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Membrane Assembly of the Triple-spanning Coronavirus M Protein

INDIVIDUAL TRANSMEMBRANE DOMAINS SHOW PREFERRED ORIENTATION*

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The M protein of mouse hepatitis virus strain A59 is a triple-spanning membrane protein which assembles with an uncleaved internal signal sequence, adopting an $N_{exo}C_{cyt}$ orientation. To study the insertion mechanism of this protein, domains potentially involved in topogenesis were deleted and the effects analyzed in several ways. Mutant proteins were synthesized in a cell-free translation system in the presence of microsomal membranes, and their integration and topology were determined by alkaline extraction and by protease-protection experiments. By expression in COS-1 and Madin-Darby canine kidney-II cells, the topology of the mutant proteins was also analyzed *in vivo*. Glycosylation was used as a biochemical marker to assess the disposition of the NH_2 terminus. An indirect immunofluorescence assay on semi-intact Madin-Darby canine kidney-II cells using domain-specific antibodies served to identify the cytoplasmically exposed domains. The results show that each membrane-spanning domain acts independently as an insertion and anchor signal and adopts an intrinsic preferred orientation in the lipid bilayer which corresponds to the disposition of the transmembrane domain in the wild-type assembled protein. These observations provide further insight into the mechanism of membrane integration of multispanning proteins. A model for the insertion of the coronavirus M protein is proposed.

Membrane proteins are integrated into the lipid bilayer in a variety of different topologies. They can either be simple, spanning the membrane only once; or complex, traversing the membrane multiple times. Their NH_2 and COOH termini may be exposed on either side of the membrane (for reviews, see Rapoport, 1986; Wickner and Lodish, 1985; Singer, 1990). Proteins acquire these topologies by the functioning of topogenic signals present in their amino acid sequences, the common characteristic of which is a core of hydrophobic residues.

In eukaryotic cells most single-spanning membrane proteins have their COOH terminus exposed cytoplasmically. This orientation is generated by the sequential insertion of an NH_2-terminal, cleavable signal sequence and a stop transfer (ST)[1] sequence. Alternatively, these functions are combined into one sequence that mediates both insertion into and anchoring within the membrane of the endoplasmic reticulum (ER). Such signal anchor (SA) sequences are generally not cleaved. Proteins containing a SA sequence can have their COOH terminus on the cytoplasmic side as in the influenza virus M_2 (Lamb *et al.*, 1985) and NB glycoprotein (Williams and Lamb, 1986) or, more commonly, have this terminus translocated into the lumen of the ER. The mechanisms by which these opposite topologies are generated have not been fully resolved, but it is clear that changes in the charged residues flanking the hydrophobic segment can influence the orientation (Haeuptle *et al.*, 1989; Monier *et al.*, 1988; Szczesna-Skorupa *et al.*, 1988; Szczesna-Skorupa and Kemper, 1989; Parks and Lamb, 1991; Beltzer *et al.*, 1991).

Similar topological elements seem to direct the membrane assembly of complex membrane proteins. Because cleaved NH_2-terminal signal sequences are rare among multispanning proteins (Laude *et al.*, 1987; Kapke *et al.*, 1988; Vennema *et al.*, 1991), they use internally located hydrophobic domains exerting SA and ST functions to achieve their disposition in the membrane. It has been proposed by Blobel (1980) and by Friedlander and Blobel (1985) that these signals act alternately and sequentially, implying that the first SA sequence determines the topology of the protein. The orientation with which the first hydrophobic domain is inserted into the membrane predestines the functioning of all the following ones as SA or as ST sequences. Any orientational preference of these domains would thus seem irrelevant.

Several studies using engineered proteins containing various combinations of topogenic sequences (Rothman *et al.*, 1988; Lipp *et al.*, 1989) or tandem repeated SA sequence (Wessels and Spiess, 1988) support the sequential insertion model. They demonstrate that such fusion proteins can assume the multispanning structures predicted, that the alternating SA and ST sequences function sequentially in time (Wessels and Spiess, 1988; Lipp *et al.*, 1989), and that signal recognition particle is involved only in the insertion of the first topogenic signal (Wessels and Spiess, 1988). The hydrophobic signals used in these studies were, however, not derived from multispanning proteins. When six of the seven hydrophobic segments of bovine opsin were constructed between reporter sequences, five of them appeared to express signal anchor features including all four segments that are supposed to function as such in the wild-type protein (Friedlander and Blobel, 1985; Audigier *et al.*, 1987). Except for the first of these natural SA sequences, they all adopted an orientation in the membrane opposite to that in wild-type opsin.

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[1] The abbreviations used are: ST, stop transfer; ER, endoplasmic reticulum; SA, signal anchor; MHV, mouse hepatitis virus; MDCK, Madin-Darby canine kidney; FIPV, feline infectious peritonitis virus; MEM, minimal essential medium; FCS, fetal calf serum.

The M protein (previously called E1) of mouse hepatitis virus strain A59 (MHV-A59) is a triple-spanning membrane protein (Armstrong *et al.*, 1984; Rottier *et al.*, 1984, 1986). Membrane insertion occurs by means of an uncleaved, internal signal sequence and requires a signal recognition particle (Rottier *et al.*, 1985). The assembled protein has its NH_2 terminus translocated to the lumen of intracellular membranes and its COOH terminus exposed in the cytoplasm ($N_{exo}C_{cyt}$). Because of its relative simplicity the M protein is an attractive model for studying the biogenesis of multispanning membrane proteins. Taking this natural protein as the starting material we carried out a mutational analysis by sequentially deleting regions potentially involved in topogenesis and investigating the topology of the mutant proteins *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells—COS-1, MDCK-II, and HeLa cells were maintained in Dulbecco's MEM containing 5% FCS.

Antibodies—The preparation of the polyclonal MHV-A59 antiserum has been described (Rottier *et al.*, 1981). A rabbit antiserum was raised to a synthetic peptide corresponding to the COOH-terminal 18 amino acids of the MHV-A59 M protein which was coupled to bovine serum albumin. Two rabbits were immunized with 3 mg of conjugate emulsified in complete Freund's adjuvant, giving 25 intradermal 50-μl injections. Rabbits were boosted similarly three times over a period of 3.5 months with 2.5 mg of conjugate emulsified in incomplete Freund's adjuvant. The animals were bled 3 weeks later, and serum was collected and stored at −20 °C. A monoclonal antiserum recognizing the amino-terminal part of the M protein was a kind gift from Drs. S. Stohlman and J. Fleming (Fleming *et al.*, 1989). Ascites from a cat infected with the feline infectious peritonitis virus (FIPV, Vennema *et al.*, 1990) was used as a source of antibodies to the FIPV-6b protein.

Oligonucleotide-directed Mutagenesis—The M gene was excised with *Hin*dIII and *Eco*RI from the vector pT3/T7-18 and was ligated into the transcription vector pTZ19R (a kind gift from Dr. D. Mead (Mead *et al.*, 1986)) cut with the same enzymes. To generate the deletion mutants, oligonucleotide-directed mutagenesis was performed with single-stranded phagemid DNA, obtained from this construct, by the method of Zoller and Smith (1982). Template DNA was produced from the pTZ vector with the helper phage M13K07 (a kind gift from Dr. D. Mead). The oligonucleotides used to generate the mutant proteins were as follows.

Mutant ΔN: 5′-ATGAGTAGTACTACTCAGCTGAAGGAATGGAACTTC-3′.

Mutant ΔC: 5′-GGTGGAGCTTCAACCCCGGGGTTAGCGGTTTTGCTG-3′.

Mutant Δa: 5′-GTTCAATTCCTTAAGGAAATGTTTATTTATGTTGTG-3′.

Mutant Δb: 5′-CACGAGCCGTAGCATGAATAATGTGTATCTTGG-3′.

Mutant Δc: 5′-GTATGCGCTAAATAATGTGAGCATAAGGTTGTTTATC-3′.

Mutant Δ(a+b): 5′-GTTCAATTCCTTAAGGAAGTGTATCTTGGATTTTCT-3′.

Mutant Δ(b+c): 5′-CCTTATGCTATTAACAAAACGGCTCGTGTAACCG-3′.

Mutant ΔaΔc was derived from mutant Δa by performing another round of mutation with the oligonucleotide used for the preparation of mutant Δc. Primer-extended DNA was transfected onto competent *Escherichia coli* NM522 cells. Mutants were identified by differential hybridization to the corresponding 5′-^{32}P-labeled oligonucleotide. Mutations were confirmed by DNA sequencing using the chain terminator method (Sänger *et al.*, 1980).

Preparation of Recombinant Vaccinia Viruses—Wild-type M and mutant M genes were excised from the pTZ19R expression vector with *Bam*HI. A *Bgl*II linker was cloned into the unique *Sma*I site of the vaccinia virus transfer vector pSC11 (Chakrabarti *et al.*, 1985) creating a unique site, into which the *Bam*HI fragments were cloned. Recombinant vaccinia viruses expressing the wild-type or mutant M proteins were prepared as described (Machamer and Rose, 1987).

In Vitro Transcription, Translation, and Membrane Integration; Alkaline Extraction; Protease Protection Assays—Transcription reactions were carried out using T7 RNA polymerase (Bethesda Research Laboratories) according to the manufacturer's instructions in 50- or 100-μl volumes containing 1 or 2 μg of *Eco*RI-linearized transcription plasmid, respectively. After a 1-h incubation template DNA was degraded for 10 min at 37 °C with RQ1 DNase (30 units/ml, Promega Corp., Madison, WI). Samples were put on ice, and EDTA and yeast tRNA were added to final concentrations of 10 mM and 20 μg/ml, respectively. RNA was isolated by phenol extraction and ethanol precipitation. Dried pellets were dissolved in half the volume of the original transcription reaction of 10 mM Tris-Cl (pH 7.4) containing 0.1 mM EDTA. Translations of the mRNAs (0.75 μl of mRNA/10-μl reaction) were done for 1 h at 30 °C in the Amersham reticulocyte lysate N.90Z in the presence of dog pancreas microsomes (a kind gift from Dr. D. I. Meyer, UCLA). To assay for membrane integration, translation reactions (10 μl) were mixed on ice with an equal volume of 0.2 M Na_2CO_3 (pH 11.5) and left on ice for at least 15 min. The samples were then layered over a sucrose step gradient (80 μl of 0.2 M on top of 20 μl of 2 M sucrose in 2 mM $MgAc_2$ and 130 mM KAc adjusted to pH 11.5 with NaOH) in tubes of the Beckman Airfuge and spun for 10 min at 25 p.s.i. and 4 °C. The upper 90 μl of the supernatant was separated from the rest ("pellet"), and the samples were diluted to 1 ml with detergent solution (50 mM Tris-Cl (pH 8.0), 62.5 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40) containing 2 mM phenylmethylsulfonyl fluoride, and 40 μg/ml aprotinin (Sigma). SDS was added to 0.2% followed by 2.5 μl of the rabbit antiserum to the carboxyl terminus of MHV-A59 M protein. After overnight incubation at 4 °C immune complexes were collected using 20 μl of a 10% suspension of *Staphylococcus aureus* (Pansorbin, Calbiochem), and after an incubation of 30 min, they were washed three times with RIPA buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Nonidet P-40) before analysis in a 20% polyacrylamide gel.

Protease protection experiments were carried out essentially as described before (Rottier *et al.*, 1984). Briefly, samples of translation reactions were diluted with 0.5 volume of proteinase K solution (1 mg/ml) and incubated in the absence or in the presence of 0.05% saponin for 1 h at room temperature. Samples were put on ice, and reactions were stopped by adding excess phenylmethylsulfonyl fluoride (final concentration 2.5 mg/ml).

Aliquots were then taken for direct analysis or for immunoprecipitation as described above.

Glycosylation Assay, in Vivo Labeling—COS-1 cells (2×10^5) plated in 16-mm dishes were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 in phosphate-buffered saline for 45 min at room temperature. Cells were incubated under Dulbecco's MEM, 5% FCS until 4.5 h postinfection, when the medium was replaced by 0.15 ml of methionine-free MEM, 2% FCS (GIBCO, Life Technologies Ltd., Paisley, Scotland) to which, when indicated, brefeldin A (6 μg/ml; a generous gift from Sandoz Ltd., Basel, Switzerland) was added. Cells were pulse labeled at 5 h postinfection for 15 min at 37 °C with 50 μCi of [^{35}S]methionine (Amersham International, Buckinghamshire, U. K.) and were either put on ice immediately or chased for 60 min in Dulbecco's MEM, 5% FCS containing 2 mM methionine. Cells were lysed on ice in 0.2 ml of lysis buffer (50 mM Tris-Cl (pH 8.0), 62.5 mM EDTA, 1% Triton X-114) containing 2 mM phenylmethylsulfonyl fluoride and 40 μg/ml aprotinin. Nuclei were spun down at 10,000 rpm for 1 min, and M proteins were immunoprecipitated as described above using the polyclonal MHV-A59 antiserum and analyzed in a 15% polyacrylamide gel.

Indirect Immunofluorescence Microscopy on Semi-intact Cells—MDCK-II cells grown to confluence on 12-mm, polylysine-coated coverslips were infected at a multiplicity of infection of 10 with recombinant vaccinia viruses and permeabilized at 16 h postinfection using the nitrocellulose method as described by Simons and Virta (1987). Cytoplasmically exposed domains were assayed by incubating the monolayers for 30 min at room temperature with either the peptide antiserum (diluted 1:150) or with the amino terminus-specific monoclonal antibody (diluted 1:40) in phosphate-buffered saline containing 8 mM $MgCl_2$, 5 mM $CaCl_2$, and 5% FCS. Cells were rinsed with phosphate-buffered saline and stained with either an affinity-purified fluorescein-conjugated goat anti-rabbit IgG (1:150) or a goat anti-mouse IgG (1:40, Kallestad Laboratories Inc., Austin, TX), both diluted in phosphate-buffered saline with $MgCl_2$, $CaCl_2$, and FCS. Cells were fixed with 3% paraformaldehyde, coverslips were mounted in 90% glycerol, 10 mM Tris-Cl (pH 8.6), containing 25 mg/ml (1,4-diazabicyclo[2.2.2.]octane, Sigma), and fluorescence was viewed using an Olympus BHS-F microscope.

Alkaline Extraction of Cellular Membranes—Confluent monolayers of COS-1 cells in 60-mm dishes were infected with recombinant

vaccinia viruses at a multiplicity of infection of 10. The 6b protein of FIPV was expressed by the double infection procedure described by Fuerst *et al.* (1986) using vaccinia virus recombinants expressing the T7 polymerase gene and the 6b gene cloned behind the T7 promoter, (Vennema *et al.*, 1992) respectively. Cells were labeled with 100 μCi of [^{35}S]methionine in methionine-free MEM, 2% FCS from 4 to 6 h postinfection, except for the 6b protein which was labeled from 6 to 7 h postinfection with 200 μCi of [^{35}S]cysteine (Amersham) in cysteine-free MEM, 2% FCS. Cells were rinsed once with ice-cold TES buffer (20 mM Tris-Cl (pH 7.4), 1 mM EDTA, 100 mM NaCl) containing aprotinin, leupeptin (both at 1 μg/ml), and phenylmethylsulfonyl fluoride (2 mM). The protease inhibitors were included in all subsequent steps. Cells were kept on ice, rinsed once with 1:10 TES, and scraped from the dishes in 1:10 TES. Cells were spun down for 5 min at 2000 rpm, the pellets were resuspended in 0.5 ml 1:10 TES. The cells were broken by 10 strokes in a Dounce homogenizer, and the nuclei and cell debris were removed by centrifugation for 10 min at 2,000 rpm. Samples were treated for 15 min on ice either at pH 11.5 by adding an equal volume of 0.2 M Na_2CO_3 or at pH 7.4 by the addition of the same amount of 1:10 TES. Microsomal and cytoplasmic fractions were separated by centrifugation for 30 min at 150,000 × *g* and 4 °C. Proteins were immunoprecipitated from supernatant and pellet fractions with the MHV-A59 antiserum or with the ascites fluid from an FIPV-infected cat as described by Vennema *et al.* (1990) and prepared for polyacrylamide gel electrophoresis.

RESULTS

Generation of Mutant Proteins—To assess the role in membrane integration of the different domains of the M protein, precise deletions were made by oligonucleotide-directed mutagenesis of the M gene. As depicted in Fig. 1*B*, a collection of mutant proteins was generated lacking either one or two transmembrane domains of which most of the hydrophilic NH_2-terminal region or a large part of the amphiphilic COOH-terminal region was deleted.

Since they were constructed in the transcription vector pTZ19R the mutant genes could be expressed directly *in vitro* by translation of the mRNAs transcribed by T7 polymerase.

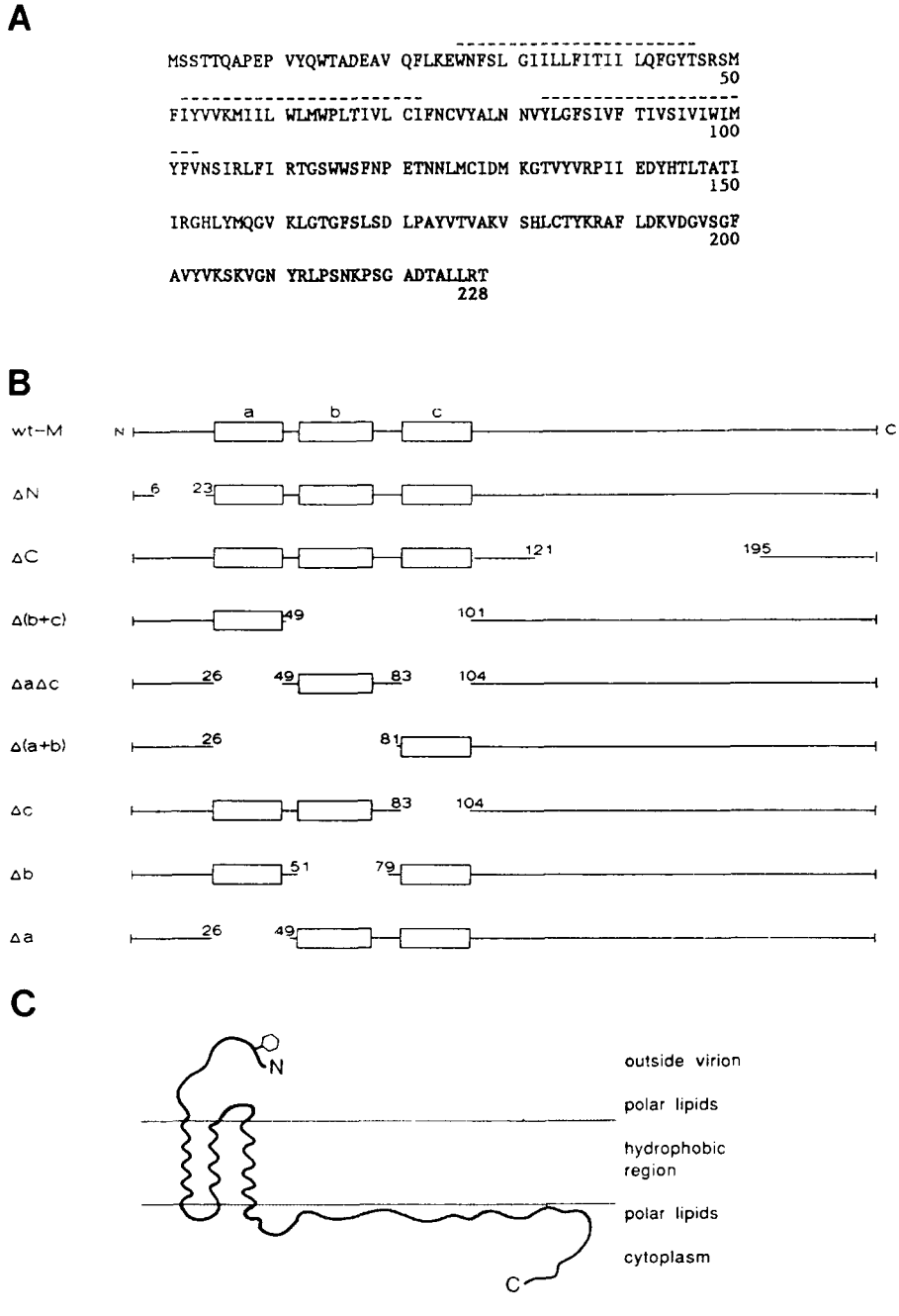

FIG. 1. *Panel A*, amino acid sequence of the M protein of MHV-A59. The *dotted lines* over the sequence indicate the predicted transmembrane domains (Rottier *et al.*, 1986). *Panel B*, schematic representation of the M deletion mutants. The *boxes* represent the transmembrane domains referred to as a, b, and c for the first, second, and third domains, respectively. The *numbers* above the *interrupted lines* indicate the first and last amino acids of the deleted part. *Panel C*, proposed disposition of the M protein in the membrane (Rottier *et al.*, 1986).

In addition, the genes were recombined into vaccinia virus for expression in eukaryotic cells.

M Proteins Lacking One or Two Hydrophobic Domains Can Still Integrate in Vitro—The effect of the deletions on the capacity the mutant proteins to integrate in membranes was investigated by translation of *in vitro* transcribed M gene mRNAs in a reticulocyte lysate in the presence of dog pancreatic rough microsomes. Membrane integration was assayed by alkaline extraction. Treatment of the membrane vesicles at pH 11.5 disrupts the vesicles, releases their content, and extracts peripheral membrane proteins. Integral membrane proteins can be collected by spinning down the membranes. As shown in Fig. 2A (*left lanes*), both the wild-type M and the mutant proteins are efficiently inserted since in each case the majority of the products cosediments with the membranes. Clearly, deletion of most of the NH_2-terminal residues (mutant ΔN) or of a major part of the COOH-terminal domain (mutant ΔC) did not affect the ability to be inserted. The proteins having only one hydrophobic domain also sedimented with the membranes. Apparently each one of these domains was individually sufficient and able to direct insertion and anchoring of the protein.

We have shown earlier that the wild-type M inserts into membranes cotranslationally (Rottier *et al.*, 1984, 1985). Indeed, the addition of microsomes to the translation mixture post-translationally, after blocking synthesis with 0.2 mM cycloheximide, showed only a background amount of sedimentable M (Fig. 2*A*, *right lanes*). The same results were obtained with the mutant proteins. Apparently, the signals for insertion and anchoring only functioned in a cotranslational manner, post-translational insertion occurring to an insignificant extent, if at all. The postsynthesis incubation gave rise to some variable degradation of the proteins. The mutant ΔaΔc was most prone to degradation under all conditions consonant with its short half-life *in vivo* (see below).

Membrane Topology of in Vitro Assembled Proteins ΔC, ΔN, and ΔaΔc—For the analysis of the topology of the mutant proteins we used antibodies specifically recognizing either the extreme NH_2 terminus or the COOH terminus of M. An antiserum to the COOH terminus was raised in rabbits with a synthetic peptide corresponding to the 18 carboxyl-terminal amino acids of the M protein (see "Materials and Methods"). The monoclonal antibody J 1.3 developed by Fleming *et al.* (1989) neutralizes MHV in the presence of complement and is NH_2 terminus-specific.

As shown in Fig. 2*B* both antibodies precipitated the wild-type M protein synthesized in a reticulocyte lysate in the presence of rough microsomes (*lanes 4* and *5*).

When treated with proteinase K, which is known to remove the COOH terminus of the integrated protein (Rottier *et al.*, 1984), only the NH_2 terminus-specific antibodies precipitated the remaining M polypeptide (*lanes 6* and *7*). Digestion from both sides of the membrane by including 0.05% saponin also abolished recognition by the monoclonal antibody (*lane 8*).

When such analyses were done with mutant proteins, identical results were obtained with mutants ΔC and ΔN, indicating that they assembled in the membranes as the wild-type protein. Apparently, the deleted parts of these two proteins

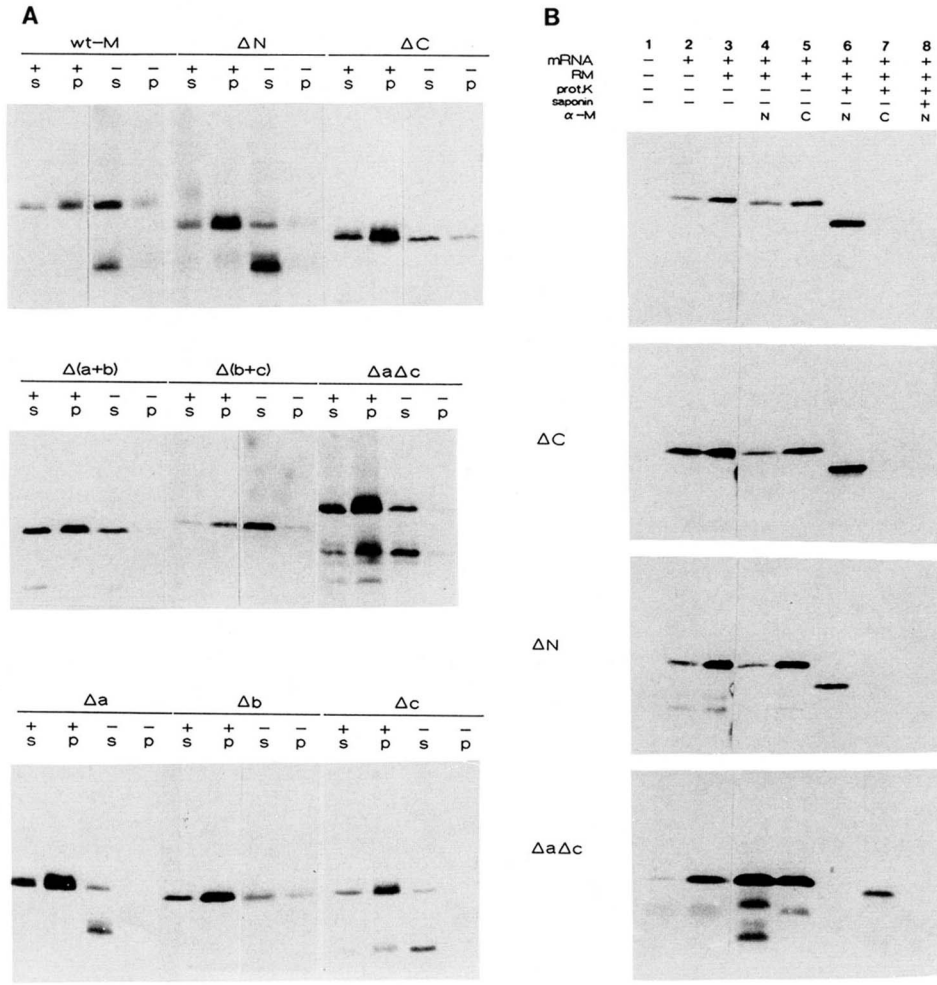

FIG. 2. *Panel A, in vitro* membrane integration of the M mutants. Translations of *in vitro* transcribed mRNAs were done either in the absence (−) or presence (+) of microsomal membranes. To assay for post-translational membrane integration, the latter translations were stopped by adding 0.2 mM cycloheximide, then microsomes were added and the incubation was continued for 45 min at 37 °C before it was stopped on ice. Samples were treated at pH 11.5 for 15 min on ice and centrifuged through an alkaline sucrose gradient. Supernatant (*s*) and pellet (*p*) fractions were separated, M proteins were immunoprecipitated and analyzed in a 20% SDS-polyacrylamide gel. *wt*, wild-type. *Panel B*, topology of the wild-type M and the mutants ΔC, ΔN, and ΔaΔc *in vitro*. mRNAs were translated in the absence (−) or presence (+) of rough microsomes. The latter were treated (+) or mock treated (−) with proteinase K (0.33 mg/ml) in the absence (−) or presence (+) of 0.05% saponin. Aliquots of reactions were subjected to immunoprecipitation with the COOH terminus-specific (*C*) or NH_2 terminus-specific (*N*) antibodies. Samples were analyzed in a 20% SDS-polyacrylamide gel.

are not involved in the topogenesis of M. In contrast, mutant ΔaΔc appeared to attain an orientation opposite to that of wild-type M. Protease treatment showed the NH_2 terminus of the polypeptide to be exposed and the COOH terminus to be protected when the membrane vesicles were intact. Similar experiments with the other mutants resulted in complex degradation patterns that could not unequivocally be interpreted. Their orientation and membrane insertion were therefore analyzed *in vivo* after expression of the mutant genes in cells.

Glycosylation as a Biochemical Marker for the Disposition of the NH_2 Terminus—In MHV-infected cells (Rottier *et al.*, 1984) and when expressed from cloned cDNA (Rottier and Rose, 1987) the M protein is glycosylated at the NH_2 terminus. As the glycosyltransferases are located in the lumina of the internal membranes, the acquisition of oligosaccharides indicates that the NH_2 terminus has been translocated to the luminal side. The M protein of MHV-A59, however, is *O*-glycosylated, an event which, in contrast to *N*-glycosylation, occurs post-translationally after the protein has left the rough ER. Since preliminary experiments showed that some of the mutants were not transported but stayed in the ER region, an assay based on *O*-glycosylation would not be conclusive. To circumvent this problem we made use of the drug brefeldin A. Brefeldin A, a fungal metabolite, has been shown to block transport of proteins from the ER but not retrograde transport. The drug causes the cis-, medial-, and trans-Golgi compartments to redistribute to the ER and to relocate the enzymes residing in the Golgi stack into the ER. These Golgi-derived enzymes can now act upon accumulated and resident ER proteins (Chege and Pfeffer, 1990; Lippincott-Schwartz *et al.*, 1989; Doms *et al.*, 1989). The effect of brefeldin A on the glycosylation of the wild-type M protein was determined in COS-1 cells expressing the protein from a recombinant vaccinia virus vector. As is shown in Fig. 3 (*panel A*), in the absence of the drug hardly any glycosylation occurred during the 15-min pulse labeling; glycosylation became apparent during the chase, giving rise to several glycosylated forms, while some nonglycosylated protein also remained. In the presence of the drug oligosaccharide addition started already during the pulse period, resulting in several intermediate forms. During the chase the protein is completely converted into one glycosylated form, which comigrates with the M_3 species made under drug-free conditions (Fig. 3, *panel A*). Apparently the relocation of the enzymes for *O*-glycosylation by brefeldin A leads to a much faster and more efficient glycosylation of the protein. The absence of the upper form M_4 in the presence of the drug probably indicates that this latter modification occurs in the trans-Golgi network (Krijnse Locker *et al.*, 1992). Next the mutant M proteins with deletions in the hydrophobic region were subjected to a similar pulse-chase experiment. When taking the unglycosylated form synthesized during a pulse without brefeldin A as a reference, it is clear from Fig. 3*B* that all of the mutants except for ΔaΔc and Δa became glycosylated. These latter proteins apparently do not translocate their NH_2-terminal domain across the ER membrane, which for the mutant ΔaΔc was also shown by the *in vitro* protease protection assay (Fig. 2*B*). Interestingly, glycosylation of the mutant Δb was incomplete. Only about half of the protein acquired oligosaccharides during the 60-min chase. Even after prolonged chase times for up to 5 h this distribution did not change (data not shown). We conclude that this mutant Δb can assume two topologies in which the NH_2 terminus is either translocated or not.

Indirect Immunofluorescence on Semi-intact Cells—Recently several procedures have been developed to permeabilize plasma membranes of culture cells while preserving the integrity and functionality of internal membranes (Beckers *et al.*, 1987; Simons and Virta, 1987; Gravotta *et al.*, 1990; Miller and Moore, 1991). One of these uses nitrocellulose to mechanically disrupt the plasma membranes of MDCK cells allowing large molecules, such as antibodies, to diffuse into the cells (Simons and Virta, 1987). We have used this permeabilization technique to set up an *in vivo* topology assay with the domain-specific antibodies. Wild-type and mutant M proteins were expressed in MDCK-II monolayers grown on coverslips. The cells were filter-stripped, and domains exposed in the cytoplasm were probed with the antibodies. As Fig. 4 shows, the wild-type M protein reacted in this assay as its known topology predicts: no immunofluorescence was observed with the NH_2 terminus-specific monoclonal antibody, but a clear staining was seen with the COOH terminus-specific peptide antiserum. When similar experiments were performed with the

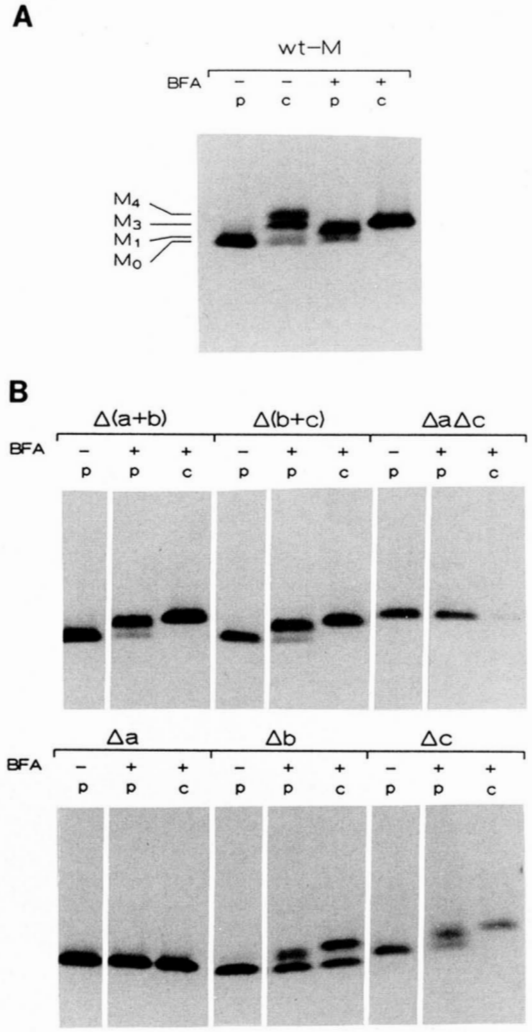

FIG. 3. **Glycosylation of the wild-type M (*wt-M*) protein (*panel A*) and mutant M proteins (*panel B*) in the absence or presence of brefeldin A.** *Panel A*, COS-1 cells infected with a recombinant vaccinia virus expressing the wild-type M protein were pulse labeled (*p*) at 5 h postinfection for 15 min in the absence (–) or presence (+) of 6 μg/ml brefeldin A and were subsequently chased (*c*) for 60 min with or without the drug. M_1 through M_4 designate the different glycosylated species, M_0 representing the unglycosylated form. *Panel B*, the mutant M proteins were analyzed in an identical experiment; the proteins were pulse labeled (*p*) with or without the drug and chased (*c*) in the presence of brefeldin A. The pulse-labeled untreated samples (*p*, –) are shown to indicate the position of the nonglycosylated form.

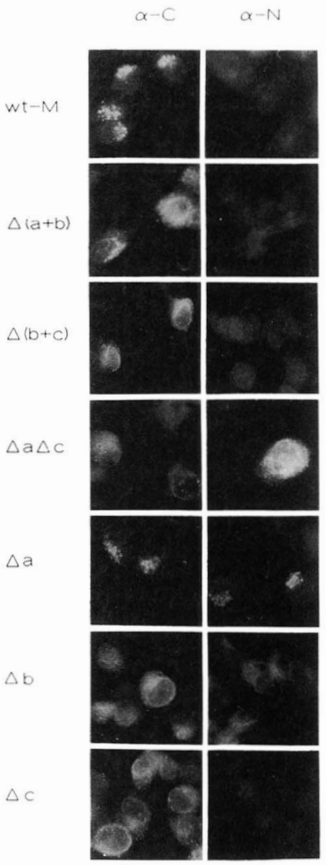

FIG. 4. **Determination of the cytoplasmically exposed domains by indirect immunofluorescence on semi-intact cells.** Confluent monolayers of MDCK-II cells grown on coverslips were infected with recombinant vaccinia viruses. The cells were permeabilized using nitrocellulose at 16 h postinfection. Domains exposed in the cytoplasm were detected by indirect immunofluorescence using the peptide-serum (α-*C*) and the monoclonal antibody (α-*N*) followed by fluorescein-conjugated goat anti-rabbit or anti-mouse IgG, respectively.

mutant proteins, ΔaΔc, Δa, and Δb appeared to stain with the monoclonal antibody, which is in agreement with the results of the glycosylation assay which showed that all but these three mutants had their NH_2 terminus located luminally. Staining with the COOH terminus-specific serum appeared to be positive with each mutant. This result was unexpected for two proteins, namely Δc and ΔaΔc. The latter mutant had been found in the *in vitro* assay to have its COOH terminus on the luminal side of the rough microsomes. The observations suggest that the translocation of the COOH-terminal domain following the hydrophobic region occurs inefficiently because of unknown structural constraints.

Membrane Association of the Mutant ΔaΔc in Vivo—To ascertain that the equivocal data of the mutant ΔaΔc did not result from its inability to insert in membranes *in vivo*, we analyzed its nature of membrane association by alkaline extraction (Fujiki *et al.*, 1982). Wild-type M, mutant ΔaΔc, and the 6b protein of FIPV, another coronavirus, were expressed in COS-1 cells. The 6b protein is a secreted glycoprotein (Vennema *et al.*, 1992), which was used as a control. After labeling with [^{35}S]methionine or [^{35}S]cysteine (the 6b protein) the cells were Dounce-homogenized and adjusted to neutral or high pH followed by centrifugation at 150,000 × *g* to separate supernatant from pellet fractions. At neutral pH integral, peripherally associated and soluble proteins (that are trapped within the vesicles) will end up in the pellet or membrane-containing fractions. At pH 11.5, however, membranes are disrupted without solubilizing them. Under these conditions only the integrated proteins sediment with the membrane sheets, while the peripherally associated and soluble proteins are released into the supernatant. Fig. 5 shows the SDS-polyacrylamide gel electrophoresis of the radiolabeled and immunoprecipitated viral proteins. The 6b protein behaved like a soluble protein; it sedimented with the membranes at neutral pH and was released at high pH (Fig. 5). The mutant ΔaΔc exhibited the same sedimentation pattern as the wild-type M protein. It was found in the pellet fraction even at high pH, a condition which gave rise to some loss of signal probably because of a decreased recognition by the antibodies. The protein was never detected in the supernatant fractions even after prolonged exposure of the autoradiographs (data not shown). This demonstrates that the protein is strongly anchored in the membranes of the cell. An identical experiment was done with the other mutants and showed these, like in the *in vitro* assay (Fig. 2*A*), also to be stably integrated in membranes (data not shown).

DISCUSSION

In this study we describe a systematic analysis of the membrane integration of a naturally occurring eukaryotic multispanning protein. Potentially topogenic regions of the triple-spanning coronavirus M protein were deleted, and the integration of the mutant proteins was assayed. The orientation of the M proteins was studied *in vitro* in protease protection experiments as well as *in vivo* by immunofluorescence on semi-intact cells and using glycosylation as an indicator of the disposition of the NH_2 terminus. The results obtained from these assays are summarized in Fig. 6.

Our data show that each of the three hydrophobic domains of the M protein can function individually as a signal anchor sequence. When two of the three transmembrane domains were removed the mutant proteins were still able to integrate stably into membranes, both *in vitro* and *in vivo*. These observations support the notion that a defined stretch of hydrophobic residues is sufficient to insert and anchor a protein in a lipid bilayer and that individual transmembrane domains of polytopic proteins fulfill this requirement (Friedlander and Blobel, 1985; Audigier *et al.*, 1987). Our approach consisted of deleting systematically one or two hydrophobic

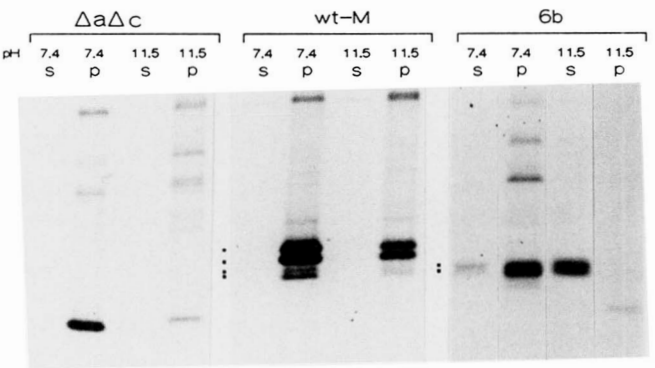

FIG. 5. **Characterization of the membrane association of the mutant ΔaΔc *in vivo*.** COS-1 cells were infected to express wild-type M (*wt-M*) (a membrane-integrated protein), the 6b protein of FIPV (a secreted glycoprotein), and the mutant ΔaΔc. Cells were labeled, Dounce-homogenized, adjusted to pH 7.4 or pH 11.5, and separated by centrifugation at 150,000 × *g* into supernatant (*s*) and pellet (*p*) fractions. The radiolabeled proteins were immunoprecipitated with the MHV-A59 antiserum or with ascites from an FIPV-infected cat. The positions of the proteins and their glycosylated forms are indicated by *small dots*.

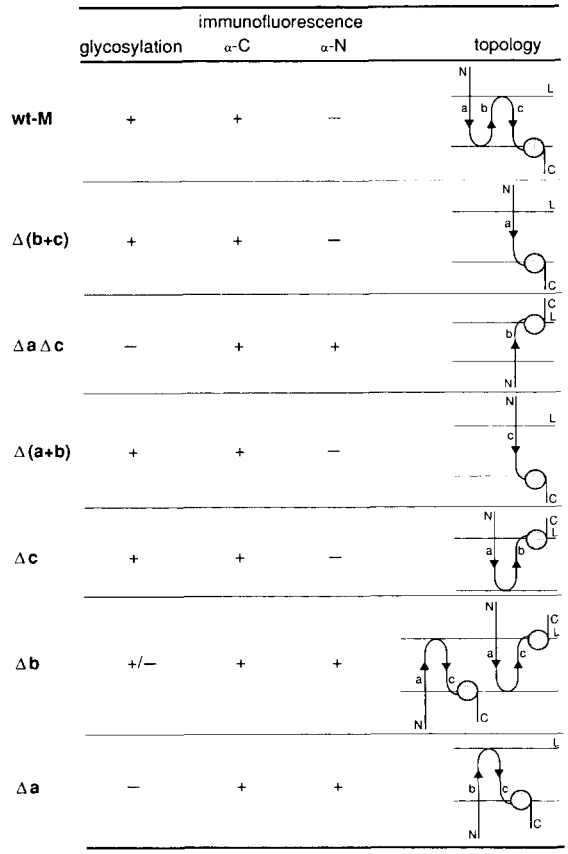

FIG. 6. **Overview of the *in vivo* results and proposed topologies of the mutant proteins.** *Arrowheads*, pointing toward the COOH terminus of the protein, indicate the direction of the hydrophobic transmembrane domains in the membrane. The luminal side is symbolized by an *L*, the *circle* represents the amphiphilic domain in the COOH terminal half of M.

domains generating mutants having the original NH_2 and COOH terminus. Interestingly, these mutants did not assume the same topologies. Mutants having only the first or the third hydrophobic domain assembled in a N-out/C-in orientation, but when only the second transmembrane domain was present it did acquire the opposite orientation. These findings suggest that each hydrophobic region integrates in a preferred orientation that corresponds to its orientation in the wild-type protein. This inference is supported by the orientations of the mutants having two hydrophobic domains. Mutants Δa and Δc appear to integrate according to the preferences of the individual hydrophobic domains. For mutant Δb, however, two different topologies were observed presumably because in this protein both hydrophobic domains have the same preferred orientation in the membrane.

The topologies we observed are in agreement with those of previously described coronavirus M proteins. Work by Machamer and Rose (1987), Mayer *et al.* (1988), and Armstrong *et al.* (1990) showed that mutant M proteins of infectious bronchitis virus and MHV-A59 containing either the first or the third hydrophobic domain inserted into membranes with their NH_2 terminus facing the lumen. In addition, Mayer *et al.* (1988) concluded that the NH_2-terminal 28 amino acids of the MHV M protein were not essential for assembly in the wild-type orientation. Interestingly, in some coronavirus M proteins translocation of the NH_2 terminus is mediated by the presence of a cleaved terminal signal sequence (Kapke *et al.*, 1988; Laude *et al.*, 1987; Vennema *et al.*, 1991). This additional signal is probably required since the mature NH_2-terminal domains in these proteins are longer and carry more charged residues.

Statistical studies of known primary sequences of membrane proteins have demonstrated that charged residues in the regions flanking SA sequences are major determinants of transmembrane orientation. The "positive-inside" rule proposed by von Heijne and Gavel (1988) states that regions facing the cytoplasm are generally enriched in positively charged residues whereas translocated regions are largely devoid of these residues. Mutational studies clearly support the importance of positive charges in topogenesis (Szczesna-Skorupa *et al.*, 1988; Szczesna-Skorupa and Kemper, 1989; Boyd and Beckwith, 1989; Nilsson and von Heijne, 1990). Using the relative net charge of the hydrophilic sequences at either end of the hydrophobic stretch Hartmann *et al.* (1989) developed the "charge-difference" rule. It predicts the orientation of the first SA sequence in eukaryotic transmembrane proteins from the difference in charge between the 15 COOH-terminal and NH_2-terminal flanking amino acids.

Although four of our M proteins including the wild-type molecule obey both rules, the orientations of three mutants (Δa, ΔaΔc, and Δb) clearly cannot be explained by either rule. Whereas the value of Δ(C-N) for the SA sequences of these mutants is positive (+1, +3, and +3, respectively), as it is for all the other M mutants, the proteins nevertheless adopt a $N_{cyt}C_{exo}$ orientation. Exceptions to the rules have been noted for a number of other integral membrane proteins (Hartmann *et al.*, 1989; Wilson *et al.*, 1990; Beltzer *et al.*, 1991; Parks and Lamb, 1991). Moreover, calculations for the single-spanning mutants of bovine opsin (Audigier *et al.*, 1987) show that in this case two of the three relevant mutants are also in conflict with predictions based on charges. These examples indicate that charged residues may not be the sole determinant in transmembrane orientation. Based on our observations we favor the idea that the hydrophobic core also contributes to the directionality of the integrating protein. How this is achieved is yet unclear.

An unexpected result of the immunofluorescence analyses was that the expressed mutants ΔaΔc and Δc reacted with the COOH terminus-specific antiserum. As the observations with the NH_2 terminus-specific antibodies in this assay were fully consistent with the results of the glycosylation assay for all the M proteins tested, we do not consider this an artifact of the method. The apparent cytoplasmic exposure of the COOH terminus of these mutant proteins can be explained in several ways. First, these proteins might simply be targeted very inefficiently to the ER membrane, leaving a substantial fraction nonintegrated. For the mutant ΔaΔc this possibility seems unlikely since we could demonstrate that the protein remained quantitatively membrane-associated at alkaline pH (Fig. 5). Second, the COOH-terminal domain in these two mutants might be inefficiently translocated because of features of the b transmembrane domain. The presence of a proline residue in the middle of this segment might represent such a feature. Third, difficulty in translocation could also be caused by the nature of the COOH-terminal domain itself. This domain is strongly protease-resistant (Rottier *et al.*, 1984; Mayer *et al.*, 1988), which has been ascribed to its folding in a very compact structure or to a close association with the polar surface of the membrane (Armstrong *et al.*, 1984; Rottier *et al.*, 1986). An interaction with the negatively charged phospholipid head groups of the bilayer can be envisioned since this domain contains 18 positively charged amino acid residues. Interestingly, an M mutant lacking all three hydrophobic regions has been shown by Mayer *et al.* (1988) to be tightly associated with membranes *in vitro*. Thus, if the

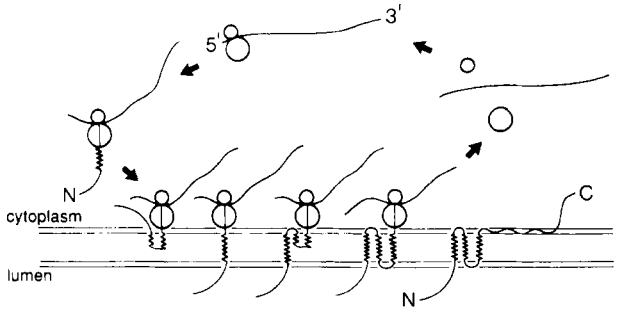

FIG. 7. **Proposed model of membrane insertion of the M protein.**

kinetics of translation and translocation would allow the COOH-terminal domain of mutants ΔaΔc and Δc to fold or to interact with the ER membrane, it might become translocation-incompetent. Finally, a fourth possible mechanism to explain the cytoplasmic exposure of the COOH terminus is that its translocation is prevented by the occurrence of a cryptic ST signal. Although the COOH-terminal half of the M protein does not contain any hydrophobic stretches likely to function as such a signal, it is still conceivable that a sequence occurs which is recognized by the translocation machinery and which leads to the abortion of protein transfer across the membrane. Apparently, such a sequence is not contained in the region spanning residues 133–207 inasmuch as deletion of this region in the mutant ΔaΔc did not change the characteristics of the protein (data not shown). Presently, we cannot distinguish among the different possibilities. It is interesting to note that similar considerations have been made to explain the release of a cleaved form of the hepatitis B virus major core protein into the cytoplasm (Garcia *et al.*, 1988).

Our data lead us to a model describing the membrane assembly of the coronavirus M protein as depicted in Fig. 7. According to this model, translation is arrested by the appearance of the first hydrophobic domain from the ribosome through the interaction with signal recognition particle (Rottier *et al.*, 1985). After transfer of the internal signal sequence to the receptor in the ER membrane the signal is inserted into the membrane as a hairpin. While translation resumes the NH_2 terminus is translocated to the lumen. Subsequently, the second insertion signal arises and is, again, inserted as a hairpin probably now without the involvement of signal recognition particle (Wessels and Spiess, 1988). As translation proceeds the third hydrophobic domain is inserted, concomitant with the translocation of the more hydrophilic region that separates the second and third domain. Finally, while the synthesis of the protein is finished the COOH-terminal part assumes its conformation. This model is in agreement with the sequential insertion mechanism proposed for multispanning membrane proteins (Blobel, 1980; Sabatini *et al.*, 1982; Friedlander and Blobel, 1985; Wickner and Lodish, 1985) and with data from artificial polytopic proteins (Rothman *et al.*, 1988; Wessels and Spiess, 1988; Lipp *et al.*, 1989).

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