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The Cytoplasmic Tail of Mouse Hepatitis Virus M Protein Is Essential but Not Sufficient for Its Retention in the Golgi Complex*

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The M protein of mouse hepatitis virus (MHV) is a triple-spanning membrane glycoprotein that is exclusively *O*-glycosylated. When expressed independently, it accumulates in late Golgi and the trans-Golgi network (TGN) (Locker, J. K., Griffiths, G., Horzinek, M. C., and Rottier, P. J. M. (1992) (*J. Biol. Chem.* 267, 14094–14101). To analyze the domains of this protein responsible for its localization, we have generated deletion mutants by site-directed mutagenesis and analyzed their intracellular transport. The intracellular distribution of the mutant proteins was determined by following the extent of *O*-glycosylation in pulse-chase experiments, by electron microscopic immunocytochemistry, and by surface immunoprecipitation. Mutant proteins lacking the first or the first and second transmembrane domains were not efficiently retained in the Golgi complex or TGN. The latter mutant proteins also localized to endocytic compartments but were not subject to rapid lysosomal degradation. Deletion of the COOH-terminal 22 amino acids, including a tyrosine residue in the context of a potential internalization signal, resulted in plasma membrane exposure of the respective mutant protein. We show that the wild-type MHV-M protein does not recycle between the plasma membrane and the TGN, but rather behaves as a late Golgi/TGN resident in our assays. We propose that the MHV-M protein is retained in the Golgi by two signals, one contained in the cytoplasmic tail and the other determined by the transmembrane domains.

Intracellular organelles are composed of specific sets of proteins that are essential for their structure and functions. After synthesis, these proteins are thought to be retained by means of sorting signals, that are often present in their primary sequence. In proteins that are retained in the Golgi complex no such signals have been identified yet and the principles that govern Golgi retention are still largely unknown. The extensive mutational analyses performed on resident Golgi proteins argue that they are retained by at least one of two possible mechanisms. For proteins residing in the cis- to trans-Golgi compartments, the transmembrane domain has been shown to be sufficient for retention (see Nilsson *et al.* (1993) for a review). Golgi-retained proteins, however, do not appear to share any obvious sequence similarity in their membrane-spanning domains. Several explanations have been put forward to explain these observations. One model has introduced the idea of "kin recognition" (Nilsson *et al.*, 1991, 1993); according to this view Golgi proteins recognize identical or related molecules forming a complex so large that it is physically unable to enter transport vesicles. Support for this hypothesis came from a study carried out by Weisz *et al.* (1993), who showed that a construct containing the first transmembrane domain of the infectious bronchitis virus (IBV)[1] M protein formed large oligomeric structures upon arrival in the Golgi complex. These data, as well as the kin recognition model, do not exclude other views on Golgi retention that state that the microenvironment of the Golgi complex or its membrane lipids may cause these proteins to aggregate (Machamer, 1993).

Proteins residing in the TGN may be retained by yet another mechanism. Here the cytoplasmic tail has been shown to be essential for TGN retention (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Wong and Hong, 1993), and within this domain short sequences appear to be important that contain a tyrosine, resembling internalization signals. Similar signals have been identified in yeast Golgi proteins (Nothwehr *et al.*, 1993; Wilcox *et al.*, 1992). It is not clear yet whether these signals merely serve for retrieval or may serve as bona fide retention signals.

It is conceivable, however, that in the TGN two mechanisms are operational, since the transmembrane domain of sialyltransferase, whose cytoplasmic tail does not contain any known internalization signal, was found to be sufficient for TGN retention (Wong *et al.*, 1992; Munro, 1991). Moreover, a more recent study on the retention of TGN38 has also attributed a role in TGN retention to the transmembrane domain of this latter protein (Ponnambalam *et al.*, 1994).

The M protein of coronaviruses has been used as a tool to study Golgi retention. The M proteins of different viruses sequenced so far have the same overall structure; they contain a short luminal domain of 20–27 amino acids that is *N*- or *O*-glycosylated, three membrane spanning domains and an approximately 100-amino acid long amphiphilic cytoplasmic domain, the most 20 COOH-terminal residues of which are exposed (Rottier *et al.*, 1984). The IBV-M protein localizes to the cis-Golgi region (Machamer *et al.*, 1990; Sodeik *et al.*, 1993;

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[1] The abbreviations used are: IBV, infectious bronchitis virus; TGN, trans-Golgi network; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MHV, mouse hepatitis virus; CI-MPR, cation-independent mannose 6-phosphate receptor; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; MVE, multivesicular endosome.

Klumperman *et al.*, 1994), while the MHV M protein localizes to trans-Golgi and the TGN (Locker *et al.*, 1992a; Klumperman *et al.*, 1994). The first transmembrane domain of IBV-M is sufficient for Golgi retention (Swift and Machamer, 1991). Studies carried out with the MHV-M protein have shown that deletion of the cytoplasmic tail caused the protein to appear at the cell surface, while deleting two of the three transmembrane domains made the protein stay in the ER or to be transported to late endosomes or lysosomes (Armstrong *et al.*, 1990; Armstrong and Patel, 1991), as assessed by indirect immunofluorescence.

In this study we have used a series of deletion mutants of MHV-M, that had been used before to study the membrane assembly of the protein (Locker *et al.*, 1992b). They were obtained by site-directed mutagenesis and expressed by vaccinia virus recombinants. Their intracellular distribution was analyzed by different biochemical assays as well as by double labeling on thawed cryosections. Collectively, our data argue that the M protein is retained by the two mechanisms that we discussed above.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies—Cos-1 cells and HepG2 cells were maintained in DMEM containing 5 and 10% fetal calf serum (FCS), respectively. The Cos cells were plated 1 day before the experiment while the HepG2 cells were done so 4–5 days before; these conditions were optimal for obtaining subconfluent monolayers. The vaccinia virus recombinants used in this study were described before (Locker *et al.*, 1992b), except for the mutant MΔCOOH. This construct was made by cloning a stop codon behind a unique *Sty*I restriction site of the wild-type M protein cDNA, deleting the COOH-terminal 22 amino acids.

Two antisera recognizing the M proteins were use: a peptide serum raised against the 18 COOH-terminal amino acids (Locker *et al.*, 1992b) and a rabbit serum raised against mouse hepatitis virus (MHV; Rottier *et al.*, 1981). The antibodies against cellular markers have been described previously, for the cation-independent mannose 6-phosphate receptor (CI-MPR) and cathepsin D by Geuze *et al.* (1985) and Klumperman *et al.* (1993) and for γ-adaptin by Ahle *et al.* (1988). Antibodies to protein disulfide isomerase were a generous gift of Dr. Steve Fuller (EMBL, Germany).

Metabolic Labeling and Immunoprecipitation—Cos-1 cells (grown in 16- or 35-mm dishes) were infected with the recombinant vaccinia viruses at a multiplicity of infection of 10 for 45 min at 37 °C. Infected cells were pulse-labeled in minimum Eagle's medium containing 2% dialyzed FCS lacking cysteine and methionine with 50–100 μCi of [^{35}S]Express label (DuPont-NEN) at 5.5 h post-infection for the indicated time and chased in DMEM, 5% FCS containing excess (2 mM was added to normal DMEM) of cold methionine and cysteine. Cells were lysed, and the labeled M proteins were immunoprecipitated with the anti-MHV serum as described elsewhere (Locker *et al.*, 1992b).

For the surface immunoprecipitation, infected and pulse-labeled cells were chased for 2 h, transferred to ice, rinsed twice with PBS, 5% FCS and incubated for 2 h at 4 °C in PBS, 5% FCS containing the anti-MHV serum (Rottier *et al.*, 1981) at a 1/20 dilution. The cells were extensively rinsed with PBS, 5% FCS to remove unbound antibody and lysed in lysis buffer (Locker *et al.*, 1992b). After spinning down the nuclei for 2 minutes at 10,000 × *g*, immunocomplexes were collected by incubating the supernatant with *Staphylococcus* A (Immunoprecipitin, Life Technologies, Inc.) for 30 min at 4 °C. The fixed bacteria were pelleted at 10,000 × *g* for 2 min, the supernatant was taken off and incubated overnight with 2 μl of anti-MHV serum for a second round of immunoprecipitation. For the "internalization assay" a normal chase was performed, but now the chase medium contained the anti-MHV serum at a 1/20 dilution. Immunocomplexes were collected as for the surface immunoprecipitation. The samples were analyzed by electrophoresis in SDS-PAGE.

The M proteins were quantitated using the method of Suissa (1983); 1 × 1-cm pieces of the autoradiogram, covering all the glycosylated forms of the protein, were cut. To correct for background, a similar piece without labeled bands was cut which was used as a blank in the subsequent measurements. The silver grains were eluted in 1 ml of filtered 1 M NaOH by incubating for 2 h at room temperature. The film was removed, and the silver grains were prevented from sedimenting by adding glycerol to 30%. The amount of silver grains was determined by measuring the absorbance at 500 nm.

Immunoelectron Microscopy—Cos cells and HepG2 cells were fixed for cryo-electron microscopy at 6 h post-infection by adding 1 ml of 0.2% glutaraldehyde and 2% acrolein in 0.1 M phosphate buffer, pH 7.4, to an equal volume of medium. After 2 h at room temperature the fixative was removed by rinsing three times with PBS. Cells were scraped with a rubber policeman and washed with PBS containing 0.15% glycine. Cells were resuspended in PBS with 10% gelatin and 1-mm^3 blocks were prepared at 4 °C. These blocks were impregnated with 2.3 M sucrose and rapidly frozen in liquid nitrogen. Cryosections were immunolabeled and double immunolabeled as described by Slot *et al.* (1988, 1991). The relative distribution over various membranes of the mutant proteins MΔ(COOH) and MΔa was determined in infected HepG2 cells in a semi-quantitative manner. Cell profiles were selected that contained a nucleus and a representative overall expression level. Per profile each gold particle within a distance of 30 nm from a membrane was counted as positive and classified into one of three categories, plasma membrane, endosomal structures, or remaining structures. The final sample number was determined by progressive mean analysis, after which the total number of gold particles was set to 100%.

RESULTS

We have recently described a study of the membrane integration of a collection of MHV-M protein deletion mutants expressed by recombinant vaccinia viruses, all of which were shown to be membrane integrated both *in vitro* as well as *in vivo* (Locker *et al.*, 1992b). These mutant proteins lacked either one or two of the three transmembrane domains or most of the COOH terminus. The mutant M proteins used in this study, including one newly prepared protein lacking the last 22 amino acids, and their topology are depicted in Fig. 1*A*. Note that in each mutant protein the orientation of the transmembrane domain is the same as in the wild-type protein.

Intracellular Localization of the M Protein Mutants by Pulse-Chase Analysis—The wild-type M protein is post-translationally *O*-glycosylated. In SDS-PAGE up to five electrophoretically different forms can be distinguished, which we have designated M_0 through M_5 (Locker *et al.*, 1992a). These forms correspond to the arrival of the M protein in different intracellular compartments (see Fig. 1*B*). Since all proteins except one (MΔa) had their NH_2 terminus on the luminal side, we could take advantage of the *O*-glycosylation as a biochemical marker to follow their transport. For this, the wild-type and mutant proteins were expressed in Cos cells and pulse-labeled for 15 min followed by a 2-h chase. Fig. 2 shows that the wild-type M protein was in the unglycosylated M_0 form after the pulse labeling but was converted completely to the glycosylated M_3 and M_4 forms during the chase. The mutant proteins MΔ(a+b), M ΔC, and MΔCOOH behaved essentially the same; they were unglycosylated after the pulse labeling and acquired the Golgi and TGN modifications during the chase. Of MΔ(a+b) and MΔC, however, a fraction was still in the unglycosylated (M_0) or GalNAc-modified (M_1) form after the 2-h chase, indicating that their transport to the Golgi complex was delayed compared to the wild-type protein. MΔ(b+c) did not acquire any detectable Golgi modifications. It remained unglycosylated for the most part, but a small portion acquired GalNAc during the chase. This indicates that the bulk of this mutant protein apparently does not leave the ER, but that a small fraction of it reached the intermediate compartment (Locker *et al.*, 1994). MΔa finally remained unglycosylated, as expected because its NH_2 terminus is on the cytoplasmic side (see Fig. 1*A*).

These data establish that at least three of the five mutant M proteins reached the Golgi complex, although with different kinetics.

Immunoelectron microscopic Localization of the M Proteins—Subsequently, the precise localization of the wild-type and mutant proteins was determined by immunogold labeling on cryosections of Cos and HepG2 cells at 6–16 h post-infection. The overall distribution of the proteins was essentially identical in

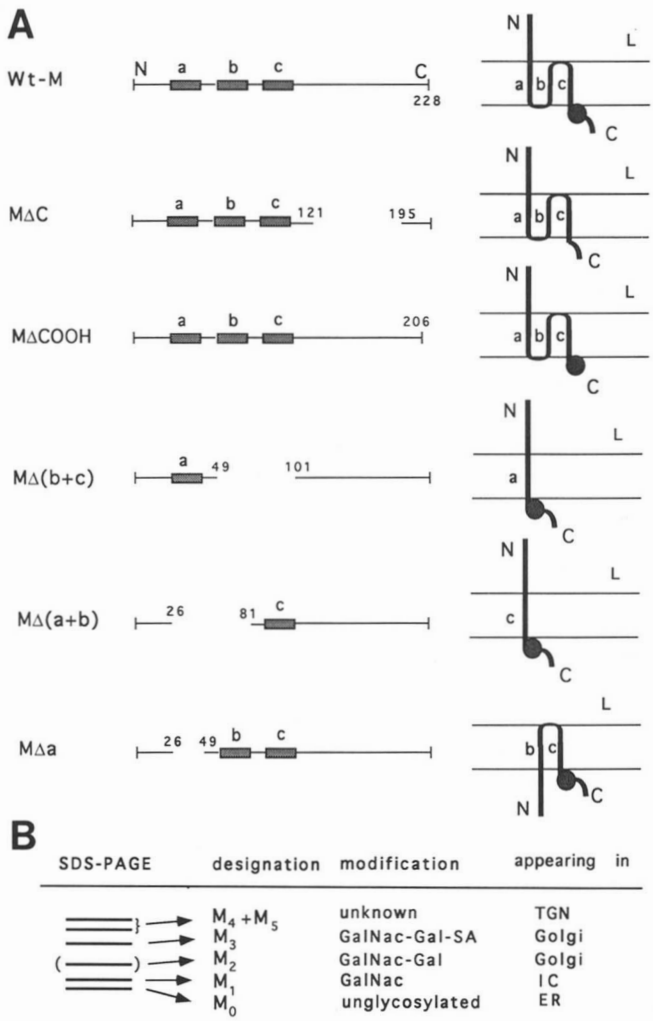

FIG. 1. *A*, schematic representation of the M protein mutants and their topology. The *filled boxes* represent the predicted transmembrane domains referred to as a, b, and c for the first, the second, and the third domains, respectively. The numbers *above* the *interrupted lines* indicate the first and the last amino acid of the deleted part. The schematic representation of the topology in the *right* part of the figure is based on earlier work (Locker *et al.*, 1992b). The *horizontal lines* represent the membrane. *L* is the luminal side of the membrane, *N* the NH_2 terminus, and *C* the COOH terminus of the wild type or mutant proteins. The *filled circle* represents the protease-resistant domain of the carboxyl-terminal half of the M protein. *B*, representation of the different *O*-glycosylated forms of the M protein as detected by SDS-PAGE, their sugar modification, and the different compartments in which the modifications have been shown to occur. The M_2 form has been placed *between brackets*, because this form does not accumulate to detectable amounts *in vivo*.

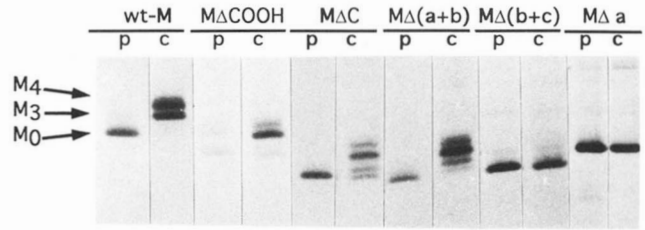

FIG. 2. **Pulse-chase analysis of the wild-type and mutant M proteins.** Infected Cos cells were pulse-labeled (*p*) for 15 min at 5 h post-infection and chased (*c*) for 2 h. The labeled proteins were prepared for immunoprecipitation and separated by SDS-PAGE. During the pulse labeling only the unglycosylated M_0 form is made, the position of which is indicated for the wild-type M protein (*wt-M*). After the chase the M_3 (the Golgi form) and M_4 (the TGN form) are formed, indicated for the wild-type M protein. Some of the mutant proteins are also converted to the two major glycosylated species, but of MΔ(a+b) and MΔC the glycosylation is delayed. Therefore these two proteins are, after the chase, also in the M_0 and M_1, the intermediate compartment derived form.

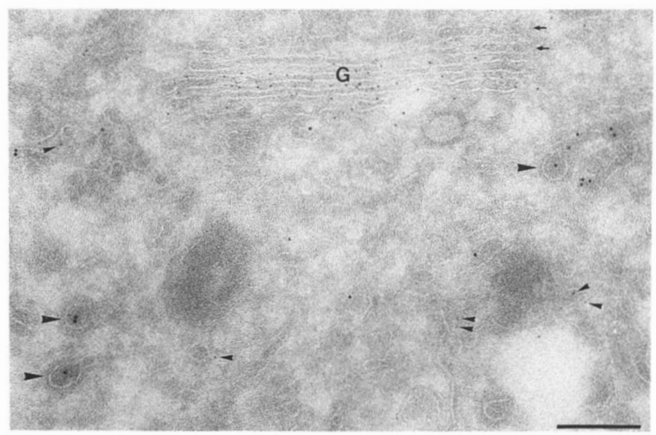

FIG. 3. **Double immunolabeling of wild-type M protein (5 nm of gold) and the CI-MPR (10 nm of gold) in the Golgi (*G*) area of infected Cos cells.** Wild-type M protein is predominantly present in the medial to trans cisternae of the Golgi, whereas the two cis-most cisternae (*arrows*) are largely devoid of label. The CI-MPR is often seen in clathrin coated areas of the TGN (*large arrowheads*). *Bars*, 200 nm.

the two cell lines. The HepG2 cells, however, were usually preferred for double labeling, since more antibodies to marker proteins are available for this cell type. The intracellular localization of the MHV-M protein in HepG2 cells has been described before (Klumperman *et al.*, 1994). The bulk of labeling was found in the intermediate compartment and the Golgi complex, and low, but specific, labeling is present in the TGN. The labeling intensity over the Golgi complex increased toward the trans side. Fig. 3 shows a similar distribution over the Golgi complex of infected Cos cells. To identify the trans side we double-labeled with antibodies to the CI-MPR (Geuze *et al.*, 1985). The plasma membrane was essentially devoid of labeling for the M protein (not shown).

Next we analyzed the distribution of the mutant proteins MΔa and MΔ(a+b). The overall distribution of these two mutant proteins appeared to be similar (see below). Since the expression level of MΔa was much higher, this protein was analyzed in more detail. When compared to the wild-type protein a larger amount of gold particles was found in the TGN (Fig. 4*A*). Within the TGN, MΔa labeling was remarkably often found in membranes containing typical electron-dense clathrin coats (Oprins *et al.*, 1993; Griffiths *et al.*, 1985; Orci *et al.*, 1984) which could be labeled with antibodies to the Golgi specific adaptor protein HAI (Fig. 4*B*) (Ahle *et al.*, 1988). In addition to being found in the Golgi region, MΔa was also found in endosome-like structures, most notably in multivesicular endosomes (MVEs), organelles apparently containing many luminal vesicles or internal membranes (Fig. 4*C*). Although their precise function is unknown (and controversial) MVEs in HepG2 cells, as well as in other cell types, have been shown to participate in some stage of the endocytic pathway (see *e.g.* Aniento *et al.* (1993), Hopkins *et al.* (1990), and Stoorvogel *et al.* (1991)). In some MVEs the protein co-localized with the CI-MPR (Fig. 4*C*). Lysosomes, identified by a high density of cathepsin D labeling and the absence of CI-MPR, were devoid of MΔa labeling (Fig. 4*D*). Despite the high level of label in endosomes, the plasma membrane was not significantly labeled. As mentioned above, MΔ(a+b) appeared to have a similar overall distribution, exhibiting prominent staining of MVEs (Fig. 4*E*).

The mutant protein MΔC showed a distribution identical to wild-type M protein; in the Golgi complex it was predominantly

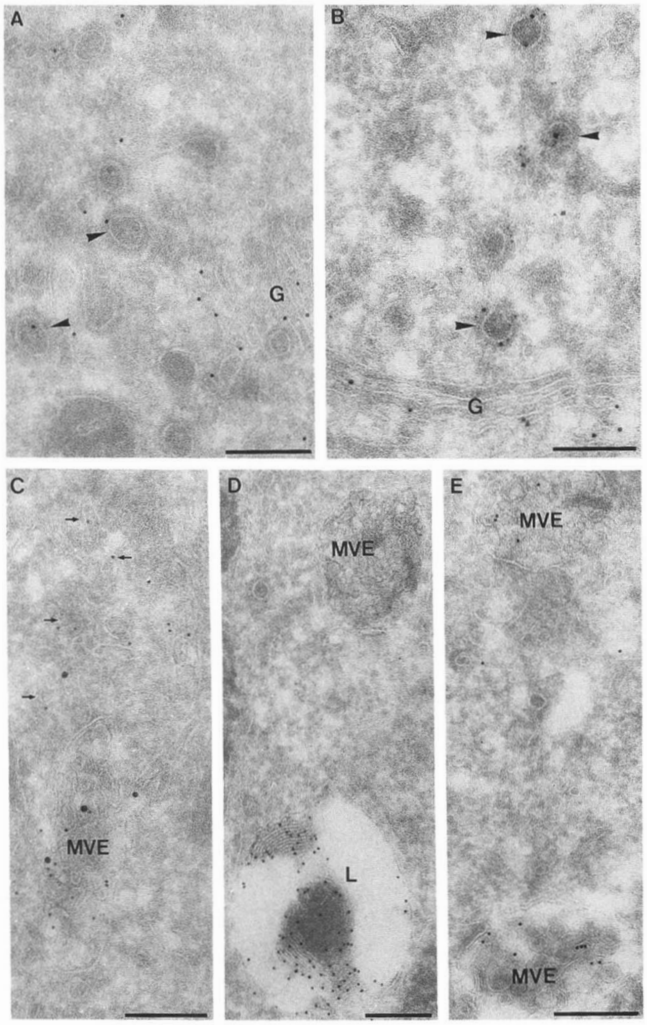

FIG. 4. **Ultrastructural localization of MΔa (*A–D*) and MΔ(a+b) (*E*) in infected HepG2 cells.** *A*, presence of MΔa in the Golgi area. Label is seen to be associated with membranes bearing a typical electron dense clathrin coat (*arrowheads*). *B*, co-localization of MΔa (10 nm of gold) and the Golgi adaptor HAI (5 nm of gold) in clathrin coated vesicles in the TGN. *C*, typical MVE, containing MΔa (7 nm of gold) and the CI-MPR (15 nm of gold). Note that MΔa is also present in the endosome-associated tubules and vesicles (*arrows*). *D*, double immunolabeling of MΔa (5 nm of gold) and cathepsin D (10 nm of gold). Cathepsin D label is restricted to lysosomes (*L*), where MΔa label is absent. *E*, presence of MΔ(a+b) in MVEs. *Bars*, 200 nm.

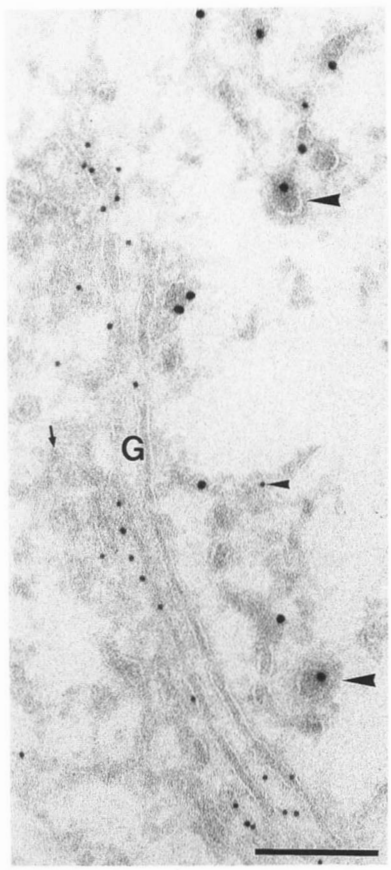

FIG. 5. **Double immunolabeling of MΔC (10 nm of gold) and the CI-MPR (15 nm of gold) in HepG2 cells.** The TGN is indicated by the presence of CI-MPR, which often localizes to clathrin coated vesicles (*large arrowheads*). The distribution of MΔC is similar to wild-type M protein; little label is found at the cis side of the Golgi (*G*) (*arrow*) and label over the TGN is low. *Bar*, 200 nm.

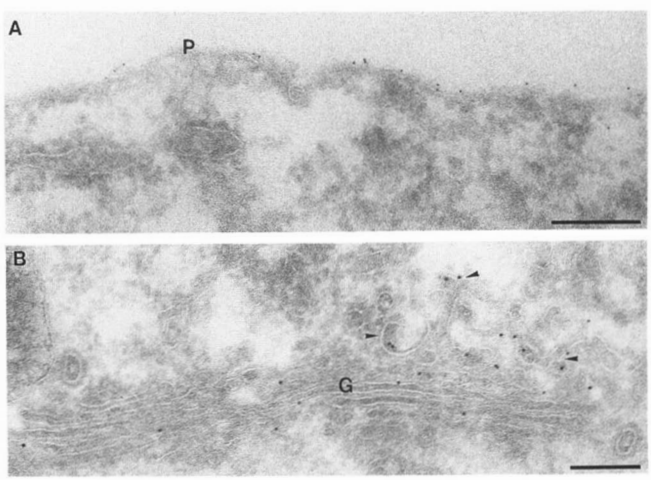

FIG. 6. **Presence of MΔCOOH in infected Cos cells (*A*) and HepG2 cells (*B*).** *A*, significant labeling of the plasma membrane (*P*). *B*, in the Golgi region MΔCOOH is found over the Golgi cisternae and in the TGN. *Bars*, 200 nm.

present at the medial to *trans* side with little label in the TGN and no label in endosomes or at the plasma membrane (Fig. 5). The mutant protein MΔCOOH, lacking the cytoplasmic tail, was readily detected at the plasma membrane (Fig. 6*A*) whereas labeling of endosomes was low or absent (not shown). Additional staining was found in the TGN and the Golgi complex (Fig. 6*B*). To get a quantitative impression of the distribution of MΔCOOH and MΔa, we determined the relative labeling of these two mutant proteins over endocytic structures and the plasma membrane (see "Materials and Methods"). Of the tail-less mutant protein, about 60% of the total labeling was associated with the plasma membrane and only 3% with endosomes. In contrast, 33% of MΔa was associated with endocytic structures and 5% with the plasma membrane.

Finally, the mutant protein MΔ(b+c) that did not acquire any Golgi modifications in the pulse-chase analysis appeared to localize to the ER, where it co-localized with protein disulfide isomerase (Fig. 7) (Hauri and Schweizer, 1992).

Collectively, the electron microscopic results are in agreement with the *O*-glycosylation patterns of the mutant proteins and add extra information about their post-Golgi localization.

Surface Immunoprecipitation and "Internalization Assay"—Since the immunocytochemistry showed that several mutant proteins localized beyond the Golgi complex, in endocytic compartments and on the plasma membrane, while the *O*-glycosylation did not allow us to distinguish between TGN and post-TGN, we attempted to obtain additional biochemical evidence for the post-TGN localization.

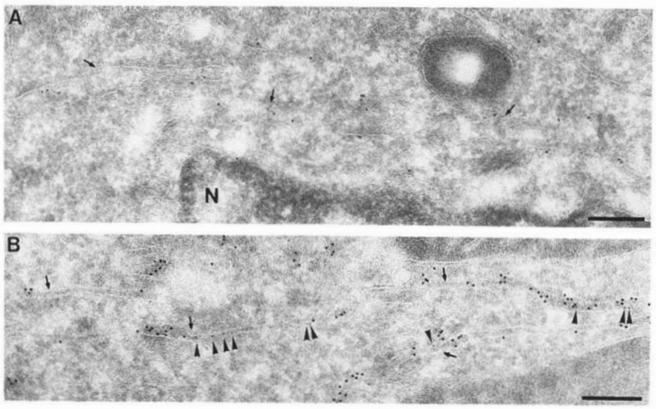

FIG. 7. **Immunolabeling of MΔ(b+c) in HepG2 cells.** *A*, MΔ(b+c) label is restricted to the membranes of the rough ER (*arrows*). *N* = nucleus. *B*, co-localization of MΔ(b+c) (5 nm of gold) and protein disulfide isomerase (10 nm of gold).

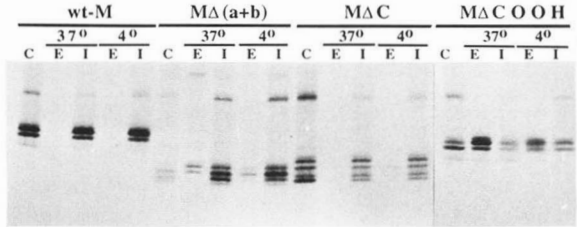

FIG. 8. **Surface immunoprecipitation and internalization assay.** M proteins were pulse labeled for 15 min and chased for 2 h (only the chase is shown). For the surface immunoprecipitation the cells were then incubated for 2 h on ice (*4°*), before lysis and immunoprecipitation. To detect wild-type and mutant proteins in endosomes or to determine whether they were rapidly internalized from the plasma membrane, the 2-h chase was also carried out in the presence of antibodies at 37 °C (*37°*). *E* is the protein that can be immunoprecipitated from the cell surface at either 0 or 37 °C, while *I* represents the remaining intracellular protein pool. The proteins were also labeled continuously for 2 h (denoted by *C*) to show all the different (unglycosylated and glycosylated) forms.

First we investigated which of the proteins reached the plasma membrane and to what extent. To this end, infected cells expressing the wild-type protein and the mutant proteins acquiring Golgi modifications (MΔCOOH, MΔ(a+b), and MΔC) were subjected to surface immunoprecipitation. MΔa was not analyzed in this assay, since its topology predicts that it has no epitopes exposed at the exterior surface. The wild-type and the three mutant proteins were expressed in Cos cells, pulse-labeled, and chased for 2 h. The amounts of the proteins that had reached the cell surface after the chase were determined by surface immunoprecipitation; the cells were cooled on ice and incubated with the anti-MHV serum. Lysates were then prepared and antigen/antibody complexes absorbed to *Staphylococcus* A. After a centrifugation step to pellet the bacteria and antibody/antigen complexes, a second round of immunoprecipitation was performed on the supernatant to determine the amount of protein that had remained intracellularly after the chase. Since immunofluorescence experiments had suggested that some of the mutant proteins were internalized after reaching the plasma membrane (not shown), the 2-h chase was also done in the presence of antibody at 37 °C to detect all the protein that had reached or passed the plasma membrane during this period. This assay may also detect antigen present in endosomes by antibody that is internalized in the fluid phase.

Fig. 8 shows that the wild-type protein was neither detected at the plasma membrane by surface immunoprecipitation, nor when the antibodies were present during the 2-h chase. Apparently, antibodies that are taken up into the cell do not reach the TGN over this period. In contrast, the mutant protein lacking the 22 COOH-terminal amino acids, MΔCOOH, could readily be detected at the plasma membrane. Quantitation (see "Materials and Methods") showed that approximately 60% of this protein had reached the plasma membrane during the chase while up to 80% could be detected after the 37 °C incubation. This shows that after the 2-h chase only little of the protein remained in an intracellular location that is not accessible to antibodies from outside. For MΔ(a+b) the situation was different since only relatively small amounts (approximately 2%) could be detected at the cell surface after the 4 °C incubation but about 7% was detected when antibodies were allowed to be internalized. Over a 2-h period, however, the bulk of this protein still remained in an intracellular location that was not accessible to antibodies from the outside. The mutant proteins MΔ(a+b) and MΔCOOH were also subjected to a time course and analyzed as above. The amount of MΔ(a+b) that could be detected by antibodies from the outside did not seem to increase after a 2-h chase (not shown). Of the tail-less mutant protein, however, essentially all of it had reached the plasma membrane after a 3-h chase (not shown).[2] In agreement with the electron microscopy data MΔC behaved essentially as the wild-type M protein; none of it was detected at the plasma membrane or in endosomes.

Collectively, these data confirm the electron microscopic results and show that MΔ(a+b) and MΔCOOH were able to arrive at the plasma membane and/or in endosomes, although the tail-less mutant protein was transported to the plasma membrane most efficiently. More importantly, both the wild-type M protein and a mutant lacking most of the amphiphilic domain of the COOH terminus, (MΔC), behaved like bona fide trans-Golgi/TGN residents in this assay.

Stability of the Mutants—Armstrong *et al.* (1990) expressing a MHV-M mutant protein identical to MΔ(a+b) showed that this protein localized to structures referred to as lysosomes by double immunofluorescence. It is important to note, however, that the antibody used in that study for the detection of lysosomes, which is directed against membrane proteins of the lamp/lgp family, labels both late endosomes and lysosomes in all cell types examined (Kornfeld and Mellman, 1989).

Since detectable levels of two of the mutant proteins appeared to be localizing to endosomes by electron microscopy (but apparently not to lysosomes), we wanted to determine whether they would also end up in degradative endocytic compartments. We therefore studied the stability by following the proteins during a long chase. For this the different proteins were expressed, pulse-labeled, and chased for up to 6 h. The cells were lysed, and after immunoprecipitation the labeled proteins were separated in SDS gels. Fig. 9 shows that over the 6-h period the labeled proteins all appeared to be stable, and none of the labeled bands seemed to disappear during the chase. Subsequently, the labeled bands were cut out of the autoradiogram, and the silver grains were eluted and quantitated by densitometry (see "Materials and Methods") (Suissa, 1983). Despite some inconsistent fluctuations in the signals, no significant decrease in the intensity of the measured bands was observed over the 6-h chase period, except for the mutant protein MΔa, which appeared to have a half-life of approximately 6 h (not shown). In agreement with the electron microscopic results, these data indicate that, rather than being degraded in lysosomes, the wild-type and mutant M proteins, MΔ(a+b), MΔCOOH, and MΔa, appeared to reside for many hours in the TGN, endosomes, and at the plasma membrane.

[2] J. K. Locker, M. Ericsson, M. C. Horzinek, and P. J. M. Rottier, manuscript in preparation.

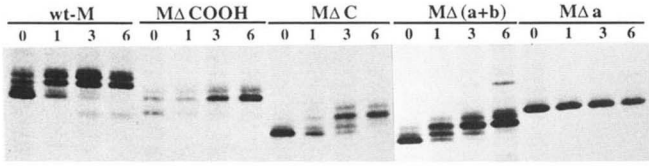

FIG. 9. **Stability of the M proteins.** The proteins were pulse-labeled (*0*) for 30 min and chased for 1, 3, and 6 h. Cell lysates were prepared, and the proteins were precipitated with the anti-MHV serum and separated by SDS-PAGE.

DISCUSSION

To investigate the molecular mechanisms of MHV-M protein retention in the Golgi complex, we generated a collection of deletion mutants lacking one or two transmembrane domains or parts of the cytoplasmic domain. We have focused on those mutant proteins that would reach the Golgi complex, including one ER mutant, MΔ(b+c). Most previous studies dealing with Golgi retention have relied on immunofluorescence for the intracellular localization and biochemical assays to detect the cell surface expression of chimeric proteins. We have analyzed the intracellular distribution of the M proteins by electron microscopy as well as by several biochemical assays. This allowed us to distinguish between localization to the Golgi complex and endosomes, a distinction that is difficult to make at the light microscopy level.

Retention of the MHV-M Protein—The MHV-M protein localizes to late Golgi and to the TGN (Locker *et al.*, 1992a; Klumperman *et al.*, 1994). Since many other studies have showed that membrane-spanning domains are important for Golgi retention (see the Introduction), it was unexpected that none of the transmembrane domains appeared to contain sufficient information for retention. Deletion of the first domain alone or of the first and second hydrophobic domain resulted in mutant proteins that localized to the TGN and endocytic compartments (see Table I for a summary). Also, the mutant protein containing the first transmembrane domain only (MΔ(b+c)) was retained in the ER. When substituted for the membrane anchor domain of the VSV-G protein, the first transmembrane domain was unable to retain the hybrid molecule intracellularly (Machamer *et al.*, 1993). The mutant protein MΔ(b+c) described in this paper, rather seemed to follow the fate of improperly folded proteins and to be degraded in the ER (not shown; Hurtley and Helenius, 1989). Deletion of most of the amphiphilic COOH terminus except for the last 22 amino acids apparently did not affect Golgi retention, as our combined electron microscopic and biochemical data showed. This implies that the approximately 100-residue long tail, with unusual properties (see *e.g.* Rottier *et al.* (1986) and Mayer *et al.* (1988)), must have another function in the M protein. Similarly, deletion of the 25 NH_2-terminal amino acids resulted in a protein that was still targeted and retained in the Golgi complex (not shown). The most striking effect was obtained when the last 22 amino acids were deleted. In our electron microscopic analysis this protein could be detected in significant amounts at the plasma membrane but not in endosomes. In agreement with this observation more than half of this protein could be immunoprecipitated from the plasma membrane after a 2-h chase. More recent data have indicated that after a 3-h chase essentially all of this protein ends up at the plasma membrane.[2]

Retention Mechanism of the Coronavirus M Proteins—The results of our study demonstrate that the retention of the MHV-M protein in the Golgi complex is not simply determined by one structural domain. Rather, the collective contributions of determinants well separated in the primary sequence of the protein seem to affect its localization. Deletion studies of MHV-M, including mutant proteins similar to the ones reported here, were carried out earlier by Armstrong *et al.* (1990) and by Armstrong and Patel (1991). In those studies the interpretation of the post-Golgi localization was based on immunofluorescence observations only. Using that approach they concluded that a mutant protein identical to our MΔ(a+b) was delivered to structures which, as explained under "Results," were probably misinterpreted as representing lysosomes. Our data show that this protein does not localize to lysosomes at the electron microscopic level, nor is it subjected to rapid lysosomal degradation. Consistent with our results a mutant quite similar to the mutant MΔ(b+c) did not leave the ER region. In addition, progressive truncations of the cytoplasmic tail resulted in progressively more plasma membrane exposure of the MHV-M protein (Armstrong and Patel, 1991). Our data support and extend the latter results. A surface immunoprecipitation approach allowed us to estimate the amount of this mutant at the plasma membrane. Furthermore, the electron microscopic data corroborated these findings by showing that, in contrast to (endosome-localized) mutant proteins that still contain the tail, the tail-less mutant protein was not detected in endosomes.

TABLE I
Summary of results

M protein	Glycosylation	Electron microscopy	Surface immunoprecipitation
Wild-type	Golgi/TGN	Golgi/TGN	–
MΔ(a+b)	Golgi/TGN	Golgi/TGN/endosomes	±
MΔa	No glycocylation	Golgi/TGN/endosomes	ND[a]
MΔ(b+c)	ER/intermediate compartment	ER	ND
MΔC	As wild type	As wild type	–
MΔCOOH	Golgi/TGN	Golgi/TGN/plasma membrane	+

[a] ND, not done.

Unlike that of the MHV-M protein, the first transmembrane domain of the IBV-M protein has been shown to be responsible for (cis) Golgi retention. In a study by Swift and Machamer (1991) this domain was shown to confer this retention to two otherwise plasma membrane-exposed proteins. Their data are largely in agreement with the results obtained with several glycosyltransferases, showing that also for the latter proteins the spanning domains are important for retention (see *e.g.* Nilsson *et al.* (1991) and Teasdale *et al.* (1992)). When comparing the cytoplasmic tails of the IBV- and MHV-M proteins it is interesting to note that the MHV-M protein has a tyrosine residue which is lacking in IBV-M (see below). Moreover, deletion of the tail of the FIPV-M protein, a protein that when expressed independently also localizes as seen by electron microscopy to the cis-Golgi region,[3] does not result in transport to the plasma membrane. Rather, the mutant protein is retained in the ER (not shown). Since the MHV-M protein and the IBV-M protein appear to be retained at different locations (intermediate compartment/cis-Golgi *versus* trans-Golgi/TGN), the different domains involved in their intracellular retention most likely reflect different retention mechanisms that operate in these compartments.

Factors That Determine TGN Retention—In our study, only one mutant was clearly expressed at the cell surface as determined by surface immunoprecipitation. This mutant lacked the cytoplasmic tail. Similar results were obtained with TGN38 where deletion of the cytoplasmic tail abolished TGN retention (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Wong and Hong, 1993).

The last 22 amino acids of the M protein that were deleted in the mutant protein MΔCOOH contain a tyrosine residue at position 211, in a context that has features expected of an

[3] J. K. Locker, J. Klumperman, V. Oorschot, M. C. Horzinek, H. J. Geuze, and P. J. M. Rottier, unpublished observations.

internalization signal; the sequence surrounding this tyrosine is rich in polar and basic residues which have been postulated to form a tight surface loop exposing the tyrosine (Ktistakis *et al.*, 1990). In addition, the YRL sequence in the M protein resembles the TGN38 YQRL signal, for which the Y and the L have been shown to be important for TGN localization (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Wong and Hong, 1993). The TGN retention of TGN38, however, is complicated by the fact that the signal needed for retention also functions in internalization from the plasma membrane. This raises the question whether recycling from the plasma membrane is part of its retention in the TGN, in a manner similar to that described for soluble ER proteins containing the KDEL sequence (see Pelham (1989) for a review). However, at steady state the bulk of KDEL proteins are in the ER and recycling from a distal compartment is only part of their retention, since deletion of the KDEL sequence does not lead to rapid release of the mutated protein from the cell (Pelham, 1989). A similar situation may apply to TGN38; the bulk of the protein is perhaps in the TGN because the cytoplasmic sequence has a high affinity for a, as yet unknown, component of the retention machinery; recycling from the plasma membrane may only serve to retrieve escaped molecules. Some evidence for this idea came from the observation that one point mutation in the YQRL sequence (the arginine replaced by an aspartic acid) abolished TGN retention without affecting internalization by coated pits. This suggests, as pointed out in the Introduction, that certain tyrosine-containing signals may be true TGN retention signals.

The MHV-M protein contains a similar cytoplasmic sequence to TGN38, but does not seem to recycle from a distal compartment (as measured by our assay), thus strengthening the idea that some internalization-like signals can be *bona fide* TGN-retention signals. The finding that progressive deletions in the cytoplasmic tail of MHV-M result in increasing loss of Golgi retention as soon as these deletions approach or include the YRL region (Armstrong and Patel, 1991) supports this view.

An alternative view on the retention of TGN38 comes from more recent data by Ponnambalam *et al.* (1994). They showed that, in addition to the cytoplasmic tail, the transmembrane domain of this protein also contains information for retention. The authors proposed that this molecule is retained in the TGN by the action of the transmembrane domain (that may serve to make oligomers; see the Introduction) and by the cytoplasmic tail that may retrieve escaped molecules. The efficient retention of the MHV-M protein, however, requires both the transmembrane domains and the cytoplasmic tail. Mutant proteins such as MΔ(a+b) and MΔa, although not readily transported to the plasma membrane, were not retained in the TGN. In addition, a hybrid construct of the normally cell surface marker CD8, whose cytoplasmic tail had been replaced by the last 22 amino acids of the M protein, was also not retained in the Golgi complex (data not shown). Thus, the simplest interpretation of our data is that the M protein retention is due to two signals, one in the transmembrane region that makes it aggregate upon arrival in the Golgi,[2] and a second that interacts with a putative TGN retention machinery. Support for the idea of an interaction of the M protein with such a "retention complex" came from the electron microscopic observation that some of the tail-containing mutant proteins localized to clathrin-coated structures in the TGN, while the tail-less mutant did not appear to localize to such regions.

Our data collectively lead to the model, suggested before by Armstrong and Patel (1991), that the retention of the M protein to late Golgi/TGN involves both the transmembrane region and the cytoplasmic tail.

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