small portion of the protein is directly involved in the process of virion assembly because only the cytoplasmically exposed fragment of M_r 1,500 would be available for interaction with the nucleocapsid. It is presumably this tiny portion that is responsible for the high affinity of E1 for RNA (44). In principle, the interaction between E1 and viral nucleocapsid could determine the localization of the E1 in infected cells. If this were the case, however, it is not clear why the majority of enveloped viruses should assemble at the cell surface. This possibility could be tested rigorously by expression of the E1 protein from a copy DNA gene in the absence of nucleocapsid protein.

Thus, the E1 glycoprotein of coronavirus has several features that distinguish it from the majority of membrane proteins. Some of these features could be clarified by analysis of the amino acid sequence of the protein (unpublished data). Which of these characteristics determine the protein's localization in the internal membranes of the cell should be established.

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