EXPRESSION OF MHV-A59 M GLYCOPROTEIN: EFFECTS OF DELETIONS ON MEMBRANE INTEGRATION AND INTRACELLULAR TRANSPORT

P.J.M.Rottier, J.Krijnse Locker, M.C.Horzinek, and W.J.M.Spaan

State University, Veterinary Faculty
Department of Infectious Diseases and Immunology
Institute of Virology
Yalelaan 1, 3584 CL Utrecht
The Netherlands

INTRODUCTION

The M protein of coronaviruses is an integral membrane protein, the general properties of which have been described elsewhere in this volume. The protein is ascribed a pivotal role in the budding process of these viruses. Its restriction to internal membranes suggests it to be a major factor in determining the intracellular site of virion assembly. In addition, M is thought to effect budding through the interaction of its cytoplasmic tail with the nucleocapsid.

Coronaviral M is a glycoprotein carrying oligosaccharides only at its amino-terminus. For most members of the family these are of the N-glycosidic type, but in the case of mouse hepatitis virus (MHV-A59) the sugars are added to serines and/or threonines through O-linkages (1). O-glycosylation is a post-translational event; the responsible enzymes do not reside in the endoplasmic reticulum (ER) but are encountered by the protein only en route to and through the Golgi apparatus.

M is a complex-type membrane protein. In most coronaviruses it has no cleavable signal sequence, yet needs signal recognition particle (SRP) for its integration in the ER (2). Three successive membrane-spanning segments anchor the protein in the ER membrane. They separate a hydrophilic N-terminus exposed to the lumen of the organelle from a C-terminal half of the molecule facing the cytoplasm (3,4,5). From the latter only the extreme terminal domain is accessible to proteases. Exactly how the protein acquires its topology is not known but the functioning as topogenic element of one or more of the hydrophobic segments seems clear. Deletion experiments with the M protein of infectious bronchitis virus (IBV) have shown both the first and the third segment to independently act as signal-anchor sequence (6). In contrast, no indication for a role in topogenesis of the N-terminal or C-terminal domain were obtained (7).

The M proteins of MHV-A59 and IBV have been expressed in cells from cloned cDNA (6,8) as well as by microinjection of mRNA (7,9). Both proteins were found by immunofluorescence to accumulate in the Golgi region. Little is known about the signals that target membrane proteins but it is clear

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that sorting information must somehow reside in the protein's structure. With respect to coronavirus M protein one interesting study has appeared supporting this view. IBV M proteins with deletions of either the first and second or the second and third transmembrane domain were assembled in cell membranes in the same orientation as the wild-type virus protein (6). It was found that deletion of the second and third domain did not alter the mutant protein's localization to the Golgi region. In contrast, the mutant protein possessing only the third domain was efficiently transported to the plasma membrane. These data suggested that the first transmembrane domain contains a Golgi specific retention signal (6).

We are interested in the M protein of MHV-A59 for its role in virus assembly and because it enables us to address fundamental questions concerning membrane assembly and protein sorting. Using in vitro mutagenesis we have prepared a number of mutant M proteins which we are now studying with respect to membrane integration and intracellular transport. Here we present the first results obtained with some of these mutants.

MATERIALS and METHODS

Plasmid constructions. The M gene was excised with HindIII and EcoRI from the vector pT3/T7-18 in which it had been cloned (8) and was ligated into the transcription vector pTZ19R (10; a kind gift from Dr.D.Mead). To generate the deletion mutants, oligonucleotide-directed mutagenesis was performed with single-stranded phagemid DNA by the method of Zoller and Smith (11). Template DNA was produced from the pTZ vector with the helper phage M13K07 (10). The following oligonucleotides, prepared by the DNA-synthesis service of Yale Medical School (New Haven) were used:

* mutant △N : 5'-ATGAGTAGTACTACTCAGCTGAAGGAATGGAACTTC-3'
* mutant △C : 5'-GGTGGAGCTTCAACCCCGGGGTTAGCGGTTTTGCTG-3'
* mutant △(a+b) : 5'-GTTCAATTCCTTAAGGAAGTGTATCTTGGATTTTCT-3'
* mutant △(b+c) : 5'-CCTTATGCTATTAACAAAACGGCTCGTGTAACCG-3'
* mutant △a△c : 5'-GTTCAATTCCTTAAGGAAATGTTTATTTATGTTGTG-3' and 5'-GTATGCGCTAAATAATGTGAGCATAAGGTTGTTTATC-3'.

Primer-extended DNA was transfected onto competent E.Coli NM522. 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 (12). For the preparation of vaccinia virus recombinants the genes were transferred into the vector pSC11 (13). The wild-type M gene was cut out of the plasmid pJCE1 (8) with BamHI, blunted with the Klenow fragment of DNA polymerase I and ligated into SmaI-digested pSC11. For the transfer of the mutant genes a BglII-site was first introduced into the SmaI-site of pSC11 by linker ligation and the genes, taken out of the pTZ vectors using BamHI, were cloned into this new site.

Preparation of recombinant vaccinia viruses. M genes were recombined into the vaccinia virus genome by transfection of infected HeLa cells with the pSC11 plasmids and recombinants were identified as described by Machamer and Rose (6).

In vitro transcription and translation; alkaline extractions; protease digestions. Transcription reactions were carried out using T7-RNA polymerase (Bethesda Research Laboratories) according to the manufacturer's instructions in 50 or 100µl volumes containing 1 or 2 µg of EcoRI-linearized transcription plasmid, respectively. After a 1h incubation template DNA was degraded for 10 min at 37°C with RQ1 DNase (30U/ml; Promega). Then samples were put on ice and EDTA and yeast tRNA were added to a final concentration of 10mM and 20µg/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 10mM Tris-HCl (pH7.4) containing 0.1mM EDTA. Translations of the mRNAs (0.75µl

mRNA/10µl reaction) were done for 1h 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). In one experiment (Fig.1) a reticulocyte lysate and microsomes purchased from New England Nuclear were used. To assay for membrane integration, translation reactions (10µl) were mixed on ice with an equal volume of 0.2M 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 (80µl 0.2M on top of 20µl 2M sucrose in 2mM $MgAc_2$ and 130mM 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 90µl of the supernatant (s) was separated from the rest (p) and the samples were diluted to 1ml with detergent solution (50mM Tris-HCl, 62.5mM EDTA, 0.4% DOC, 1% Nonidet P-40, pH8.0) containing 2mM PMSF and 100U kallikrein inhibitor. SDS was added to 0.2% followed by 2.5µl of rabbit antiserum to the carboxy-terminus of M. After an overnight incubation at 4°C immune complexes were collected using 20µl of a 10% suspension of Staph. aureus (Pansorbin; Sigma) and washed three times with RIPA buffer (10mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% DOC, 1% Nonidet P-40, pH7.4) before analysis in a 20% polyacrylamide gel.
Protease protection experiments were carried out essentially as described before (4). Briefly, samples of translation reactions were diluted with half a volume of proteinase K solution (1mg/ml) and incubated in the absence or in the presence of 0.05% saponin for 1h at room temperature. Samples were put on ice and reactions stopped by adding excess PMSF. Aliquots were then taken for direct analysis, for immunoprecipitation as described above or for extraction with Triton X-114 as described previously (4). Final analyses were done in 20% polyacrylamide gels.

In vivo labeling. COS cells were infected with recombinant vaccinia viruses at a m.o.i. of 20. Proteins were labeled from 4 to 7 hours p.i. with ^{35}S-methionine (30µCi/ml) in methionine-free medium. Cells were lysed and the M proteins were immunoprecipitated with 2 µl of a polyclonal MHV-A59 antiserum and 15µl Staph. aureus suspension. The samples were electrophoresed without heating in 15 % polyacrylamide gel.

Indirect immunofluorescence microscopy. Infected COS cells were fixed with 3 % paraformaldehyde at 6h p.i. and permeabilized with 1 % Triton X-100 in PBS. Cells were stained with polyclonal MHV-A59 antiserum (1:150) followed by affinity-purified fluorescein-conjugated goat anti-rabbit IgG (1:150, Kallestad) according to Rose and Bergman (14). For cell surface fluorescensce, infected COS cells were incubated at 10h p.i. with polyclonal MHV-A59 antiserum (1:150 in PBS + 5 % FCS) and then with fluorescein-conjugated anti-rabbit IgG (1:150), both for 30 min at room temperature. Cells were then fixed, washed and visualized with a Zeiss microscope.

Antisera. The preparation of polyclonal antiserum to purified MHV-A59 has been described (15). Antiserum to the carboxy-terminal tail of the MHV-A59 M protein was prepared in 2 rabbits with a synthetic peptide corresponding to the C-terminal 18 amino acids coupled to BSA. Both animals were immunized with 3mg of conjugate emulsified in complete Freund adjuvant, giving about 25 intradermal 50µl injections. Rabbits were boosted similarly 3 times over a period of 3.5 months with 2.5mg of conjugate now emulsified in incomplete Freund adjuvant. The animals were killed 3 weeks later; blood was collected and serum stored at -20°C. The N-terminus specific monoclonal antibody J.1.3 (16) was a kind gift from Drs.S.Stohlman and J.Fleming.

RESULTS

Generation of mutant proteins. As a first approach to test for a role of potentially relevant domains of the M molecule in membrane integration

and transport, precise deletions were made by in vitro mutagenesis of the M gene. The following mutants were constructed:
* ΔN: deletion of 16 of the 25 N-terminal amino acids, residues A(7) through F(22)
* ΔC: deletion of 75 amino acids from the cytoplasmic domain, residues E(121) through D(195)
* Δ(a+b): removal of the first two transmembrane domains, residues W(26) through N(81)
* Δ(b+c): removal of second and third transmembrane domain, residues S(49) through Y(101)
* ΔaΔc: deletion of first and third domain, residues W(26) through S(49) and Y(83) through N(104).
Being constructed in the transcription vector pTZ19R (10) the mutant genes could be expressed directly in vitro by translation of the T_7-polymerase derived mRNAs. In addition, the genes were recombined into vaccinia virus for expression in eukaryotic cells.

In vitro membrane integration of mutant M proteins. The effect of the mutations on the protein's ability to be stably integrated into membranes was investigated by translation of in vitro transcribed M gene mRNAs in a reticulocyte lysate in the presence of rough microsomes. Integration was assayed by alkaline treatment of the reaction mixture followed by separation of integrated and unintegrated translation products by centrifugation of the membrane sheets. As shown in Fig. 1 (left lanes) both wild-type M and the mutant proteins are efficiently inserted: in each case the majority of the products cosedimented with the membranes. Deletion of most of the N-terminal residues or of a major part of the C-terminal domain was without effect. M proteins lacking two of the three hydrophobic segments also sedimented with the membranes. Apparently, each transmembrane domain can individually direct insertion into the lipid bilayer as well as anchor the protein.

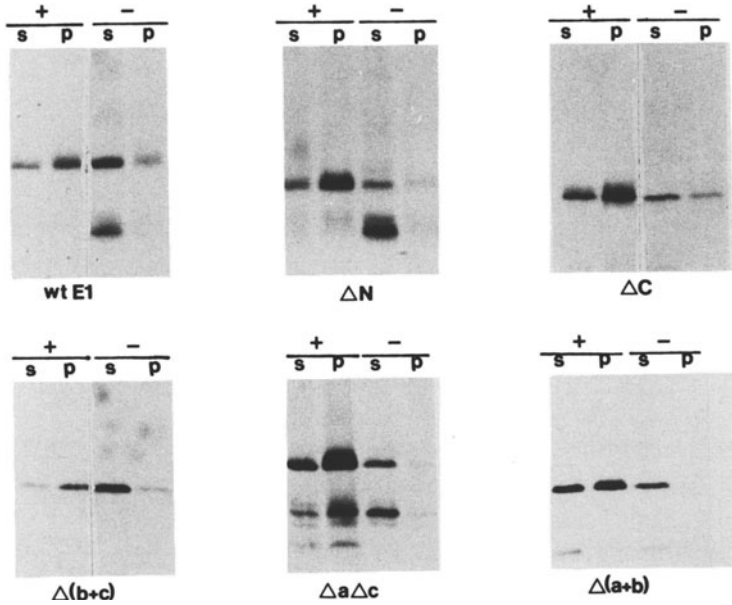

Fig.1. In vitro membrane integration. Wild-type and mutant M mRNAs were translated in a reticulocyte lysate. Microsomes were present during the translation (+) or added post-translationally (-). After treatment of the samples with alkali the membranes were sedimented through a sucrose gradient. Both the supernatant (s) and the membranes (p) were analyzed.

As we have shown earlier, wild-type M inserts into membranes co-translationally (2,4). Indeed, addition of microsomes to the translation mixture post-translationally, after having blocked further polypeptide synthesis with cycloheximide, showed only a background amount of sedimentable M (Fig.1, right lanes). When similar post-synthesis incubations with microsomes were applied to the mutant polypeptides, the same pictures were obtained. Thus, the insertion signals only function in a co-translational manner, post-translational insertion occurring to an insignificant extent, if at all.

Membrane topology of integrated proteins. Two approaches were taken to analyze the organization of the assembled proteins. In one assay we made use of antibodies specifically recognizing the extreme N-terminus and C-terminus, respectively, of M. The former was the monoclonal antibody J.1.3 developed by Fleming et al. (16) which neutralizes MHV in the presence of complement, the latter an antiserum raised in rabbits to a synthetic peptide corresponding to the 18 carboxy-terminal amino acids of M protein. As shown in Fig. 2 both antibodies precipitated the wild-type M protein synthesized in a reticulocyte lysate in the presence of rough microsomes. When treated with proteinase K, which is known to remove the C-terminus of the integrated protein (4,7), only the N-terminus specific antibodies brought down the M-fragment. Digestion from both sides of the membranes by including 0.05% saponin also abolished precipitation with this monoclonal antibody. When such analyses were done with mutant proteins, identical results were obtained with mutants ΔN and ΔC. Apparently, the deletions

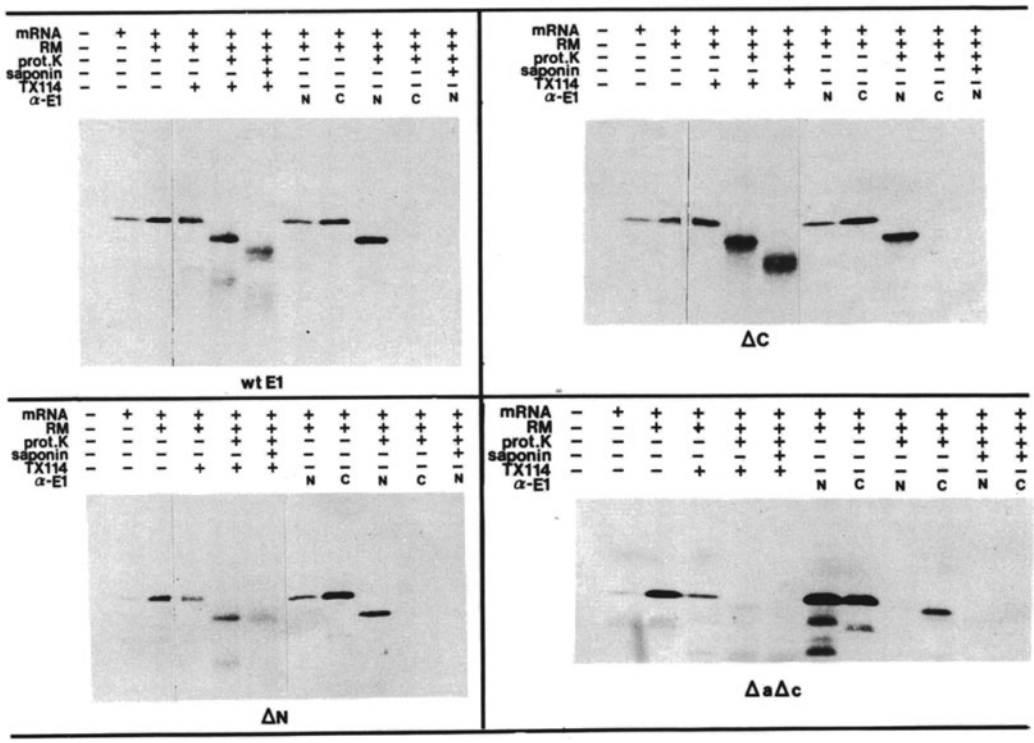

Fig.2. Topology of integrated proteins. Translations were done in the presence or absence of rough microsomes (RM). Aliquots were then taken for direct analysis and for proteinase K treatment in the presence or absence of saponin. Some samples were extracted with Triton X-114 and the detergent phase analyzed; others were immuno-precipitated with antibodies specific for the N- or C-terminus of M.

introduced into these proteins are not involved in the topogenesis of M. Of the single-spanning mutants, which lack two of the three hydrophobic domains, △(a+b) and △(b+c) did not yield unequivocal results. Mutant △a△c, however, appeared to attain an orientation opposite to that of wild-type M. Protease treatment now showed that the C-terminus of the polypeptide was protected, but only when the membrane vesicles were intact.

In order to confirm and extend these findings, a second approach was taken; the mutant proteins were expressed in cells and glycosylation was used as a marker to assay for translocation of the protein's N-terminus across the membrane. It should be kept in mind, however, that O-glycosylation is a post-translational event, the first step of which is supposed to occur in the transitional "budding compartment" followed by additional glycosylation steps in the Golgi apparatus (17). In Fig. 3 immune precipitates of proteins expressed from a vaccinia virus vector in COS cells after a 3h labeling period are compared with the respective primary translation products obtained in a reticulocyte lysate in the absence of microsomes. Clearly, glycosylated products were obtained in the case of the wild-type protein and with mutants △(a+b) and △C. While this came as no surprise for △C, it indicates that the N-terminus of △(a+b) is translocated. As could also be expected, the mutants △a△c and △N did not acquire oligosaccharides. In the former case this is in agreement with its reversed orientation, in the latter case the mutation in the N-terminal region apparently affected the functioning of this domain as a substrate for glycosyl transferases. Mutant △(b+c) protein comigrated with the in vitro synthesized product; however, a small amount of material migrating slightly slower was also observed. This band may well represent polypeptides to which only the initial N-acetylgalactosamine has been added as the protein seems not to be transported to the Golgi compartments (see below).

Intracellular transport. In order to extend the information obtained from the in vivo labeling experiments, immunofluorescence staining of COS cells infected with the different recombinant vaccinia viruses was performed to visualize the location of the respective proteins. Figure 4 shows the results of indirect staining of the cells at 6h p.i. with a polyclonal MHV-A59 antiserum followed by a fluorescein-conjugated second antibody. Wild-type M was localized in the same juxtanuclear region as we have observed earlier using a SV40-derived vector and which was shown to co-localize with a Golgi marker (8). This typical fluorescence distribution is not specific for COS cells; it was also seen in various other cell types such as HeLa, AtT20, CHO, Ratec, and Sac(-) (not shown). A similar staining pattern was observed with the mutants △N, △C and △(a+b). These proteins apparently

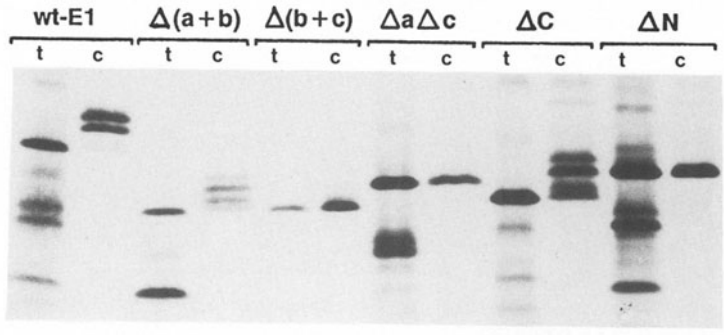

Fig.3. Expression of M proteins in culture cells. Wild-type and mutant proteins synthesized in recombinant vaccinia virus infected COS cells (c) during a 3h labeling period were compared by PAGE with the respective in vitro translation product (t).

reach the Golgi apparatus. Mutants △a△c and △(b+c) showed up clearly differently. Staining now extended into the cell from all around the nucleus. Though somewhat diffuse, the stained material was of a reticular appearance.

Indirect immunofluorescence microscopy on non-permeabilized cells was performed to analyze transport of M proteins to the plasma membrane. As shown in Fig.4, surface staining was positive with mutant △(a+b). All other mutants as well as wild-type M were negative (not shown).

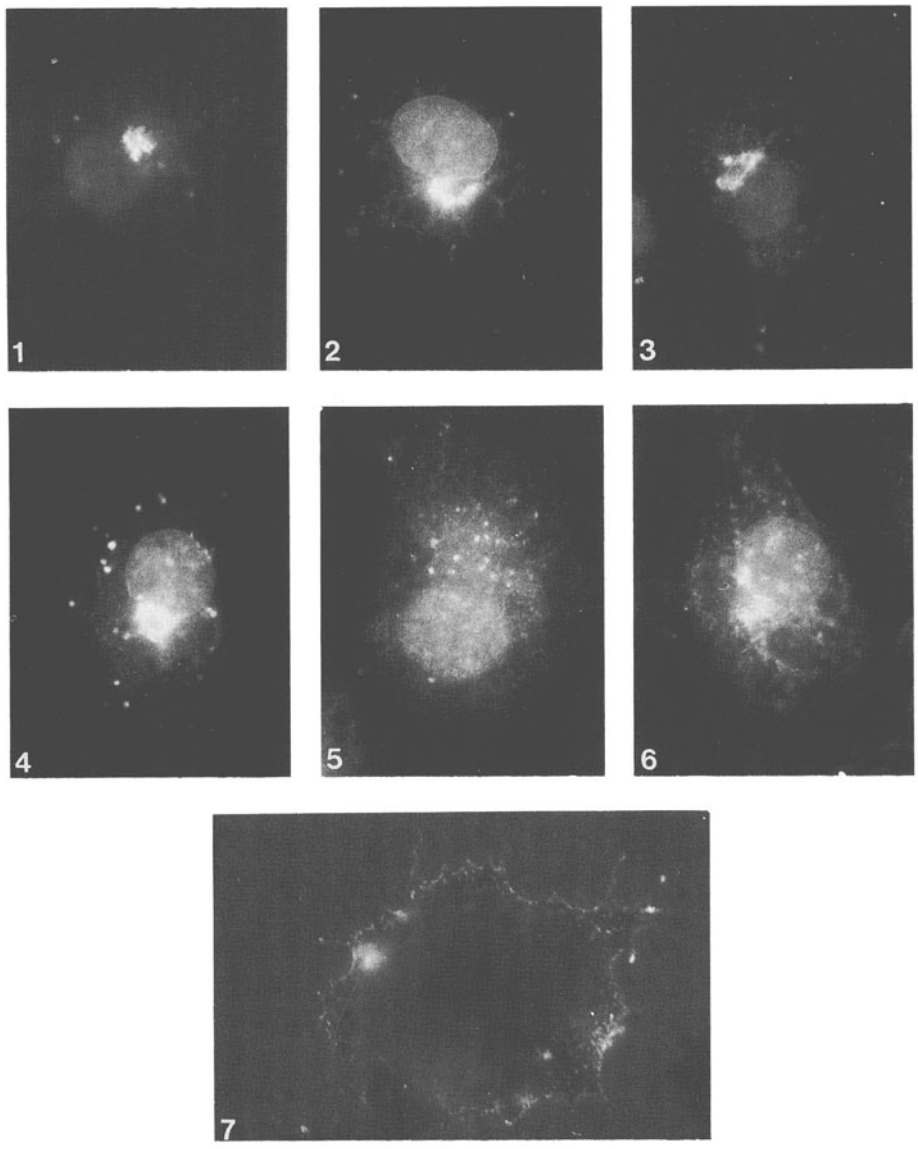

Fig.4. Localization of M proteins by indirect immunofluorescence. Internal staining of COS cells infected with recombinant vaccinia viruses expressing wild-type M (1) and the mutants △N (2), △C (3), △(a+b) (4), △a△c (5) and △(b+c) (6). Cell surface staining of cells expressing the mutant △(a+b) protein (7).

DISCUSSION

The general conclusion from the data reported here is that the region of the coronavirus M protein comprising the three membrane-spanning domains determine both its membrane topology and its intracellular location. Neither the amino-terminal domain nor the cytoplasmic part of the polypeptide seem to be involved in directing membrane assembly or intracellular transport. A role, however, for the regions immediately flanking the hydrophobic domains cannot be excluded yet.

The N-terminus of the MHV M protein is not cleaved (4,7). Its hydrophilic nature strongly argues against a role as a membrane insertion signal (3,5). The unchanged assembly of the protein lacking 16 out of the 25 N-terminal residues confirms this idea and is in agreement with conclusions drawn from similar work by Mayer et al. (7). Interestingly, the M proteins of porcine transmissible gastroenteritis virus (18,19) and feline infectious peritonitis virus (H. Vennema, pers. comm.) do have a cleaved signal peptide. This peptide precedes the hydrophilic domain and can thus be considered an extension of the N-terminus. It is not clear why these very proteins have an extra signal sequence, but it might be required to aid in translocating the hydrophilic region, which in these viruses has a higher positive charge.

Each of the possible single-spanning M mutants was found to stably integrate into the lipid bilayer. This indicates that each membrane-spanning domain can act as a signal-anchor sequence by itself. This is not so surprising, as the overall hydrophobicity seems to be the major requirement. Having identical amino-terminal and carboxy-terminal domains and thus differing only in the hydrophobic region, these mutant proteins are particularly interesting with respect to their orientation in the membrane. It appeared that the mutant possessing only the third membrane-spanning region has its N-terminus translocated to the lumen, as has been found with a comparable IBV M mutant (6). In contrast, when only the middle hydrophobic domain is present, the N-terminus is in the cytoplasm and the C-terminus exposed to the lumen. No clear conclusion could be drawn about mutant Δ(b+c) but it is likely to also translocate its N-terminus across the membrane. Such an observation has been made with a similar IBV M mutant (6) as well as with a MHV-A59 M protein lacking residues 45-132 which includes the second and third transmembrane domain (7). This implies that the orientation of the hydrophobic domain in each mutant is identical to the orientation of this domain in the assembled wild-type M protein. In other words: each transmembrane domain has an intrinsic preferred orientation in the membrane. It remains to be assessed whether this preference is determined by the hydrophobic segments or by the sequences directly flanking them.

Targeting of the M protein in cells to the Golgi apparatus was affected neither by deleting most of the hydrophilic N-terminus nor by taking about one-third of the polypeptide out of the C-terminal half. Apparently, these domains do not contain sorting information. In contrast, deletions in the hydrophobic cluster generating single-spanning mutant proteins had drastic effects. Deletion of the first and second transmembrane domain generated a mutant protein that was no longer retained in the Golgi apparatus but was transported to the cell surface. A similar deletion in the IBV M protein had the same effect (6). Deletion either of the first and third domain or of the second and third domain both yielded polypeptides that appeared to be retained in the ER region. In the case of the mutant having only the second transmembrane domain this may be explained by its reversed orientation, possible targeting signals no longer being presented to the proper compartment. The location of the mutant with only the first domain is surprising since a similar mutation in IBV M did

not change its Golgi accumulation (6). The latter result combined with the cell surface expression of the IBV mutant having only the third transmembrane domain suggested the presence of a Golgi retention signal in the first membrane-spanning segment. Our data do not support such a conclusion. We can only speculate about the reason why the mutants anchored by the first transmembrane region behave differently. The most plausible explanation is the difference in the mutations themselves: in the case of the IBV mutant the deletion comprised 59 residues which is 6 more than was the case with our MHV mutant. Clearly, more work will be required to straighten out this interesting discrepancy since this might give further clues about the signals involved in the targeting of this complex protein.

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