CHAPTER 6

The Coronavirus Membrane Glycoprotein

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I. INTRODUCTION

Coronaviruses have a simple protein composition. While there is some variation among different members, a basic set of four protein species universally occurs: the nucleocapsid protein (N), the spike protein (S), a small membrane protein (SM), and the membrane glycoprotein (M). Some coronaviruses have an additional membrane glycoprotein (HE). The M protein, previously also called E1, is the subject of this chapter. As will become clear, M is a peculiar glycoprotein, different from all other viral glycoproteins in its structural and biochemical features. These unique features may be responsible for important biological properties of coronaviruses, in particular for their intracellular budding.

M is the most abundant virion protein. In murine hepatitis virus (MHV) it was estimated by isotopic labeling to comprise some 40% of the particle's protein mass, exceeding the nucleocapsid protein on a molar basis at a ratio of 2:1 (Sturman *et al.*, 1980). A similar ratio was obtained from incorporation studies with avian infectious bronchitis virus (IBV) (Stern *et al.*, 1982), while equimolar ratios were determined for bovine coronavirus (BCV) (King and Brian, 1982) and human coronavirus (HCV) OC43 (Hogue and Brian, 1986). In the latter case, M and N were calculated to be present at a rate of 726 molecules per virion.

The M gene, together with the genes for the other structural proteins, is located in the 3′ one third of the coronaviral genome, downstream from the spike protein gene and upstream from the nucleoprotein gene. As a conse-

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quence of the specific mode of transcription of coronaviruses, the M protein is expressed from a mRNA that, in addition to the M sequence, carries extra genetic information including the N sequence. This additional information is located 3′ from the M gene and is functionally redundant; the M protein is equally well translated from a mRNA derived from a cloned copy of the M gene (see Machamer and Rose, 1987; Mayer *et al.*, 1988; Rottier and Rose, 1987).

II. PHYSICOCHEMICAL PROPERTIES

A. Covalent Modifications

The M protein is usually found as a family of differentially glycosylated proteins, including the unglycosylated precursor. These proteins span a M_r range of 20 to 38kDa. The M protein of a particular coronavirus carries either N- or O-linked oligosaccharides (Table I). The only other modification identified so far is the addition of sulfate (Garwes *et al.*, 1976), but it is unknown whether this constituent is attached to the oligosaccharide side chains or bound directly to the polypeptide through tyrosine. M is not acylated (Niemann and Klenk, 1981; Schmidt, 1982), nor does it contain phosphate (e.g., Stohlman and Lai, 1979; Rottier *et al.*, 1981a; King and Brian, 1982; Hogue and Brian, 1986).

The structures of the N-linked oligosaccharides carried by coronavirus M proteins have not been studied in detail, but are probably not different from those found in other viral and cellular N-glycosylated proteins. Carbohydrates bound to a polypeptide through O-linkage to serine or threonine residues are quite uncommon among viral proteins. Of the O-glycosylated coronaviral M proteins, the side chains of the M protein of MHV strain A59 grown in 17Cl1 cells, a spontaneously transformed BALBC/3T3 line, have been analyzed (Niemann and Klenk, 1981; Niemann *et al.*, 1984). Two size classes of oligosaccharides were released from the protein by β-elimination. Their structures, as

TABLE I. Type of Glycosylation of M Proteins of Coronaviruses

Common name	Designation
N-glycosylation	
Canine coronavirus	CCV
Feline infectious peritonitis virus	FIPV
Feline enteric coronavirus	FECV
Human coronavirus strain 229E	HCV-229E
Infectious bronchitis virus	IBV
Turkey coronavirus	TCV
Transmissible gastroenteritis virus	TGEV
O-glycosylation	
Bovine coronavirus	BCV
Diarrhoea virus of infant mice	DVIM
Human coronavirus strain OC43	HCV-OC43
Mouse hepatitis virus	MHV

FIGURE 1. Structures of the O-linked oligosaccharide side chains of the MHV-A59 M glycoprotein.

determined by a combined gas chromatographic and mass spectrometric analysis, are shown in Fig. 1. The branched form was most abundant, comprising about 65% of the carbohydrate structures. Both forms were attached to the M polypeptide via *N*-acetylgalactosamine. Of the sialic acids (*N*-acetylneuraminic acid, Neu5Ac), some 20% were identified as the O-acetylated derivative Neu5,9Ac_2 (Niemann *et al.*, 1984).

The oligosaccharides are attached to the N-terminal region of the M molecule and exposed at the virion surface. Treatment of MHV (Sturman, 1977; Sturman and Holmes, 1977; Rottier *et al.*, 1984) and BCV (King and Brian, 1982) with various proteases removed some 5kDa from the polypeptide, including all the O-linked sugars. Similarly, the N-linked oligosaccharides of the IBV M protein were all detached by hydrolysis of the exposed domain of the molecule (Cavanagh, 1981; Cavanagh *et al.*, 1986a). Using [^{35}S]formyl-methionine to terminally label the polypeptide, this ectodomain was identified as the N-terminus (Stern *et al.*, 1982; Rottier *et al.*, 1984).

B. Solubility

The M protein has unusual solubility properties. This was demonstrated for MHV M in the pioneering studies of Sturman and Holmes (Sturman, 1977, 1981; Sturman and Holmes, 1977; Sturman *et al.*, 1980). When isolated at 4 °C from virions after nonionic detergent solubilization of the viral membrane, the protein formed globular, irregular aggregates of various sizes. Raising the temperature to 37 °C induced a conformational change in the molecules that led the aggregates to associate with the nucleocapsid through interaction with the viral RNA (Sturman *et al.*, 1980). Thermosensitivity of the M protein was also prominent in sodium dodecyl sulfate (SDS) solution: heating from 25 °C or 37 °C, in which range the protein is in a monomeric state, to 100 °C resulted in the formation of various self-aggregates, an effect that was even more pronounced in the presence of reducing agents (Sturman, 1977; Sturman and Holmes, 1977). Unlike the M protein of the MHV-A59 strain, the M protein of MHV-JHM additionally appeared to form heterogeneous complexes with the S glycoprotein under these conditions (Wege *et al.*, 1979; Siddell *et al.*, 1981). The

tendency of the MHV M protein to aggregate was also observed after various other denaturing treatments (Sturman, 1977). Apparently, any condition that promotes the unfolding of the M protein can expose a (probably hydrophobic) domain of the molecule which then engages in interactions.

Aggregation of M in SDS has been described for a number of other coronaviruses such as hemagglutinating encephalitis virus (HEV) (Callebaut and Pensaert, 1980), HCV-OC43 (Schmidt and Kenny, 1982; Hogue and Brian, 1986), and BCV (Niemann and Klenk, 1981; Deregt *et al.*, 1987). On the other hand, it is not a universal feature as the effect was not observed after heat denaturation of the M polypeptide of IBV (Cavanagh, 1981; Stern and Sefton, 1982b) or HCV-229E (Schmidt and Kenny, 1982), not even in the presence of 2-mercaptoethanol. The reason for this variable behavior is not understood. The effect seems to be independent of the degree and type of glycosylation. Even complete removal of the N-terminal ectodomain did not abolish the aggregation properties of the polypeptide (Sturman, 1977).

III. PROTEIN STRUCTURE

A. Primary Structure

During the past years, the M genes of several coronaviruses have been sequenced. The deduced amino acid sequences of a number of M proteins are compiled in the alignment presented in Fig. 2. The primary structures are 225–230 amino acids long, with the exception of the transmissible gastroenteritis virus (TGEV) and feline infectious peritonitis virus (FIPV) sequences which amount to 262 residues, due to an extension at the NH_2-terminus. The polypeptides are slightly basic with net charges at neutral pH ranging from +4 (HCV-229E) to +9 (BCV). Their cysteine content is quite variable, with only two such residues in the HCV-229E sequence and nine residues in IBV M. These numbers do not explain the effects of reducing agents on the solubility of the coronavirus M proteins.

Pairwise sequence comparisons support the long-standing classification of coronaviruses made on the basis of antigenic relationships (McIntosh *et al.*, 1969; Bradburne, 1970; Pedersen *et al.*, 1978; Horzinek *et al.*, 1982). The M proteins of MHV and BCV, viruses of the same antigenic subgroup, are very closely related (86% identity), but differ largely from all the others (e.g., MHV/IBV 29%, MHV/HCV-229E 32%, MHV/FIPV 36%). Similarly, the M sequences of TGEV and FIPV, also antigenically related viruses, show a strong homology (84% identity), but are only distantly related to the others (e.g., TGEV/IBV 17%). The avian IBV M polypeptide has only low homologies to the mammalian proteins (e.g., IBV/FIPV 20% identity), in agreement with it being classified separately. HCV-229E has previously been placed into the TGEV group on the basis of weak serological cross-reactivities (Pedersen *et al.*, 1978; Macnaughton, 1981). The M protein of this virus has only little sequence similarity with the TGEV or FIPV protein (e.g., HCV-229E/TGEV 44% identity) or with any of the other M proteins. The same holds true for the S proteins (Wesseling *et al.*, 1994). The data therefore support the recent proposition by Sanchez *et al.* (1990) to

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          1                                                                      70
MHV       .......... .......... .......... .MSSTTQAPE PVYQWTADEA VQFLKEWNFS LGIILLFITI
BCV       .......... .......... .......... ..MSSVTTPA PVYTWTADEA IKFLKEWNFS LGIILLFITI
HCV       .......... .......... .......... .......... MSNDNCTGDI VTHLKNWNFG WNVILTIFIV
IBV       .......... .......... .......... ..MPNETNCT LDFE....QS VQLFKEYNLF ITAFLLFLTI
FIPV      MKYILLILAC IIACVYGERY CAMQ.DSGLQ CINGTNSRCQ TCFE..RGDL IWHLANWNFS WSVILIVFIT
TGEV      MK.ILLILAC VIACACGERY CAMKSDTDLS CRNSTASDCE SCFN..GGDL IWHLANWNFS WSIILIVFIT
Consensus ---------- ---------- ---------- ---------- --F-----D- ---L--WNF- --IIL-----

          71                                                                    140
MHV       ILQFGYTSRS MFIYVVKMII LWLMWPLTIV LCIFN..CVY ALNN.VYLGF SIVFTIVSIV IWIMYFVNSI
BCV       ILQFGYTSRS MFVYVIKMII LWLMWPLTII LTIFN..CVY ALNN.VYLGF SIVFTIVAII MWIVYFVNSI
HCV       ILQFGHYKYS RLFYGLKMLV LWLLWPLVLA LSIFDTWANW D.SNWAFVAF SFFMAVSTLV MWVMYFANSF
IBV       ILQYGYATRS KVIYTLKMIV LWCFWPLNIA VGVIS..CTY PPNTGGLVA. AIILTVFACL SFVGYWIQSI
FIPV      VLQYGRPQFS WLVYGIKMLI MWLLWPIVLA LTIFNAYSEY QVSRYVMFGF SVAGAVVTFA LWMMYFVRSV
TGEV      VLQYGRPQFS WFVYGIKMLI MWLLWPVVLA LTIFNAYSEY QVSRYVMFGF SIAGAIVTFV LWIMYFVRSI
Consensus ILQ-G----S -FVY--KM-I LWLLWP---- L-IF-----Y ---------F SI-------- -WI-YFV-SI

          141                                                                   210
MHV       RLFIRTGSWW SFNPETNNLM CIDMKGTVYV RPIIEDYHTL TATIIRGHLY MQGVKLGTGF SLSDLPAYVT
BCV       RLFIRTGSWW SFNPETNNLM CIDMKGRMYV RPIIEDYHTL TVTIIRGHLY MQGIKLGTGY SLSDLPAYVT
HCV       RLFRRARTFW AWNPEVNAIT VTTVLGQTYY QPIQQAPTGI TVTLLSGVLY VDGHRLASGV QVHNLPEYMT
IBV       RLFKRCRSWW SFNPESNAVG SILLTNGQQC NFAIESVPMV LSPIIKNGVL YCEGQWLAKC EPDHLPKDIF
FIPV      QLYRRTKSWW SFNPETNAIL CVNALGRSYV LPLDGTPTGV TLTLLSGNLY AEGFKMAGGL TIEHLPKYVM
TGEV      QLYRRTNSWW SFNPETKAIL CVSALGRSYV LPLEGVPTGV TLTLLSGNLY AEGFKIADGM NIDNLPKYVM
Consensus -LF-R--SWW SFNPE-N--- -I---G--Y- -P-------- T-T---G-LY --G-----G- ----LP-YV-

          211                                                        272
MHV       VAKVSHLCTY K...RAFLDK VDGVSGFAVY VKSK....VG NYRLPSNKPS G..ADTALLR I.       228
BCV       VAKVSHLLTY KR.GF..LDK IGDTSGFAVY VKSKV....G NYRLPSTQKG SGMDTALLRN NI       230
HCV       VAVPSTTIIY SRVGR..SVN SQNCTGWVFY VRVKH....G DFSAVSSPMS NMTENERLLH FF       225
IBV       VCTPDRRNIY RMVQKYTGDQ SGNKKRFATF VYAKQSVDTG ELESVATGGS SLYT...... ..       225
FIPV      IATPSRTIVY TLVGK..QLK ATTATGWAYY VKSKA....G DYST.EARTD NLSEHEKLLH MV       262
TGEV      VALPSRTIVY TLVGK..KLK ASSATGWAYY VKSKA....G DYST.EARTD NLSEQEKLLH MV       262
Consensus VA--S----Y ---------- -----G-A-Y V--K-----G -Y-------- -------L-- --
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FIGURE 2. Sequence alignment of the M proteins of MHV-A59 (Armstrong *et al.*, 1984), BCV (Lapps *et al.*, 1987), HCV-229E (Raabe and Siddell, 1989), IBV (Baudette strain; Boursnell *et al.*, 1984), FIPV (Vennema *et al.*, 1991a), and TGEV (Laude *et al.*, 1987). Not included are the recently determined sequences of TCV (Verbeek and Tijssen, 1991), HCV-OC43 (Mounir and Talbot, 1992), CCV (Horsburgh *et al.*, 1992), and FECV (A. Herrewegh, H. Vennema, R. de Groot, and P. Rottier, unpublished data). Comparison shows the former two to be very similar to the sequences of MHV and BCV, while the latter have a high similarity to the sequences of FIPV and TGEV.

classify HCV-229E in a distinct taxonomic cluster; this suggestion was based on an extensive antigenic comparison of coronaviruses using monoclonal antibodies (see Chapter 1).

As indicated by its solubility, M is a very hydrophobic protein. It contains 44–51% of hydrophobic amino acids which are concentrated in the NH_2-terminal half of the molecule (Fig. 3). Despite the high degree of sequence variation between the coronavirus M polypeptides, the hydropathicity profiles are remarkably similar. The dominant common feature is the occurrence of three hydrophobic domains alternating with short hydrophilic regions. In the TGEV and FIPV sequence a fourth hydrophobic domain is present at the NH_2-terminus, which functions as a cleavable membrane insertion signal (Kapke *et al.*, 1988; Vennema *et al.*, 1991b). In the other M proteins, no such signal appears to be operative; rather, these proteins have a hydrophilic amino terminus. The carboxy terminal half of all M proteins is amphiphilic, with a hydrophilic domain at the carboxy end.

In view of the large differences in primary sequences, the surprising conser-

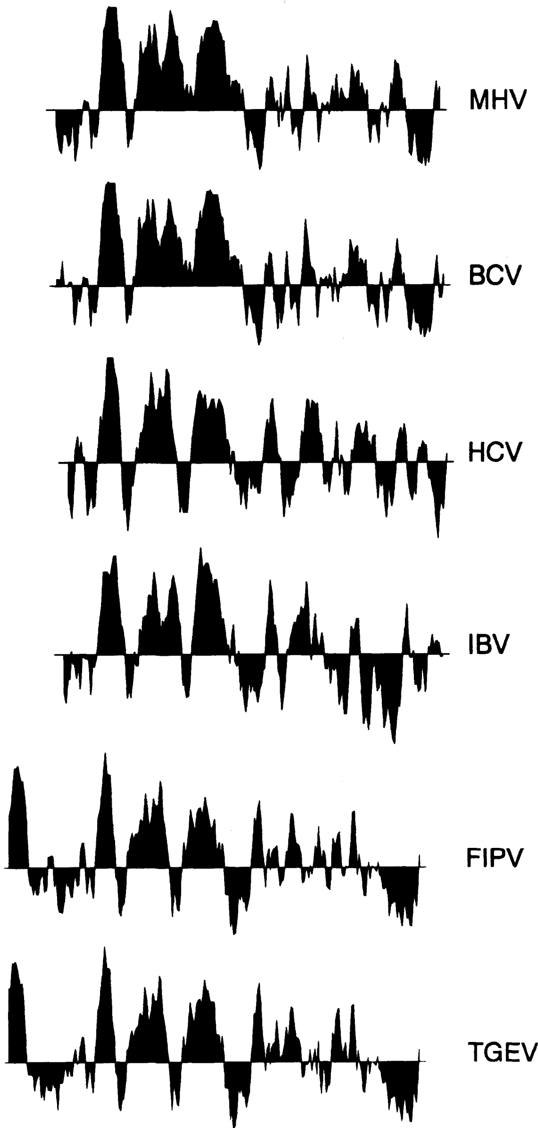

FIGURE 3. Hydropathicity profiles of M proteins from MHV-A59, BCV, HCV-229E, IBV, FIPV, and TGEV determined according to Kyte and Doolittle (1982) using a seven residue moving window. Peaks extending upward indicate hydrophobic regions, downward peaks correspond to hydrophilic regions.

vation of the overall chemical features indicates that there are rigid structural constraints on M as a result of functional requirements. The highest levels of sequence conservation appear in the hydrophobic regions and in the center of the polypeptide directly adjacent to the third internal hydrophobic region, where a stretch of 8 amino acids is extremely well conserved (see consensus in Fig. 2). This suggests a selective pressure on these domains for the maintenance of important structural characteristics.

As mentioned above, coronavirus M proteins are glycosylated in their NH_2-terminal ectodomain, i.e., the hydrophilic domain preceding the first internal

hydrophobic region (see Section III.B). In HCV-229E, IBV, FIPV, and TGEV, the protein is N-glycosylated. Potential oligosaccharide attachment sites occur once in the relevant part of the HCV-229E M sequence (position 45 in Fig. 2), twice in the case of IBV M (positions 35 and 38), and once in the cases of FIPV and TGEV M (position 33). These sites are indeed used as the numbers of side chains are in agreement with those experimentally observed (Stern and Sefton, 1982b; Kapke *et al.*, 1988; Vennema *et al.*, 1991b). Another N-glycosylation consensus sequence (positions 58–60 in Fig. 2) occurs at the start of the first internal hydrophobic region of some M proteins but appears not to be used. Apparently, this sequence is not exposed to the modifying enzymes in the endoplasmic reticulum and resides within the membrane.

It is not known which of the serine and threonine residues are substituted in the O-glycosylated coronavirus M proteins. The structural features that determine a functional O-glycosylation site have not yet been established. In MHV, a cluster of four hydroxy amino acids is located next to the initiating methionine which presumably is removed posttranslationally as has been shown for IBV M (Cavanagh *et al.*, 1986b). The resulting NH_2-terminal tetrapeptide sequence (Ser-Ser-Thr-Thr) is identical to the O-glycosylated aminoterminus of glycophorin A, the major glycoprotein of the human erythrocyte membrane. It was shown by Niemann *et al.* (1984) that the O-linked sugar structures of MHV M are identical to those found in glycophorin. As the coronavirus protein also appeared to exhibit blood group M activity, as does glycophorin, the authors inferred that the hydroxy amino acid cluster contains the functional oligosaccharide acceptor sites in MHV M. On the basis of the heterogeneity in the glycosylation of the M protein, they concluded that up to three of the four residues in the cluster are modified by oligosaccharide side chains. In BCV a slightly different cluster of hydroxy amino acids occurs at the NH_2-terminus due to the presence of a valine residue (Fig. 2). Assuming that the sequence requirements for glycosylation allow for this difference, Lapps *et al.* (1987) suggested, by analogy, that the additions of the glycans take place in this terminal segment. They argued that, in the case of BCV, up to two O-linked oligosaccharide side chains per M molecule are attached.

Recent sequence information shows that considerable genetic diversity exists among different strains or isolates of coronaviruses in their spike protein (e.g., for MHV, see Luytjes *et al.*, 1987; Schmidt *et al.*, 1987; Parker *et al.*, 1989; Gallagher *et al.*, 1990; Wang *et al.*, 1992). The limited data available for the M gene suggest that variations are less extensive. Comparison, for instance, of the A59 and JHM strains of MHV (Pfleiderer *et al.*, 1986) revealed 21 nucleotide changes scattered over the entire molecule, which result in only seven conservative amino acid changes (3.5%). Cavanagh and Davis (1988) analyzed the NH_2-terminal domain, including the first hydrophobic region, of the M sequence of 23 strains of IBV. Both base substitutions and small deletions and insertions were detected. A fourfold greater extent of amino acid variation was found in the ectodomain of the protein as compared to the membrane-embedded segment. Notably, one of the N-glycosylation sequences (positions 38–40 in Fig. 2) was highly conserved while the other (positions 35–37) was not. Also, based on a complete comparison of M sequences of two IBV strains (Binns *et al.*,

1986), it was concluded that the exposed NH_2-terminal domain is the most variable part of the molecule. The M proteins of TGEV and PCRV (Rasschaert *et al.*, 1990), two closely related porcine coronaviruses, also differ in their amino acid sequences at 13 positions, 8 of which occur in the amino terminal part preceding the first hydrophobic region.

B. Membrane Topology

The disposition of the M molecule in the lipid bilayer was studied through protease protection analyses. Digestion of *in vitro* assembled MHV-A59 M protein showed the bulk of the polypeptide to be resistant to proteolysis: only a small (1.5-kDa) portion was removed when the membranes were intact while another 2.5-kDa fragment was digested after detergent permeabilization (Rottier *et al.*, 1984). The latter fragment is located luminally and represents the NH_2-terminus of the molecule, as was shown by selective labeling. In virions, this 2.5-kDa fragment is exposed on the outside and is glycosylated to a variable extent, while the 1.5kDa COOH-terminal end protrudes from the inner face of the membrane. Experiments with IBV (Cavanagh *et al.*, 1986a) suggest that the tertiary structure of the M protein of this virus, and probably of other coronaviruses as well, is very similar.

The results of the biochemical studies, combined with a theoretical analysis of the primary structure of the M polypeptide, have led to a general topological model of the assembled protein as shown in Fig. 4A (Armstrong *et al.*, 1984; Rottier *et al.*, 1986). The structure is characterized by the presence of three membrane-spanning helices in the NH_2-terminal half that anchor the protein in the lipid bilayer. This segment is flanked on the one side by the hydrophilic NH_2-terminus and on the other side by a region that contains the extremely well-conserved 8 amino acids domain (Fig. 2) and in which a surface helix is predicted for some (but not all) M proteins (Rottier *et al.*, 1986). The bulk of the carboxy-terminal half of the M molecule is supposedly embedded in the polar surface of the membrane. In line with this, a mutant M protein lacking all the

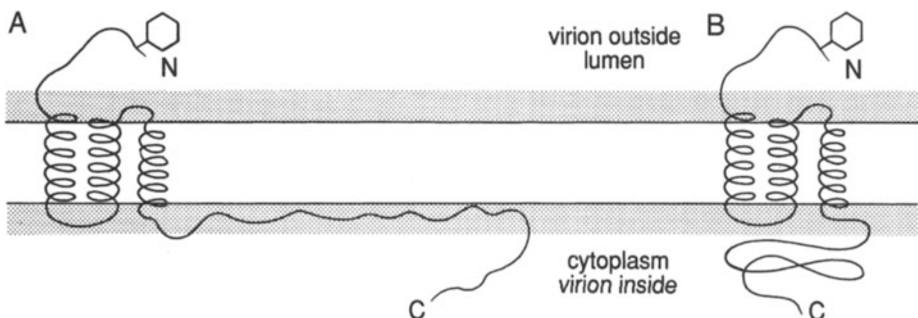

FIGURE 4. Topological models of the membrane-assembled M protein. Note that the two models differ only in the disposition of the protease-resistant region in the carboxy-terminal half. The hexagon symbol attached to the NH_2-terminal region indicates the potential glycosylation sites.

membrane-spanning domains was found to associate with membranes *in vitro* (Mayer *et al.*, 1988). However, a location of this extremely protease resistant region adjacent to the membrane, as in Fig. 4B, cannot be excluded.

IV. ASSEMBLY IN THE ENDOPLASMIC RETICULUM

Viral proteins use mechanisms and intracellular pathways as do normal cellular proteins. The process by which a nascent polypeptide is directed to and assembled in the endoplasmic reticulum occurs quite rapidly. Therefore, these processes are most conveniently studied in *in vitro* systems, which are easier to manipulate and in which the reaction kinetics are inherently slower. Though these systems have given us insight into the processes involved in the insertion and translocation of simple membrane and secretory proteins, little is known about the events that generate the more complex, multispanning membrane proteins. The coronavirus M protein, relatively simple as it is with only three transmembrane domains, thus represents an attractive model.

The MHV-A59 M protein is translated on membrane-associated polysomes (Niemann *et al.*, 1982). Its membrane insertion involves the action of the ribonucleoprotein complex called the signal-recognition particle (SRP). In a wheat germ translation system devoid of membranes, the synthesis of the protein could be specifically and stably blocked by the addition of SRP. Subsequent addition of salt-washed microsomes fully released the translation arrest and resulted in the correct membrane integration of the polypeptide (Rottier *et al.*, 1985).

In agreement with the absence of a hydrophobic NH_2-terminal peptide, the protein is assembled without a cleavable signal sequence (Rottier *et al.*, 1984). The polypeptide chain is inserted into the lipid bilayer in a cotranslational manner. Time course experiments in a synchronized *in vitro* translation of the M mRNA showed that the nascent polypeptide chain was able to integrate when membranes were added before 140–150 of the 228 residues had been polymerized. Later additions no longer allowed membrane insertion. Once beyond a critical point in synthesis, the domain containing the insertion signal(s) is apparently no longer accessible to the insertion machinery. Additional time course experiments revealed that such signal(s) may be located anywhere within the hydrophobic NH_2-terminal half of the molecule. During a synchronized translation in a wheat germ extract, SRP was able to induce an arrest until the most C-terminal hydrophobic domain had emerged from the ribosome (Rottier *et al.*, 1985).

Direct evidence that the topogenic signals in the M protein reside within the hydrophobic part of the polypeptide came from expression studies with cloned cDNA copies of M genes. *In vitro* mutagenesis followed by transcription and translation in the presence of microsomal membranes showed that each hydrophobic domain can individually insert and anchor the polypeptide in the membrane (Mayer *et al.*, 1988; Rottier *et al.*, 1990; Krijnse Locker *et al.*, 1992b). Similar results were obtained by expression of mutant M genes of MHV-A59 M (Mayer *et al.*, 1988; Armstrong *et al.*, 1990; Krijnse Locker *et al.*, 1992b) and IBV

M (Machamer and Rose, 1987). The mutant proteins with only the first or the third transmembrane domain integrate in membranes such that their NH_2-terminus is translocated into the lumen, while their COOH-terminus remains on the cytoplasmic side. Large deletions in the hydrophilic NH_2-terminal region did not affect this ability, nor did the mutations alter the topology of the assembled protein (Mayer *et al.*, 1988; Krijnse Locker *et al.*, 1992b). This part of the protein consequently plays no role in the membrane integration process. As expected, similar conclusions could be drawn from studies of mutations in the carboxy-terminal domain (Armstrong *et al.*, 1990; Rottier *et al.*, 1990; Krijnse Locker *et al.*, 1992b).

These observations lead to the model shown in Fig. 5. SRP interacts with the first hydrophobic domain as soon as it appears from the ribosome; elongation halts until the complex has attached to the membrane where the hydrophobic domain interacts with the signal sequence receptor and is inserted into the membrane, probably as a hairpin, while SRP is released. Presumably, the hydrophilic NH_2-terminus is translocated and the two following hydrophobic domains are then sequentially inserted. Completion of the polypeptide chain accompanied by further cotranslational folding finally leads to the fully assembled protein.

Interestingly, the deduced amino acid sequences of the TGEV (Laude *et al.*, 1987; Kapke *et al.*, 1988), FIPV (Vennema *et al.*, 1991a), and canine coronavirus (CCV) M protein (Horsburgh *et al.*, 1992) have a hydrophobic amino terminal extension (see Section III.A). Sequencing of the mature M polypeptide from purified TGEV showed that the first 17 residues were absent, indicating a cleavable signal sequence (Laude *et al.*, 1987). Translation studies with mRNA specifying TGEV M showed the signal-directed membrane insertion of the protein *in vitro*, although cleavage did not occur under these conditions (Kapke *et al.*, 1988). These authors also tested the expression of a truncated version of

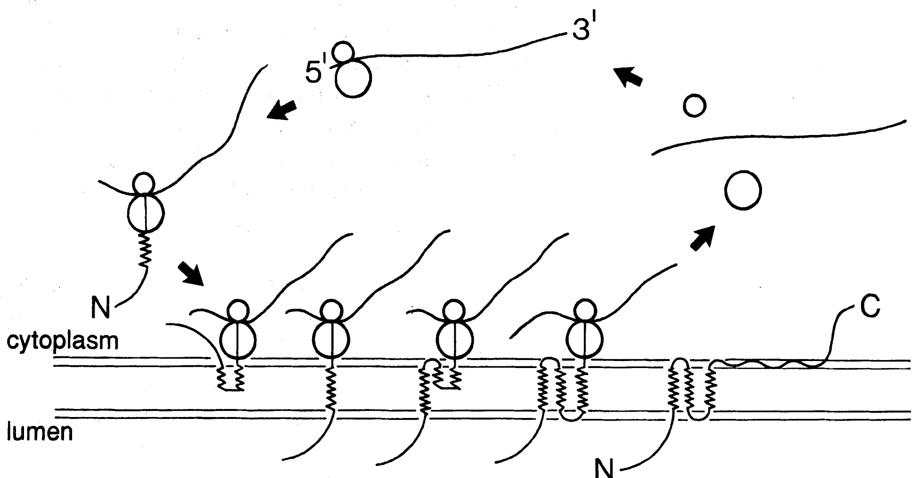

FIGURE 5. Model of the membrane assembly process of the coronavirus M protein.

the gene which lacked the information for the signal-containing first 21 residues. Though the polypeptide appeared to be integrated efficiently into microsomal membranes, translocation of the NH_2-terminal domain was poor, as judged from the small extent of N-glycosylation of the protein. In contrast, deletion of the signal sequence had no effect when the FIPV M protein was expressed in a vaccinia virus/cell system (Vennema *et al.*, 1991b). The mutant protein was glycosylated as efficiently as the wild-type protein. Why, in these viruses, the M protein contains a cleavable amino-terminal signal sequence thus remains unclear.

V. INTRACELLULAR TRANSPORT AND MATURATION

A. Transport and Processing in Coronavirus-Infected Cells

Intracellular transport of the coronavirus M protein differs from that of most other viral glycoproteins, including the coronaviral spike protein. Whereas these proteins are usually targeted to the cell surface, migration of the M protein is limited to the perinuclear region of the cell. This restricted mobility correlates with the intracellular location of coronavirus assembly in the infected cell.

1. Intracellular Budding of Coronaviruses

Already from the early studies of the coronavirus infection process, it became clear that the entire replication cycle takes place in the cytoplasm. Ultrastructural observations with different coronaviruses demonstrated that morphogenesis occurs at intracellular membrane-bound compartments as virions were seen in the lumina of the rough endoplasmic reticulum, smooth-walled vesicles and Golgi apparatus (e.g., David-Ferreira and Manaker, 1965; Becker *et al.*, 1967; Chasey and Alexander, 1976; Holmes and Behnke, 1981; Dubois-Dalcq *et al.*, 1982, 1984). Virus budding never takes place at the plasma membrane. A thorough investigation by J. and S. A. Tooze *et al.* (J. Tooze *et al.*, 1984, 1985, 1987; S. A. Tooze *et al.*, 1988), using a combination of biochemical and morphological techniques, has elucidated the temporal sequence of events in the maturation of MHV-A59 in Sac(−) cells, a line of transformed murine fibroblasts. Early in infection, shortly after the appearance of M and S, the first progeny virions are seen by electron microscopy in the perinuclear region. Budding of these early particles occurs into small, smooth vesicles or tubules lying between the rough endoplasmic reticulum and the *cis* side of the Golgi stack. This smooth membrane compartment, termed *budding compartment* by the authors (S. A. Tooze *et al.*, 1988), is distinct from and does not form part of the Golgi complex but is connected to the rough endoplasmic reticulum (Krijnse Locker *et al.*, 1994a). At early times, budding does not occur in either of these two organelles. Later, however, budding also starts to occur into the rough membranes of the endoplasmic reticulum, and this compartment becomes the major site of virus assembly late in infection. Budding into the Golgi cisternae

is a rare event. Virions are transported from their site of synthesis to and through the Golgi complex by vesicular carriers. In the Golgi complex they are usually seen only at the rims of the stacks. At the *trans* side of the Golgi system, particles are collected into vesicles of the constitutive exocytic pathway (J. Tooze *et al.*, 1987) and released from the cell.

The budding compartment also exists in uninfected Sac(−) cells, where it is associated with transitional elements and vesicles of the rough endoplasmic reticulum and the *cis* side of the Golgi complex. Presumably, it is equivalent to the intermediate or salvage compartment which plays a crucial role in the sorting of resident proteins of the endoplasmic reticulum (for review, see Hauri and Schweizer, 1992). As virions accumulate in the budding compartment during MHV-A59 infection, it becomes strongly dilated.

Budding into the intermediate compartment seems to be a general feature of coronaviruses. An ultrastructural analysis of cells infected with IBV, TGEV, or FIPV revealed that these viruses also use the smooth perinuclear membranes (Klumperman *et al.*, 1994). Budding into such tubulovesicular membrane structures has also been described in neural cells infected with either MHV-A59 or the JHM strain of MHV (Dubois-Dalcq *et al.*, 1982).

2. Localization and Transport of the M Protein

After its synthesis on membrane-bound polyribosomes, the M protein is transported from the endoplasmic reticulum and through the Golgi complex to finally appear outside the cell as part of virions. The rate and extent of this process differs depending on the particular virus–cell system and on the time of infection. Pulse-chase experiments early in MHV-A59 infection showed the M protein to be chased almost quantitatively from 17C11 cells within about 2 hr (Holmes *et al.*, 1981a). In contrast, very inefficient clearance of M was observed, both from 17C11 cells (Holmes *et al.*, 1981b) and from Sac(−) cells (Rottier *et al.*, 1981b) during a similar period somewhat later in the infection cycle. It appears that in early stages the synthesis of the protein is balanced with its release in virions.

To relate the localization of the MHV-A59 M protein to the site of budding, J. and S. A. Tooze *et al.* (J. Tooze *et al.*, 1984; S. A. Tooze *et al.*, 1988) labeled infected Sac(−) cells for indirect immunofluorescence with a monoclonal antibody to the protein. Early in infection, a perinuclear fluorescence pattern was observed that was similar to but more extensive than that obtained with antibodies specific for the Golgi apparatus. As infection proceeded, a more reticular staining throughout the cell appeared. No labeling of the plasma membrane occurred until late in infection when patches of fluorescence indicated association of released virions with the cell surface. Immunoperoxidase staining confirmed that the M protein had accumulated in the budding compartment and not in the endoplasmic reticulum early in infection. There was, however, also some labeling of the stacked cisternal membranes of the Golgi complex, indicating that some M protein reaches the Golgi apparatus as free integral membrane protein. As no virus assembly is observed in these compartments, it was

inferred that the density of the protein in this organelle apparently does not reach the critical threshold level required for budding.

Similar data were obtained by Klumperman *et al.* (1994) in a study of the subcellular localization of M proteins in coronavirus-infected cells. Using immunogold labeling and electron microscopy, they demonstrated that in MHV and IBV infection, both in Sac(−) cells, free M protein is present in the membranes of the budding compartment as well as in the Golgi complex. Within the Golgi complex, the distribution patterns of MHV-M and IBV-M were found to be different. MHV-M was localized more toward the *trans* side, while IBV-M was concentrated on the *cis* side, similar to the patterns found when these proteins are expressed independently (Machamer *et al.*, 1990; Krijnse Locker *et al.*, 1992a; Klumperman *et al.*, 1994).

The combined data indicate that at early times the M protein is transported as an integral membrane protein to the smooth membranes of the budding compartment where it accumulates and is incorporated into virions and from where it is exported through the Golgi complex out of the cell. At later times, when the rate of M synthesis in the rough endoplasmic reticulum exceeds the rate of its exit to the budding compartment, the protein allows virion assembly also in these reticular membranes. A fraction of M apparently escapes from being incorporated into virions and accumulates in the Golgi complex.

Interestingly, when the temperature of Sac(−) cells infected with MHV-A59 was lowered to 31 °C, release of virions was strongly inhibited (S. A. Tooze *et al.*, 1988). Electron microscopic analysis showed that virion formation at this temperature occurred normally, but that the particles accumulated in the budding compartment and in the rough endoplasmic reticulum. Entry of virions into the Golgi complex appears to be inhibited at 31 °C, as few virions were observed in or beyond this compartment and the M protein did not undergo the oligosaccharide modifications known to occur there.

MHV M protein is not transported as free integral membrane protein to the plasma membrane of infected cells (Dubois-Dalcq *et al.*, 1982; J. Tooze *et al.*, 1984, 1987; S. A. Tooze *et al.*, 1988; S. A. Tooze and Stanley, 1986). The protein is detected there only in extracellular virions that have readsorbed in clusters to the cell surface (Sugiyama and Amano, 1981; Dubois-Dalcq *et al.*, 1982; S. A. Tooze *et al.*, 1988). This is in keeping with the Golgi localization of MHV-M when expressed from its cloned gene (see Section VB). It was surprising, therefore, that free M protein was observed at the surface of cells infected with TGEV. This was shown both biochemically (surface iodination) and serologically using M-specific monoclonal antibodies by Laviada *et al.* (1990). The protein was detected at the plasma membrane as early as 4 hr after infection, i.e., before any infectious virus had been released from the cells. In another study, however, Pulford and Britton (1990) could not confirm these findings.

3. Oligosaccharide Modifications during Transport

Transport of glycoproteins through the biosynthetic pathway of the cell is accompanied by modifications of their glycan moieties as they encounter the

modifying enzymes. Due to the specific locations of these enzymes, the modifications occur sequentially in time and place. As a consequence, the state of glycosylation is an indicator of a glycoprotein's progress on the exocytic route. Many of the oligosaccharide-processing enzymes occur in the Golgi apparatus. The formation of coronavirions in a pre-Golgi compartment thus implies that these enzymes act on glycoprotein molecules which present themselves as parts of huge macromolecular structures.

The M protein of a number of coronaviruses is glycosylated only by O-linked carbohydrates (see Table I). This type of linkage is rare among viral glycoproteins, but is found quite frequently in various cellular glycoproteins. Usually, O-linked oligosaccharides occur on a polypeptide in combination with N-glycosidically linked side chains. Sometimes the O-glycosylation pattern is very extensive and complex such as in mucin-type molecules. For these and other reasons, O-glycosylation has been difficult to study, and our knowledge lags far behind that of N-glycosylation. Because of its relative simplicity, the coronavirus M protein seems an attractive tool to catch up.

The M protein of MHV-A59 has been resolved into a number of differentially O-glysosylated forms. As mentioned previously, two types of oligosaccharide side chains are bound to M in the virions produced by 17Cl1 cells (see Fig. 1) (Niemann *et al.*, 1984). The same structures were found to predominate when the virus was grown in Sac(−) cells (S. A. Tooze *et al.*, 1988), but at least two additional glycosylated species were detected in these cells. One was identified as carrying only *N*-acetyl-galactosamine (GalNAc); the other was not identified. Two glycosylated forms were observed in AtT20 cells, a pituitary tumor cell line, but their structures were not clearly resolved (J. Tooze *et al.*, 1987). In all cases, a significant proportion of the M molecules remained unglycosylated. These observations indicate that the extent and possibly also the nature of O-glycosylation varies and is determined by the host cell.

Early studies on the biosynthesis of MHV-A59 M, both in infected Sac(−) cells (Rottier *et al.*, 1981b) and in 17Cl1 cells (Holmes *et al.*, 1981b; Niemann *et al.*, 1982), suggested that the addition of O-linked oligosaccharides is a post-translational event. This idea is now well established (S. A. Tooze *et al.*, 1988; Krijnse Locker *et al.*, 1992a, 1994a). The M protein is synthesized in a non-glycosylated form in the rough endoplasmic reticulum and is transported to the intermediate compartment where the first sugar, GalNAc, is added. Due to the membrane-continuities between these compartments, no vesicular transport step is required for this addition (Krijnse Locker *et al.*, 1994a). All subsequent modifications occur in the Golgi apparatus. First, a galactose unit is added, followed immediately by sialic acid. The transferase enzymes responsible for these additions are both located in Golgi cisternae, proximal to the *trans*-Golgi network. Finally, further modifications can lead to the appearance of two more forms of the M protein, the oligosaccharides of which have not been characterized. These modifications occur in the *trans*-Golgi network as they can be inhibited specifically by treatment of the cells with the drug brefeldin A (Krijnse Locker *et al.*, 1992a).

Coronavirus M proteins that are modified by N-linked oligosaccharides acquire their sugars cotranslationally in the endoplasmic reticulum. Core-

glycosylation is, however, not a very efficient process with these proteins. Unglycosylated and partially glycosylated molecules occur in infected cells as well as in the virions released from them (Stern and Sefton, 1982a, b; Stern *et al.*, 1982; Cavanagh, 1981, 1983; Vennema *et al.*, 1990b; Jacobs *et al.*, 1986). Incomplete glycosylation does not correlate with the involvement of a cleavable NH_2-terminal signal sequence during biosynthesis; it was observed both with IBV and with FIPV and TGEV M proteins which have an internal and an amino terminal insertion signal, respectively. During transport to the budding compartment, and on passing through the Golgi complex en route to the plasma membrane, the high-mannose oligosaccharides are trimmed and processed, having been converted to the complex type as they are released from the cell. Again, these processes occur very inefficiently, since a significant proportion of the M molecules in extracellular virions remains sensitive to the enzyme endoglycosydase H which only recognizes immature glycans (Stern and Sefton, 1982b; Cavanagh, 1983; Vennema *et al.*, 1990b). The incomplete maturation of coronavirus glycoproteins is not specific for the M protein. The same applies to the S protein (Stern and Sefton, 1982b; Cavanagh, 1983; Vennema *et al.*,1990b). The reasons for these findings are unclear, but it is plausible that they are caused by steric effects. Since the proteins move through the Golgi compartments as part of and protruding from the viral envelope, access of the modifying enzymes to the oligosaccharides might well be severely hindered. Incomplete maturation of N-linked carbohydrates is not unprecedented and has occasionally been observed with other glycoproteins (Doyle *et al.*, 1986; Geyer *et al.*, 1988; Earl *et al.*, 1991).

B. Transport of the Expressed M Protein

The interesting membrane structure and the intracellular restriction in coronavirus-infected cells prompted studies of the biogenesis and transport of the M protein in the absence of other coronaviral proteins. The M proteins of MHV-A59, IBV, TGEV, and FIPV have been expressed in cells from cloned cDNA (e.g., Machamer and Rose, 1987; Rottier and Rose, 1987; Machamer *et al.*, 1990; Klumperman *et al.*, 1994) as well as by microinjection of an *in vitro* transcribed mRNA (Armstrong *et al.*, 1987, 1990; Mayer *et al.*, 1988). These studies unequivocally demonstrate that the protein accumulates in the Golgi apparatus and does not reach the plasma membrane. Analysis of the expressed MHV-A59 M protein by immunofluorescence in various cell types revealed the perinuclear appearance typical for the Golgi complex (Armstrong *et al.*, 1987; Rottier and Rose, 1987; Mayer *et al.*, 1988; Krijnse Locker *et al.*, 1992a; Klumperman *et al.*, 1994 (see also Fig. 6). This localization was confirmed by electron microscopy using immunogold labeling and Golgi-specific markers (Krijnse Locker *et al.*, 1992a; Klumperman *et al.*, 1994), showing that within the Golgi complex the MHV M protein is concentrated in the *trans*-most compartments. Consistently, biochemical labeling revealed that, although a proportion of the molecules usually remained unglycosylated, as in coronavirus-infected cells, the large majority acquired the O-linked oligosaccharides added in the Golgi

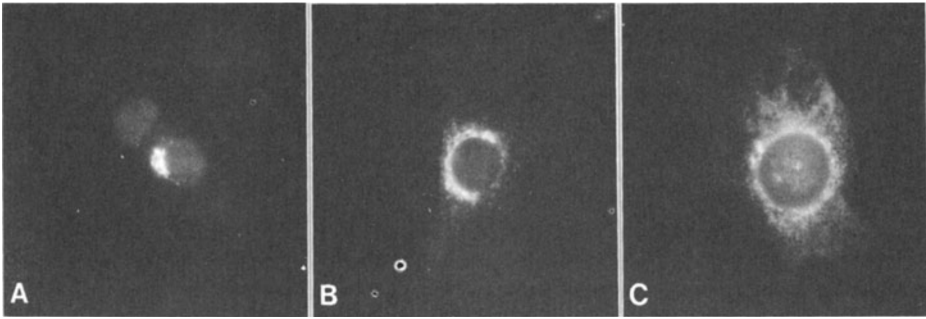

FIGURE 6. Localization of the MHV-A59 M and S protein by indirect immunofluorescence. Sac(−) cells expressing the M protein from (A) a vaccinia virus vector or, (B, C) during infection with MHV-A59 were stained with a M-specific antipeptide serum (A, B) or an S-specific monoclonal antibody after permeabilization.

apparatus (Armstrong *et al.*, 1987; Rottier and Rose, 1987; Krijnse Locker *et al.*, 1992a; Klumperman *et al.*, 1994). In contrast, the IBV M protein expressed in AtT20 cells (Machamer *et al.*, 1990) or in HepG2 cells (Klumperman *et al.*, 1994) was localized by immunoelectron microscopy to the *cis* side of the Golgi complex. Accordingly, its N-linked sugars remain largely immature. Taken together, these studies indicate that the intracellular restriction of coronavirus M is an intrinsic property of the protein and is not dependent on other coronaviral factors.

Its accumulation in the Golgi apparatus, midway on the exocytic pathway, has made the M protein an attractive tool for studying the principles of targeting to and retention in this organelle. For the IBV protein, Machamer and coworkers (Machamer and Rose, 1987; Machamer *et al.*, 1990, 1993; Swift and Machamer, 1991) have shown that the first transmembrane domain carries the signal necessary and sufficient for localization in the *cis*-Golgi region. This was demonstrated most convincingly by transferring the hydrophobic domain into reporter molecules normally transported to the plasma membrane and showing their Golgi retention (Swift and Machamer, 1991). Several uncharged polar residues were found to be critical for the functioning of the domain. These residues line one face of a predicted α-helix formed by the transmembrane domain (Swift and Machamer, 1991; Machamer *et al.*, 1993). Surprisingly, no such role is played by the first transmembrane domain of the MHV M protein. A mutant M protein with only this transmembrane domain did not leave the endoplasmic reticulum (Armstrong *et al.*, 1990; Rottier *et al.*, 1990), although inserting this domain in place of the membrane-spanning domain of a plasma membrane protein still allowed this chimeric molecule to reach the cell surface (Machamer *et al.*, 1993). Mutation studies with the MHV M protein indicate that, in this case, the carboxy-terminal domain, probably in combination with an internal domain, determines the Golgi localization (Armstrong and Patel, 1991; Krijnse Locker *et al.*, 1994b). It was suggested that different principles may act in the differential localization of the IBV and MHV M proteins in the Golgi complex (Weisz *et al.*, 1993).

VI. BIOLOGICAL FUNCTIONS

Glycoproteins of enveloped viruses are involved in a number of biological activities. Envelope proteins function in the binding of virions to cell receptors. They mediate the introduction of the nucleocapsid into the cytoplasm by triggering the fusion of viral and cellular membranes. Viral membrane proteins are also essential at the end of the infection cycle during the process of virion assembly. In some viruses, envelope glycoproteins exhibit receptor-destroying activities. Finally, the proteins generally induce immunological responses in the host by eliciting neutralizing antibodies and cytotoxicity. In coronaviruses, several of these properties have been attributed to the S and HE proteins. The M protein has been demonstrated to play a key role in coronavirus budding and to induce immunological reactions during infection in the host.

A. Role of M in Coronavirus Budding

There are two types of findings pointing to a predominant function of the M protein in the intracellular formation of progeny virus particles. First, growth of coronaviruses in the presence of tunicamycin gave rise to the production of spikeless, noninfectious virions (Holmes *et al.*, 1981b; Rottier *et al.*, 1981b; Stern and Sefton, 1982b; Mounir and Talbot, 1992). These particles were indeed devoid of S and HE protein but contained M, suggesting that M is the only envelope glycoprotein required for virus budding. Second, throughout the infection there is a correlation between the intracellular sites at which progeny virions bud and the perinuclear location of the M protein (see Section V). Concentration and location of M apparently are decisive in determining where and when budding occurs.

Molecular details of the budding process have not yet been elucidated. Conceivably, the M protein is transported as an integral membrane protein to the budding compartment or, later in infection, backs up in the endoplasmic reticulum. The protein accumulates locally to a concentration high enough to be recognized by nucleocapsids formed in the cytosol. The nucleocapsids associate with the M protein by interacting with its cytoplasmic domain. These interactions initiate budding into the lumen of the membrane compartment, whereby M is sequestered into progeny virions. An affinity of M for nucleocapsids has been observed *in vitro* with several coronaviruses. Subviral particles prepared by Nonidet P-40 disruption of purified MHV (Wege *et al.*, 1979; Sturman *et al.*, 1980), HEV (Callebaut and Pensaert, 1980), IBV (Lanser and Howard, 1980), or BCV (King and Brian, 1982) still contained M protein associated with the nucleocapsids. The significance of these interactions remains to be assessed. Binding of MHV-M to nucleocapsids did occur through an interaction with the RNA but was not specific for viral RNA and was dependent on a temperature-induced conformational change in the M protein (Sturman *et al.*, 1980). The distribution of positive charges over the M molecule certainly seems to favor an association with the viral RNA. For instance, all but one of the 18 arginines and lysines present in the MHV M protein are located at the cyto-

plasmic face of the intracellular membrane; of these, 5 occur in the carboxy-terminal 24 amino acids.

Little is still known about where and how the other viral membrane proteins are assembled into coronavirions. Electron microscopic observations have shown that the envelopes of budding virions were covered with peplomers (Chasey and Alexander, 1976; Dubois-Dalcq *et al.*, 1982). Apparently, budding involves the collective incorporation of all viral envelope proteins. This implies that the spike glycoproteins convene with the M proteins at the sites of budding and, by consequence, that the different viral envelope proteins recognize each other and interact. Association of the M and S protein in MHV-A59 infected cells has indeed been demonstrated (Opstelten *et al.*, 1993a, 1994). After their synthesis, M molecules rapidly engage in formation of noncovalently linked heteromultimeric structures with S proteins, probably already in the endoplasmic reticulum. In contrast, newly synthesized S protein first has to undergo a number of folding reactions before it reaches a conformation competent to interact with M. If proper folding of S is prevented, e.g., by inhibiting the formation of disulfide bonds, no complexes between the two proteins are formed and the M protein is transported to the Golgi complex (Opstelten *et al.*, 1993b).

These data suggest a model of virus assembly in which the viral envelope proteins form complexes that accumulate in the budding compartment to generate a microenvironment where nucleocapsids can bind and assemble into virions. Such a process would explain the exclusion of host cellular membrane proteins from virions as the molecular selection for viral membrane proteins would preclude their incorporation into the heteromultimeric complexes. It might also explain why the M and S proteins are efficiently assembled into viral particles in the budding compartment, while their intrinsically preferred destinations are the Golgi complex and the plasma membrane, respectively. Consistent with such a model, the MHV M and S proteins when coexpressed in cells were found to associate and form complexes (D.-J. Opstelten and P. Rottier, unpublished observations). Surprisingly, however, these complexes were not retained in the budding compartment, but accumulated in the Golgi complex. Apparently, other viral factors are required to localize budding in pre-Golgi membranes. One possibility is that the nucleocapsid plays an organizing role. Alternatively, the small nonglycosylated virion membrane protein (SM) might be important. So far, this protein has largely been neglected. Further studies are warranted, however, because the protein was recently shown to play a key role in the formation of viruslike particles (H. Vennema, G.-J. Godeke, and P. Rottier, unpublished observations).

While the M protein is required for budding, its glycosylation is not. Nonglycosylated coronavirus M proteins are able to efficiently direct the formation of virions as was shown using tunicamycin (Stern and Sefton, 1982b) and monensin (Niemann *et al.*, 1982), inhibitors of N- and O-glycosylation, respectively. Moreover, in the absence of drugs no particular form of the differentially glycosylated M species is preferentially incorporated into virions. Their relative abundance in extracellular viral particles correlates with the ratio of their

appearance in the infected cell (Stern and Sefton, 1982b; Holmes *et al.*, 1981a). Additional evidence that glycosylation of M is not essential for virus assembly comes from the demonstration by Laude *et al.* (1992) that disruption of the consensus sequence of the sole N-glycosylation site in the M protein of TGEV results in a viable mutant virus. The authors mention that the mutation did not affect the specific infectivity of the virus nor its multiplication rate. Apparently, the oligosaccharides attached to the coronaviral M proteins are not important for the infection process at the level of the cell. They probably play a role in the interaction with the host at the level of the organism.

B. Induction of Immunological Responses

With the exception of FIPV (Vennema *et al.*, 1990a), the S glycoprotein of coronaviruses is believed to be the prime inducer of protective immunity. Nevertheless, several observations indicate that immune responses to other viral proteins, including the M protein, may also play a role. Though largely buried within the membrane, the M protein does elicit specific antibodies during infection in the host and also when expressed separately through a live carrier virus (e.g., Pulford and Britton, 1990; Vennema *et al.*, 1991a; Wesseling *et al.*, 1993).

Monoclonal antibodies to the M protein can neutralize infectivity *in vitro*, but only in the presence of complement, as was shown for some monoclonal antibodies to MHV-M (Collins *et al.*, 1982; Fleming *et al.*, 1989) and TGEV-M (Woods *et al.*, 1988). In most cases, however, anti-M antibodies appear non-neutralizing *in vitro* (Buchmeier *et al.*, 1984; Laude *et al.*, 1986; Deregt and Babiuk, 1987; Fiscus and Teramoto, 1987; Fleming *et al.*, 1989).

Little is known about the protective effects of antibodies to the M protein in animals. Monovalent antibodies elicited by immunization of mice with purified MHV M protein failed to protect against a virus challenge (Hasony and Macnaughton, 1981). In contrast, two of four monoclonal antibodies to MHV-JHM M did protect mice from a normally lethal challenge after passive transfer (Fleming *et al.*, 1989). This protection was not associated with a particular antigenic determinant in the M protein nor was it mediated by complement. Interestingly, one of the two antibodies was not neutralizing *in vitro*, not even in the presence of complement. Some level of protection against another coronavirus was also observed in cats after immunization with a recombinant vaccinia virus expressing the FIPV M protein (Vennema *et al.*, 1991a). Although all animals seroconverted after a challenge with a lethal dose of FIPV and developed clinical signs, three of eight kittens survived.

The M protein of TGEV can induce the production of α-interferon in lymphocytes as was shown by the inhibiting effect of anti-M monoclonal antibodies on interferon induction by fixed TGEV-infected cells (Charley and Laude, 1988). By analysis of mutant viruses with reduced interferogenic potential, this biological activity was mapped to the exposed amino-terminal region

of the M molecule (Laude *et al.*, 1992). The significance of α-interferon action for immunity to viral infection needs to be established.

Little is known about the cellular immune response to coronavirus infections. A cytotoxic T-cell response to the M protein has not been described. It has been suggested, however, that cell-mediated recognition of the M protein might be an important part of an effective immune response to MHV-JHM (Mobley *et al.*, 1992).

VII. SUMMARY AND PERSPECTIVES

The coronavirus M protein has a unique molecular structure. The different domains in its structure may explain the specific properties of the protein and reflect its biological features in infection.

The N-terminal hydrophilic virion ectodomain carries the N- or O-linked oligosaccharides. It is the most variable part of the molecule which presumably contains the major antigenic determinants. It is responsible for immunological reactions such as antibody and interferon induction.

The hydrophobic region containing the three transmembrane helixes constitutes the core of the protein and is probably responsible for its peculiar physical properties. This region dictates the protein's topology in the membrane and its intracellular transport. It is the part of the protein most likely to be engaged in the intermolecular interactions that must occur at the membrane during the virion assembly process, both mutually between M molecules and with other viral membrane proteins.

The carboxy-terminal half of the molecule consists of an amphiphilic portion and an exposed tail facing the cytoplasm in infected cells. Virion budding is accomplished by the association of nucleocapsids with this part of the M protein.

The data reviewed in this chapter demonstrate that the coronavirus M protein has a number of interesting features both as a virion protein and as a model membrane protein. Though much has already been learned, many fundamental questions remain to be answered. Clearly, more needs to be known about the precise mechanism by which the protein is assembled in the membrane, about the disposition of the amphiphilic region of the molecule, and about the signals governing its intracellular transport. Little is known about the molecular details of the assembly of coronaviruses, the interactions of the M proteins with the nucleocapsid, or the interactions with the other membrane components. Nothing is known about the importance of the differential glycosylation among coronaviral M proteins. Some of these questions may be approached through mutagenesis and expression of the M gene. The answer to most questions, however, will require the generation of mutant viral genomes, either by the manipulation of infectious cDNA clones or by RNA recombination. This holds true not only with respect to the role of the M protein but for the molecular analysis of coronavirus infection in general. Evidently, this will be the major challenge for coronavirologists in the years to come.

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