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Oligomerization of a *trans*-Golgi/*trans*-Golgi Network Retained Protein Occurs in the Golgi Complex and May Be Part of Its Retention*

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The mouse hepatitis virus M protein is a triple spanning membrane glycoprotein that, when expressed independently, localizes to *trans*-Golgi as well as to the *trans*-Golgi network (TGN). Passage of this protein from the endoplasmic reticulum through the intermediate compartment to the late Golgi and TGN can be conveniently followed by analyzing its *O*-linked sugars.

Using pulse-chase analyses we studied the oligomerization of the M protein in sucrose gradients. The Golgi and TGN forms migrated as large heterogeneous complexes, whereas the endoplasmic reticulum and intermediate compartment forms of the protein appeared to migrate as monomer. Moreover, a mutant of the M protein lacking the 22 COOH-terminal amino acids, that is transported to the plasma membrane, gave rise to similar complexes, albeit smaller in size, that persisted at the plasma membrane.

We propose that the *trans*-Golgi/TGN retention of the MHV-M protein is governed by two mechanisms: oligomerization possibly mediated by the transmembrane domains and binding of its cytoplasmic tail to cellular factors in trans Golgi/TGN.

Proteins that are retained at intracellular locations may contain signals present in their primary sequence that specify their retention. Two well documented examples of such a signal are the KDEL sequence (ER;[1] Pelham, 1988; Tang *et al.*, 1992) and the signal consisting of two positively charged amino acids located in the cytoplasmic tails of both type I and II resident ER proteins (Jackson *et al.*, 1990; Schutze *et al.*, 1994).

No such signal has yet been identified for Golgi proteins. Mutational analyses have suggested that the transmembrane domain of many Golgi proteins contains sufficient information for retention (see *e.g.* Munro, 1991; Nilsson *et al.*, 1991; Swift and Machamer, 1991; Burke *et al.*, 1992; Colley *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992). However, these membrane-spanning domains appear not to share sequence homology.

One way to explain retention by means of transmembrane regions is by "kin recognition" (Nilsson *et al.*, 1993, 1994). According to this model, upon their arrival at the appropriate cisterna of the stack, Golgi proteins will form large heterooligomers possibly mediated by their membrane-spanning domains. Within any one cisterna these oligomers would comprise the different proteins that localize to this particular cisterna. Nilsson *et al.* (1993) have argued that the retention may also be facilitated by the cytoplasmic tails of these proteins possibly by binding to a cytosolic intercisternal matrix. A consequence of this retention mechanism is that the ensuing complexes are thought to be so large that they are physically excluded from transport vesicles (Nilsson *et al.*, 1993, 1994). Although not mutually exclusive, the putative formation of oligomers in the Golgi complex may also be induced by the microenvironment of a specific Golgi cisterna, for instance its lipid composition (Machamer, 1993). So far, only one example of complex formation or detergent insolubility of a Golgi-retained protein has been described *in vivo* (Weisz *et al.*, 1993).

Other studies have emphasized that Golgi membrane proteins have generally shorter and less hydrophobic transmembrane domains compared with plasma membrane proteins and that this may explain retention of the former (Masibay *et al.*, 1993; Bretscher and Munro, 1993). Bretscher and Munro (1993) pointed out that the plasma membrane is relatively enriched in cholesterol, a lipid that tends to make the bilayer "thicker." Golgi-resident proteins, according to this view, will be retained simply because they are sorted away from the thick bilayers. In three cases experimental evidence appeared to be consistent with this hypothesis (Munro, 1991; Swift and Machamer, 1991; Masibay *et al.*, 1993).

The M protein of coronaviruses has been extensively used as a tool to study Golgi retention (see above; Swift and Machamer, 1991; Armstrong *et al.*, 1990; Armstrong and Patel, 1991). The overall structure is well conserved among different coronaviruses; it invariably contains a short luminal domain of about 25 amino acids that is either *O* (mouse hepatitis virus, MHV)- or *N*-glycosylated (IBV-M), three membrane-spanning domains and a long amphiphilic cytoplasmic domain (see *e.g.* Rottier *et al.*, 1986). The *O*-glycosylation of the MHV-M protein has been analyzed extensively (Niemann *et al.*, 1984; Tooze *et al.*, 1988). In SDS-PAGE one can distinguish up to five electrophoretically different forms, which we have referred to as M_0 through M_5. Since these forms correspond to the arrival of the protein in different intracellular compartments (see Fig. 1; Krijnse Locker *et al.*, 1992a, 1994a), the extent of *O*-glycosylation can be used as a marker of transport of the M protein. When expressed independently the IBV-M protein localizes to the

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[1] The abbreviations used are: ER, endoplasmic reticulum; MHV, mouse hepatitis virus; PAGE, polyacrylamide gel electrophoresis; IC, intermediate compartment; TGN, *trans*-Golgi network; VV, vaccinia virus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic; BFA, brefeldin A.

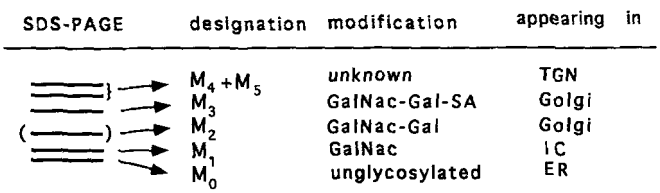

FIG. 1. **Representation of the different *O*-glycosylated forms of the M protein as detected by SDS-PAGE, their sugar modification, and the different compartments in which the modifications have been shown to occur.** The M_2 form has been placed between *parentheses*, because this form does not accumulate to detectable amounts *in vivo*.

cis-Golgi region, where it co-localizes with markers of the IC (Machamer *et al.*, 1990; Sodeik *et al.*, 1993; Klumperman *et al.*, 1994). In contrast, and for reasons that remain elusive, the MHV-M protein localizes to the trans side of the Golgi and the TGN (Krijnse Locker *et al.*, 1992a; Klumperman *et al.*, 1994). This difference in localization also seems to have implications for their retention signals. In the IBV-M protein, but not in MHV-M, the first transmembrane domain appears to be sufficient for retention (Swift and Machamer, 1991), whereas the cytoplasmic tail of the MHV-M protein, in contrast to that of IBV-M, appears to contain information for retention (Armstrong and Patel, 1991; Krijnse Locker *et al.*, 1994b). In this study we have analyzed the oligomerization of the MHV-M protein with the aim of testing the hypothesis that retention of this protein may correlate with the formation of detergent-insoluble aggregates.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies—COS-1 cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum. SA:48 cells, a kind gift of Tommy Nilsson (Imperial Cancer Research Foundation, London), were kept in Dulbecco's modified Eagle's medium, 10% FCS containing 200–400 μg/ml Geneticin (Life Technologies, Inc., Paisley, Scotland). The vaccinia virus (VV) recombinants expressing the wild-type MHV-M protein (vvMHV-M) and the M protein lacking the cytoplasmic tail (vvMHV-MΔCOOH) have been described (Krijnse Locker *et al.*, 1992b; Krijnse Locker *et al.*, 1994b). The VSV-G protein was expressed using a VV recombinant expressing the tsO45 mutant of the G protein under the control of an early, 7.5 kDa, VV promotor (vvVSV-GtsO45; a kind gift of Robert Doms, University of Pennsylvania).

The VV recombinant expressing the IBV-M protein (vvIBV-M) was a kind gift of Carolyn Machamer (John Hopkins University, Baltimore) and has been described before (Machamer *et al.*, 1990). The following antibodies were used to detect these proteins: the polyclonal anti-MHV serum (Rottier *et al.*, 1981) and a peptide serum recognizing the 18 COOH-terminal amino acids of the MHV-M protein (Krijnse Locker *et al.*, 1992b); a rabbit serum raised against the VSV-G protein was a kind gift of Jean Gruenberg (University of Geneva, Switzerland); the rabbit peptide serum recognizing the IBV-M protein (Machamer *et al.*, 1990) was kindly provided by Carolyn Machamer. To detect sialyltransferase in the SA:48 cells we used the monoclonal antibody P5D4 recognizing the VSV-G P5D4 epitope (Kreis, 1986).

Metabolic Labeling and Velocity Gradient Centrifugation—The M and G proteins were expressed by infecting COS-1 cells grown in 35- or 60-mm dishes with the respective VV recombinants as described (Krijnse Locker *et al.*, 1992b).

Except for the IBV-M protein (see below), infected cells were pulse-labeled at 5.30 h post-infection with 100–200 μCi of ^{35}S Express-label (NEN Dupont GmbH, Dreiech, Germany) for the indicated time. The cells were either lysed immediately or chased for 2 h before lysis. The lysis buffer consisted of 50 mM Tris-Cl, pH 8.0, 62.5 mM EDTA, 0.5% Nonidet P-40 (Sigma-Aldrich, Deisenhofen, Germany), 0.5% sodium deoxycholic acid (Sigma). After lysis on ice, the samples were layered on top of a continuous 15–30% (w/v) sucrose gradient layered on top of a cushion of 0.2 ml of 60% (w/v) sucrose. The sucrose solutions were in 50 mM Tris-Cl, pH 8.0, 62.5 mM EDTA, 0.1% of Nonidet P-40, and 0.1% sodium deoxycholic acid. The gradients were spun for 15–16 h at 40,000 rpm and 4 °C in a SW 60 rotor. Each gradient was fractionated in ten fractions of 450 μl, each fraction was diluted with 500 μl of lysis buffer and the labeled proteins they contained were immunoprecipitated with 1–3 μl of the respective antibody. The immunoprecipitation was carried out as described (Krijnse Locker *et al.*, 1992b), and the proteins were analyzed in a 10 or 15% SDS-PAGE. The treatment with brefeldin A was carried out as described (Krijnse Locker *et al.*, 1992a). The endo-β-*N*-acetylglucosaminidase H treatment of the VSV-G protein was as described earlier (Klumperman *et al.*, 1994).

Western Blotting and Enhanced Chemiluminescence (ECL)—Since the IBV-M protein was difficult to detect by immunoprecipitation, this protein was analyzed by ECL. COS cells infected with vvIBV-M were lysed as above at 9 h post-infection. The lysate was layered on a sucrose gradient and spun as described above, but the M protein present in the collected fractions was trichloroacetic acid-precipitated as follows; each fraction of the gradient was adjusted to 10% sucrose with lysis buffer, and an equal volume of 20% trichloroacetic acid, 50% acetone, and 10 μg of myoglobin as carrier was added. The samples were left on ice for 60 min, spun for 15 min at 14,000 rpm at 4 °C, and the pellets were dissolved in Leammli sample buffer. The precipitates were run in a 15% SDS-PAGE and transferred to nitrocellulose (ECL, Hybond, Amersham International, Buckinghamshire, United Kingdom). The blot was extensively blocked with 5% milk powder in phosphate-buffered saline containing 0.1% Tween 20. The blot was incubated for 1 h with the anti-IBV-M peptide serum at a 1/1000 dilution. The secondary antibody, goat anti-rabbit conjugated to horseradish peroxidase was from Cappel (Cooper Biomedical, West Chester, PA). The IBV-M protein was detected by ECL (Amersham) according to the instructions of the manufacturer.

Estimation of the Size of the Complexes—For the estimation of the size of the M protein complexes two proteins with known $s_{20,w}$ were used; catalase (11.3 $s_{20,w}$) and thyroglobulin (19.3 $s_{20,w}$) were centrifuged as described above, the proteins in the gradient fractions were trichloroacetic acid-precipitated, run in SDS-PAGE, and stained with Coomassie Blue. The size estimation of the M protein and the mutant protein MΔCOOH complexes was carried out according to Martin and Ames (1961).

Surface Immunoprecipitation—The surface immunoprecipitation of the mutant M protein MΔCOOH was done as described (Krijnse Locker *et al.*, 1994b).

Electron Microscopy—SA:48 cells were infected with the recombinant VV expressing the MHV-M protein as described (Krijnse Locker *et al.*, 1992b). At 6 h post-infection 16 nm bovine serum albumin-gold at an OD of 4 was added to the culture medium, as well as 100 μg/ml cycloheximide. Before fixation the cells were permeabilized with streptolysin O and fixed as described (Krijnse Locker *et al.*, 1994a). The cells were prepared for cryosectioning and subsequently double-labeled as described (Slot *et al.*, 1988, 1991).

Quantitation of the Labeled Bands—Each gradient fraction was subjected to immunoprecipitation and the labeled proteins were separated in 15% SDS-PAGE. The gel was fixed for 1 h in 20% methanol, 7% acetic acid and dried. The quantitation of the bands was performed by PhosphorImager. In the first fraction from the bottom of the gradient as well as in the pellet we consistently observed a labeled band of approximately 25 kDa, most likely derived from VV, that almost co-migrated with the M_3 form of the M protein. We therefore quantitated the 25-kDa protein in the pellet fraction and substracted its value from that of the first fraction of the gradient.

RESULTS

Intracellular Localization of the MHV-M Protein—We have recently shown that the M protein, when expressed using a vaccinia virus recombinant localized to late Golgi and the TGN (Krijnse Locker *et al.*, 1992a). Moreover, we observed that the M protein, unlike TGN38 and furin (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Molloy *et al.*, 1994), does not appear to recycle between the plasma membrane and the TGN (Krijnse Locker *et al.*, 1994b). For the present study we wanted to determine in more detail how much of the M protein would reach the TGN after a 2-h chase with cycloheximide. For this we made use of a HeLa cell line stably transfected with the human α-2,6 sialyltransferase tagged on its COOH terminus (the luminal side) with the P5D4 epitope of the VSV-G protein.[2] These cells were infected with the recombinant VV expressing the M protein. Six hours after infection we treated the cells for 2 h with the

[2] C. Rabouille, K. Hunte, B. Kiekbusch, E. Berger, G. Warren, and T. Nilsson, submitted for publication.

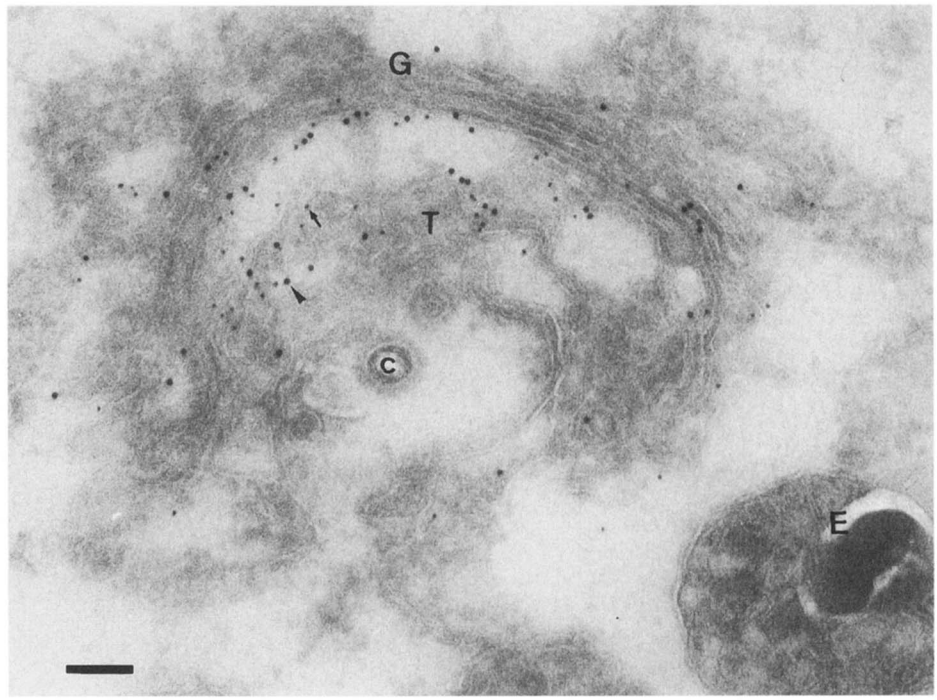

FIG. 2. **Localization of the MHV-M protein in SA:48 cells.** The M protein was expressed using vvMHV-M. The infected cells were treated with bovine serum albumin-gold and cycloheximide to "chase" the M protein. Before fixation the cells were permeabilized with streptolysin O. The M protein (10 nm gold, *arrowhead*) localizes mainly to one side of the Golgi, where it co-localizes with sialyltransferase (detected by anti-P5D4, 5 nm gold, *small arrow*) in the TGN. On this side of the stack typical electron-dense clathrin coats are visible (*c*), as well as tubular elements (*T*). E, endosome. *Bars*: 100 nm.

endocytic marker bovine serum albumin-gold in the presence of cycloheximide. Under these conditions, by blocking protein synthesis we chased the M protein to its final location, whereas the cells internalized the gold to essentially all endocytic compartments. The intracellular distribution of the M protein was determined using the P5D4-tagged sialyltransferase as a marker of the TGN (Roth *et al.*, 1985). In most of the cells the M protein was clearly located on one side of the stack, co-localizing in part with sialyltransferase in the TGN (Fig. 2). Only very little label for the M protein was found in endosomes that were labeled with the internalized gold (not shown). In some cells considerable amounts of the M protein were also found throughout the Golgi stack extending beyond the label for sialyltransferase, probably due to accumulation of the protein as a result of overexpression. These data confirm earlier results showing that the MHV-M protein localizes to the trans side of the Golgi stack and to the TGN. They show in addition that under our experimental conditions we were unable to chase the M protein quantitatively into the TGN (see below).

The M Protein Occurs in Heterogeneous Complexes in the Golgi Complex—In MHV infected murine cells the two viral membrane glycoproteins, M and the spike (S), interact. This interaction can be followed by co-immunoprecipitation under specific detergent conditions (Opstelten *et al.*, 1994a). When analyzed in sucrose gradients the complexes of the M and S proteins appeared to consist of large heterogeneous heterooligomers of M and S (Opstelten *et al.*, 1994b). Since the S protein, when expressed independently as well as in infected cells, is assembled into trimers (Delmas and Laude, 1990)[3] it seemed likely that the M protein was responsible for this multimer formation. We decided, therefore, to analyze the oligomeric structure of the M protein in more detail.

COS-1 cells were infected with vvMHV-M expressing the M protein. The cells were pulse-labeled and chased and lysed with the same detergent combination used previously to demonstrate the M/S complexes, consisting of 0.5% Nonidet P-40 and 0.5% sodium deoxycholic acid (Opstelten *et al.*, 1994a; see also "Materials and Methods"). The lysates were loaded onto 15–30% sucrose gradients, centrifuged, and after fractionation the M protein was immunoprecipitated from each fraction. The unglycosylated ER form of the M protein (M_0; see Fig. 1) labeled during the pulse, stayed at the top of the gradient (in the upper four fractions). However, the glycosylated Golgi forms, formed during the 60-min chase, were heterogeneously distributed over the gradient (Fig. 3*A*). This heterogeneous pattern did not change after a 120-min chase or after chasing for up to 6 h (not shown). It also remained unchanged when intact, or streptolysin O permeabilized cells were treated with the cross-linker dithiobis(succinimidylpropionate), a membrane-permeable cross-linker, before lysis (not shown). It is significant to note in Fig. 3*A* that the residual fraction of the M protein that did not acquire Golgi-specific modifications during the chase (M_0 and M_1) remained in the upper fractions of the gradient. To show the M_0 and M_1 forms more clearly, an overexposed autoradiogram of the chase is shown in Fig. 3*B*.

When testing the effect of different detergents, CHAPS (20 mM) appeared to give a similar heterogeneous distribution of the glycosylated forms, whereas non-ionic detergents such as Triton X-100 and Nonidet P-40 failed to preserve any complexes (not shown).

We next quantitated the distribution of the immunoprecipitated forms of the M protein over the different fractions by PhosphorImager (see "Materials and Methods"). As expected from Fig. 3, after the pulse labeling the bulk of the label was present at the top of the gradient, whereas after the chase the M protein was found more or less evenly distributed over the gradient with a slight increase toward the bottom (Fig. 4). In the bottom fraction we consistently noted a 25-kDa protein (as well as two other proteins that were not co-migrating with the M protein), probably derived from the vaccinia virus infection, that almost co-migrated with the M_3 form of the MHV-M protein. Since some of this 25-kDa protein was apparently also present in fraction number 1, we quantitated the 25-kDa protein in the bottom fraction and substracted this amount from that of the first fraction (see Fig. 3 and "Materials and Methods"). In order to estimate the size of the M protein complexes, two proteins with known $s_{20,w}$ value were used as sedimenta-

[3] H. Vennema, H. Blaak, G. J-Godeke, M. Horzinek, P. Rottier, manuscript in preparation.

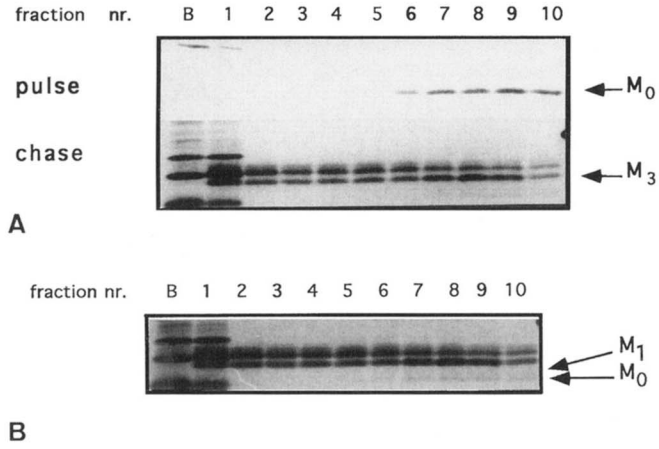

FIG. 3. *A*, pulse-chase analysis of the M protein. The M protein was expressed by vvMHV-M in COS cells. The cells were pulse labeled for 12 min and chased for 60 min. Pulse-labeled and chased cells were lysed and lysates were layered on top of a 15–30% continuous sucrose gradient. After centrifugation the gradients were fractionated from the bottom (*fraction 1*) to the top (*fraction 10*). The bottom (*B*) of the tube was resuspended in lysis buffer to dissolve pelleted material. The M protein was immunoprecipitated from each fraction and analyzed in a 15% PAGE. After the pulse labeling only the unglycosylated ER form (M_0; indicated) is apparent at the top of the gradient. After the chase the Golgi (M_3; indicated) and the TGN (not indicated) forms are observed and these are heterogeneously distributed over the gradient. In *B* the chase part of *A* has been overexposed to emphasize the M_0 and M_1 forms (indicated) that remain at the top of the gradient.

tion markers. From Fig. 4 it is clear that the largest complexes of the glycosylated M protein migrated with sedimentation values larger than 19 $s_{20,w}$; the largest complexes near the bottom of the gradient migrated around 30 $s_{20,w}$. Assuming that these multimers consist of the M protein only (see "Discussion") the complexes in the first fraction of the gradient corresponded to approximately 1000 kDa, equivalent to about 40 molecules of the M protein. Collectively these data indicate the M protein occurs in heterogeneous complexes in the Golgi complex.

The VSV-G Protein Does Not Occur in Large Complexes—The specificity of the above described observations was investigated by making use of another well characterized viral membrane protein, the VSV-G protein. This latter protein, like the M protein, was expressed using a recombinant VV (see "Materials and Methods"). The trimerization of the VSV-G protein has been described in detail, and the preservation of the trimers analyzed using sucrose gradients requires a low pH (Doms *et al.*, 1987). Likewise, using the detergent mixture used in this study that requires a neutral to alkaline pH (Helenius and Simons, 1975), the G protein remained at the top of the gradient both after the pulse labeling as well as after the chase, peaking in the second and third fraction from the top (Fig. 5). It seemed thus that the G protein migrated as a monomer, both after the pulse and after the chase. Clearly, the protein was transported through the Golgi complex during the chase, since it became almost completely endo-β-*N*-acetylglucosaminidase H-resistant (Fig. 5). When transport to the plasma membrane was prevented and the G protein was accumulated in the TGN by chasing at 20 °C (Griffiths *et al.*, 1985), no higher order oligomeric structures were observed under our experimental conditions (not shown).

A Tail-less Mutant of the M Protein That Fails to be Retained in the Golgi Complex Also Occurs in Complexes—From the preceding results it is clear that the M protein occurs in heterogeneous complexes in the Golgi complex, whereas a plasma membrane protein failed to do so. Therefore, it was of interest to analyze the behavior of a mutant M protein that fails to be retained in the TGN but rather is transported to the plasma membrane. Deletion of the 22 COOH-terminal amino acids of the M protein results in the delivery of the mutant protein to the plasma membrane (Armstrong and Patel, 1991; Krijnse Locker *et al.*, 1994b).

This tail-less mutant protein (called MΔCOOH) was pulse labeled and chased for 3 h to accumulate more than 90% of the labeled protein at the plasma membrane as determined by surface immunoprecipitation (Fig. 6). The cells were lysed and the protein analyzed as before. From Fig. 6 it is clear that the mutant protein behaved similar to the wild-type protein appearing in heterogeneous complexes that now occurred at the cell surface. Upon closer inspection, however, the complexes of this particular protein appeared to be consistently smaller than those of the wild-type protein, since they were absent from the two lowest fractions from the bottom. Also, they appeared to be more homogeneous, since they were largely concentrated in the fractions 4 through 6. This enabled us to estimate their size in more detail. By running the marker proteins catalase and thyroglobulin and MΔCOOH in one gradient the average size of the mutant protein's complexes appeared to be about 19 $s_{20,w}$ corresponding to about 650 kDa or 30 molecules of the mutant protein.

Determination of the Initial Site of Complex Formation—From Fig. 3, *A* and *B*, it is clear that the unglycosylated ER form of the M protein (M_0) and the M_1 form, which carries the IC modification *N*-acetylgalactosamine (GalNAc; Krijnse Locker *et al.*, 1994a), did not appear in large complexes. Also, when the M protein was labeled continuously for 2 h the M_0 and M_1 species were not found in complexes (not shown). This suggests that complex formation of the M protein occurs upon, or after, arrival in the Golgi complex. In an attempt to determine where in the Golgi complex, the complexes would first be detected, we labeled the M protein in the presence of BFA, thereby preventing it from reaching the TGN (Chege and Pfeffer, 1990; Krijnse Locker *et al.*, 1992a).

The M protein was pulse-labeled for 15 min and chased for 1 h both in the presence of BFA. Consistent with our earlier data (Krijnse Locker *et al.*, 1992a, 1992b) the *O*-glycosylation was much faster in the presence of the drug and the M protein was already mainly converted to the M_2 and M_3 form after the pulse. After the chase only the M_3 form was made, whereas the TGN forms (M_4 and M_5) were not apparent (Fig. 7; see also Krijnse Locker *et al.*, 1992a). From Fig. 7 it is clear that in the presence of BFA the M protein was not incorporated into complexes and instead migrated as a monomer. In order to find out whether this lack of complex formation was due to a general effect of BFA or to the failure of the M protein to reach the compartment where the large complexes are formed, *e.g.* the TGN, we carried out the following experiment. The M protein was pulse-labeled and chased for 3 h without BFA to enable the bulk of the protein to reach the Golgi and the TGN. The cells were then treated for 1 h with BFA before lysis and analysis of the M protein in sucrose gradients. As shown in Fig. 8 the M protein complexes that were formed before the BFA treatment were partially resistant to the BFA treatment, since it appeared that less of the M protein was now detected in the bottom five fractions. Since the distribution of the Golgi and TGN forms of the M protein over the gradient does not change with increasing times of chase (see above), the effect of the 1 h BFA treatment must be due to a partial dissociation of the complexes.

The IBV-M Protein Does Not Form Large Oligomers—We next analyzed whether the IBV-M protein, a protein that has previously been localized to the *cis*-Golgi region (Machamer *et al.*, 1990; Sodeik *et al.*, 1993; Klumperman *et al.*, 1994) did

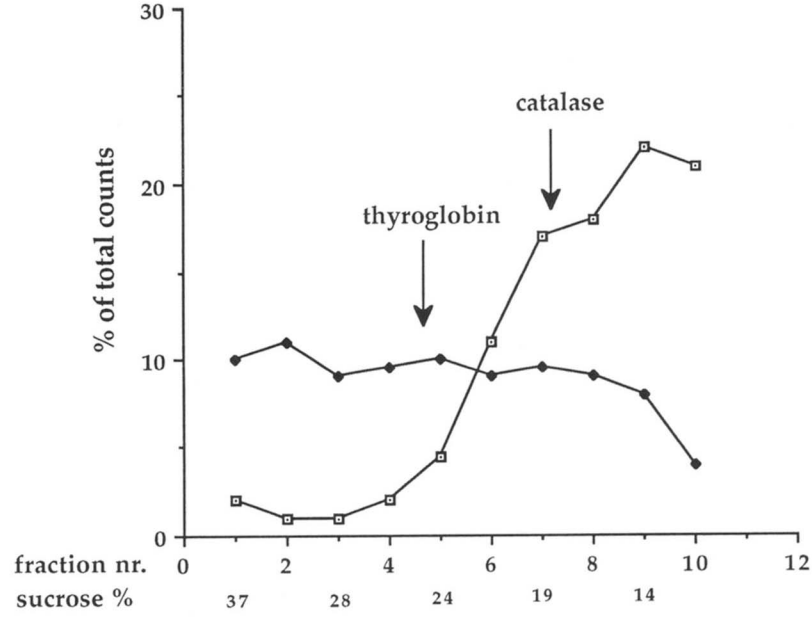

FIG. 4. **Distribution of the M protein over the gradient.** The labeled M protein in each gradient fraction of Fig. 3 was quantitated by PhosphorImager analysis. The amount of radioactivity in each fraction was plotted as a percentage of the total counts of the M protein present in all fractions of the gradient. The sedimentation of two marker proteins catalase (11.3 $s_{20,w}$) and thyroglobin (19.3 $s_{20,w}$) is indicated. ⊡, pulse; ♦, chase.

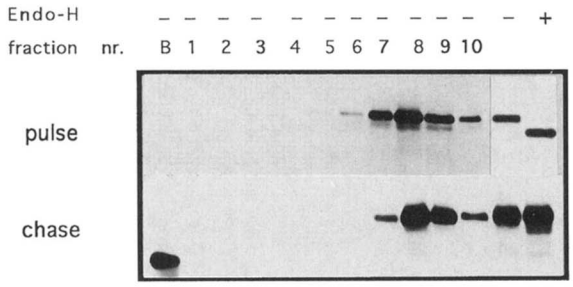

FIG. 5. **Pulse-chase analysis of the VSV-G protein.** COS cells were infected with vvVSV-GtsO45 and pulse-labeled for 30 min at 39 °C, followed by a chase of 2 h at 32 °C. Cells were lysed and the G protein analyzed after centrifugation, the same as for Fig. 3. The labeled protein is associated with the upper part of the gradient in fractions 7 through 10. Part of the lysates was analyzed without centrifugation; it was aliquoted in two and treated or not treated with endo-β-*N*-acetylglucosaminidase H (*two right lanes* of the panel).

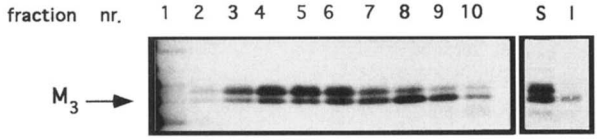

FIG. 6. **Analysis of MΔCOOH.** COS cells infected with vvMHV-MΔCOOH were pulse labeled for 30 min and chased for 3 h (only the chase is shown). The protein was analyzed in a sucrose gradient and immunoprecipitated from each fraction. The Golgi (M_3; indicated) and the TGN forms (not indicated) are heterogeneously distributed over the gradient, but only a minor fraction of the protein is associated with the bottom two fractions, 1 and 2. To estimate the amount of this protein present at the plasma membrane, a surface immunoprecipitation was carried out. The *right panel* shows that most of the protein was at the plasma membrane (*S*) and only a small amount of the M_3 form had remained intracellularly (*I*) after the 3-h chase.

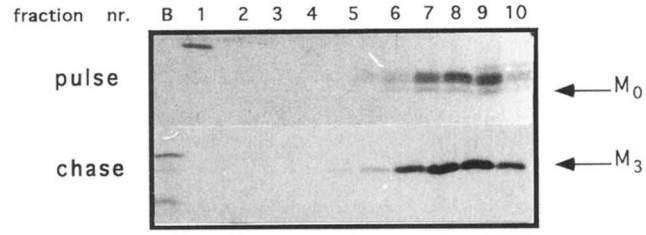

FIG. 7. **Analysis of the M protein after BFA treatment.** Infected COS cells were pulse-labeled for 15 min and chased for 60 min in the presence of 5 μg/ml BFA. The M protein was analyzed as described in the legend to Fig. 3. In the presence of BFA only the M_3 form (indicated) is made during the chase.

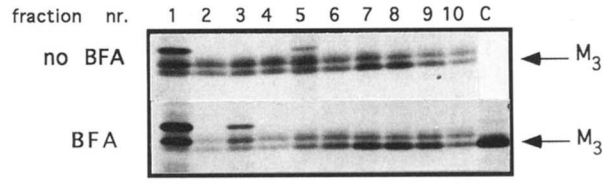

FIG. 8. **The M protein complexes partially resist treatment with BFA.** Infected COS-1 cells were pulse-labeled for 30 min at 5 h 30 min post-infection and subsequently chased for 3 h. The cells were then treated (+ BFA) or not treated (− BFA) for 1 h with 5 μg/ml BFA before lysis and analysis on sucrose gradient as described in the legend to Fig. 3. As a control infected cells were also pulse-labeled and chased with BFA (*lane C*: + BFA). Under these conditions, as described before, only the M_3 form is made (see text).

appear in large complexes under our conditions. Previous experiments had shown that the wild-type IBV-M protein does not form oligomers when using Triton X-100, whereas a chimeric protein containing the first transmembrane domain of this latter protein did (Weisz *et al.*, 1993). We wanted to know whether this failure to detect oligomers of the wild-type IBV-M protein was due to the experimental conditions used in the latter study. However, consistent with the results of Weisz *et al.* (1993), when the IBV-M protein expressed in COS cells, was sedimented in sucrose gradients like the MHV-M protein and detected by ECL the bulk of the protein was found in the first four fractions from the top (Fig. 9) and apparently failed to form multimers.

DISCUSSION

Oligomerization of the MHV-M Protein—The data from this study show that the newly synthesized M protein of MHV occurs in large detergent-insoluble complexes following its arrival in the Golgi complex. Importantly, this complex formation differed in many respects from the oligomerization that has been extensively described for the VSV-G and influenza HA protein (Doms *et al.*, 1987; Copeland *et al.*, 1988). In contrast to the latter process that occurs in the ER/IC (Doms *et al.*, 1993), the formation of the M protein complexes appeared to start only after the protein had left both the rough ER and the IC, as

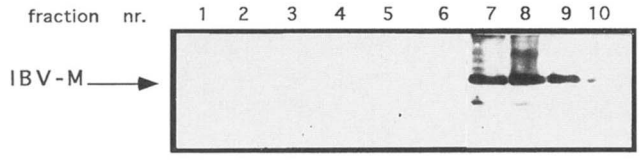

FIG. 9. **Analysis of the IBV-M protein.** vvIBV-M-infected COS cells were lysed at 9 h post-infection and the lysate centrifuged as described in the legend to Fig. 3. The proteins in each fraction were trichloroacetic acid-precipitated and separated in a 15% SDS-PAGE. The proteins were transferred onto nitrocellulose, and the IBV-M protein was detected by ECL.

judged by its state of *O*-glycosylation. In an attempt to determine where in the Golgi complex these complexes would occur, we used BFA. In the presence of this drug the M protein failed to oligomerize, suggesting that the oligomerization may start in the TGN. However, already formed complexes seemed to partially dissociate upon BFA treatment. From the EM data (see Fig. 2) and from previous data (Krijnse Locker *et al.*, 1992a; Krijnse Locker *et al.*, 1994b), it seems clear that after a 3-h chase period the M localizes to the stack as well as to the TGN. Since newly synthesized M does not form complexes in the presence of BFA, this result may suggest that the M oligomers that are in the Golgi stack after the chase dissociate upon subsequent BFA addition, whereas the complexes in the TGN may resist the BFA treatment. This may imply that the complex formation starts in the Golgi and persists in the TGN. Alternatively, the drug may destabilize the complexes, and their partial dissociation caused by BFA, may be nonspecific and not related to their intracellular location.

Another striking difference between the M protein and molecules such as VSV-G was that complex-formation did not result in dimers or trimers, but rather in heterogeneous and large oligomers. We estimate that the largest complexes are roughly 1000 kDa. this would argue that they may contain about 40 molecules of the M protein and are therefore considerably larger than the trimers of the VSV-G and the influenza HA protein. At present it is not known whether the heterogeneity we observed is due to the fact that these complexes are unstable under our experimental procedures or whether it is an inherent characteristic of the M complexes. The sizes of the aggregates we describe were not appreciably different after cross-linking, nor were they affected by pH (not shown). Also, when we tested different detergents, the M protein migrated as a monomer in the presence of all (that were tested like Nonidet P-40, Triton X-100, sodium deoxycholic acid, and octyl glucoside) except one. The exception was CHAPS in which the M protein behaved identical as in our sodium deoxycholic acid/Nonidet P-40 mixture.

The formation of the complexes was neither affected by nocodazole nor by cytochalasin D, suggesting that the cytoskeletal elements sensitive to these drugs are not important for this process (not shown).

Comparison to the Oligomerization of the IBV-M Chimera—The formation of detergent insoluble complexes has been described before for a Golgi-resident protein. A chimeric construct consisting of the VSV-G protein that had its anchor domain replaced by the first transmembrane domain of the IBV-M protein (called Gm1; Swift and Machamer, 1991) has been shown to form Triton X-100-insoluble complexes upon arrival in the Golgi complex (Weisz *et al.*, 1993). It is not clear, however, whether these observations are comparable with ours. First, the IBV-M complexes were found for the chimeric construct only and not for the wild-type IBV-M protein, whereas the MHV-M complexes were obtained with the wild-type protein as well as with some of our mutant M proteins (see below). Moreover, under our experimental conditions we were unable to detect complexes of the wild-type IBV-M protein. Second, the complexes of Gm1 were stable in Triton X-100, a detergent that failed to preserve MHV-M complexes. It is also not clear whether the Gm1 complexes described by Weisz *et al.* (1993) were heterogeneous as are the MHV-M complexes. Moreover, the different analytical conditions (5–20% *versus* 15–30% sucrose gradients) do not allow direct comparison. The Gm1 oligomers appeared to be smaller, consisting of approximately 12 molecules of the chimera, whereas the largest oligomers of the MHV-M protein contained at least 40 molecules. Finally, the MHV-M complexes persisted at the plasma membrane, whereas oligomers of the Gm1 protein were no longer observed with mutants that were transported to the cell surface (Weisz *et al.*, 1993). It seems, therefore, that the phenomenon of complex formation as observed for Gm1 and for MHV-M reflects different cellular processes.

Detergent Insolubility of Other Membrane Proteins—The phenomenon of detergent insolubility of membrane proteins destined for the plasma membrane has been documented before. Thus, the influenza virus HA protein has been shown to become partially detergent-insoluble in Triton X-100 and CHAPS (Skibbens *et al.*, 1989; Kurzchalia *et al.*, 1992; Fiedler *et al.*, 1993). When the insoluble complexes of the HA protein that are seen with both these detergents were compared, it appeared that they were qualitatively similar but the Triton X-100 complexes were richer in (glyco)lipids (more than 95% (w/w) lipid content) than those formed in CHAPS (~40%, w/w). Based on these observations it was concluded that the CHAPS complexes may be dominated by protein-protein interactions (Fiedler *et al.*, 1993). In our study the MHV-M protein complexes were not preserved in Triton X-100 but were formed in the presence of both CHAPS and the sodium deoxycholic acid/Nonidet P-40 mixture. It is, therefore, tempting to speculate that the MHV-M complexes are also relatively more enriched in protein rather than lipids (see below). This view is consistent with our finding that the M protein complexes do not flotate in sucrose gradients (not shown).

Retention Mechanism of the MHV-M Protein—We and others (Armstrong and Patel, 1991; Krijnse Locker *et al.*, 1994b) have shown previously that deletion of the cytoplasmic tail of the MHV-M protein resulted in transport to the plasma membrane of the mutant protein. This tail has features of an internalization signal, but unlike TGN38 or furin (Humphrey *et al.*, 1993; Bos *et al.*, 1993; Molloy *et al.*, 1994) the MHV-M does not seem to recycle from a distal compartment. The cytoplasmic tail alone, however, was not sufficient for TGN retention; mutant MHV-M proteins that still contained the tail but lacked one or two transmembrane domains were now detected in endosomes, but were not subjected to rapid lysosomal degradation. The amphiphilic cytoplasmic tail, except for the last 22 amino acids, appeared to have no information for Golgi retention, since deletion of this part resulted in a mutant protein that localized in an identical fashion to the wild-type protein (late Golgi and TGN). The same appears to apply for the luminal amino terminus, since deletion of this part did not affect its retention in the Golgi and the TGN. Based on these results we have concluded that the retention of the MHV-M protein is determined by two components, one contained in the transmembrane domains and one in the cytoplasmic tail (Krijnse Locker *et al.*, 1994b). When some of these mutant proteins were analyzed using sucrose gradients, we found that deletions in the cytoplasmic tail (deleting either the extreme 22 COOH-terminal amino acids or the complete cytosolic tail except for these 22 amino acids) or deletion of the 25 NH_2-terminal residues, resulted in complex formation that was similar to the wild-type protein (see *e.g.* Fig. 7; not shown). However, mutant proteins

with only one or two transmembrane domains showed significantly less or no oligomerization (not shown). This strongly suggests that the presence of all three transmembrane domains is required for the formation of the multimers.

Recent data from our laboratory have shown that when the M and S protein are co-expressed, the formation of multimers (now containing both M and S) may start as early as the IC (*i.e.* pre-Golgi). Subsequently, these (preformed) M/S complexes appear to be transported in a multimeric form to the Golgi and possibly to the TGN, where they are retained. These data imply, as for the M protein alone, that the complex formation is not the sole determinant of their retention.[4] Our data, therefore, clearly differ from that of Weisz *et al.* (1993) and Schweizer *et al.* (1994) that suggested that oligomerization alone is sufficient for retention. They also differ from the recent results on TGN38 that showed that either the transmembrane domain or the cytoplasmic tail themselves were sufficient for retention (Ponnambalam *et al.*, 1994). It is interesting to note, however, that the complexes of the mutant M protein, that is transported to the plasma membrane, were clearly smaller than those of the wild-type protein. We have estimated their size to be around 650 kDa or 30 molecules. Perhaps a complex of this size might not be excluded from a 50–100-nm transport vesicle en route to the plasma membrane (Nilsson *et al.*, 1994).

The medial Golgi enzymes NAGT I and Man II can functionally interact *in vivo*. Although their intracellular distribution partially overlaps with GalTf, they appear not interact with this latter enzyme (Nilsson *et al.*, 1994). The coronavirus M protein may have no cellular template to interact with. If one assumes that our complexes are predominantly due to protein-protein interactions (see above), it seems most likely that the MHV-M oligomers we detect are composed of the M protein only. The simplest interpretation of our available data is that the intracellular retention of this protein is dependent on both this "self-association" and on the interaction of the cytoplasmic tail with components on the cytosolic side of the TGN, consistent with the kin recognition model (Nilsson *et al.*, 1993).

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