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O-Glycosylation of the Coronavirus M Protein

DIFFERENTIAL LOCALIZATION OF SIALYLTRANSFERASES IN N- AND O-LINKED GLYCOSYLATION*

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It has previously been shown that the M (E_1) glycoprotein of mouse hepatitis virus strain A59 (MHV-A59) contains only *O*-linked oligosaccharides and localizes to the Golgi region when expressed independently. A detailed pulse-chase analysis was made of the addition of *O*-linked sugars to the M protein; upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, three different electrophoretic forms could be distinguished that corresponded to the sequential acquisition of *N*-acetylgalactosamine (GalNAc), galactose (Gal), and sialic acid (SA). A fourth and fifth form could also be detected which we were unable to identify. Following Brefeldin A treatment, the M protein still acquired GalNAc, Gal, and SA, but the fourth and fifth forms were absent, suggesting that these modifications occur in the *trans*-Golgi network (TGN). In contrast, in the presence of BFA, the G protein of vesicular stomatitis virus (VSV), which contains *N*-linked oligosaccharides, acquired Gal and fucose but not SA. These results are consistent with earlier published data showing that Golgi compartments proximal to the TGN, but not the TGN itself, relocate to the endoplasmatic reticulum/intermediate compartment. More importantly, our data argue that, whereas addition of SA to *N*-linked sugars occurs in the TGN the acquisition of both SA on *O*-linked sugars and the addition of fucose to *N*-linked oligosaccharides must occur in Golgi compartments proximal to the TGN. The glycosylation of the M protein moreover indicates that it is transported to *trans*-Golgi and TGN. This was confirmed by electron microscopy immunocytochemistry, showing that the protein is targeted to cisternae on the *trans* side of the Golgi and co-localizes, at least in part, with TGN 38, a marker of the TGN, as well as with a lectin specific for sialic acid.

In eukaryotic cells, membrane proteins are synthesized in the rough endoplasmatic reticulum (RER).[1] Here they enter the exocytic pathway along which they are transported to different locations in the cell or to the plasma membrane. During their synthesis and while being transported through compartments of the biosynthetic pathway, the proteins undergo various modifications including proteolytic cleavages, disulfide bond formation, glycosylation, and acylation. Glycosylation of membrane proteins is a dynamic process involving additions, removals, and modifications of oligosaccharides by compartment-specific enzymes such that the structure of the side chains always reflect the compartments the protein has reached or passed. *N*-linked glycosylation to asparagine has been extensively investigated. It starts with the co-translational transfer in the ER to the nascent polypeptide chain of an oligosaccharide chain from a lipid-linked intermediate. Biosynthesis proceeds by sequential modifications in the ER and Golgi complex, consisting of trimming of glucose and mannose residues and of the subsequent addition of *N*-acetylglucosamine (GlcNAc), fucose, galactose (Gal), and sialic acid (SA). While the sequence of enzymatic reactions involved in this process is now well established, the precise location of most of these enzymes within the Golgi complex is far from clear. The difficulty of allocating the enzymes to distinct Golgi compartments is closely coupled to the unsolved problem of defining both the boundary between the ER and the Golgi and the actual number of Golgi compartments (Simons and Mellman, 1992). In fact, only the localization to the TGN of the last enzyme in sequence, the α2,6-sialyltransferase that acts on *N*-linked oligosaccharides, is now well established (Roth *et al.*, 1985; Chege and Pfeffer, 1990). Noteworthy is the fact that in no case two enzymes have been localized by double-labeling at the ultrastructural level (Simons and Mellman, 1992).

When it comes to the synthesis of *O*-linked oligosaccharides, there are even greater gaps in our understanding. This process is initiated by the post-translational addition of *N*-acetylgalactosamine (GalNAc) which is proposed to be added as early as in the RER (Pathak *et al.*, 1988; Strous, 1979), in the intermediate compartment (Tooze *et al.*, 1988), or in an early Golgi region (Roth, 1984; Abeijon and Hirschberg, 1987; Elhammer and Kornfeld, 1984). Glycosylation then proceeds by the addition of Gal and SA, most probably in the *trans*-Golgi region (Elhammer and Kornfeld, 1984; Cummings *et al.*, 1983) and may sometimes be completed by linkage to the oligosaccharide chain of fucose, GalNAc, GlcNAc (Roth, 1984, 1987), or sulfate (van Beurden-Lamers *et al.*, 1989).

Brefeldin A (BFA), a fungal metabolite, causes rapid redistribution of Golgi proteins into the ER probably by blocking anterograde but not retrograde membrane traffic (Lippincott-Schwartz *et al.*, 1990). Enzymes normally residing in the Golgi complex are relocated to the ER, and these can process both resident ER proteins and newly synthesized proteins now retained in this organelle (Lippincott-Schwartz *et al.*, 1989;

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[1] The abbreviations used are: RER, rough endoplasmic reticulum; ER, endoplasmic reticulum; BFA, Brefeldin A; GalNac, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; IBV, infectious bronchitis virus; MHV-A59, mouse hepatitis virus strain A59; NA, neuraminidase; SA, sialic acid; TGN, *trans*-Golgi network; VMM, vaccinia virus M recombinant; VSV, vesicular stomatitis virus; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EM, electron micoscopy; PBS, phosphate-buffered saline; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum essential medium.

Doms *et al.*, 1989). Under these conditions, *N*-linked glycoproteins were found to become Endo H-resistant and to acquire Gal but no SA (Chege and Pfeffer, 1990; Lippincott-Schwartz *et al.*, 1989; Doms *et al.*, 1989; Nuchtern *et al.*, 1989). It was concluded that the latter addition occurs in a compartment not relocated to the ER and that the addition of Gal and SA to *N*-linked sugar chains thus occurs in separate compartments (Chege and Pfeffer, 1990). In this way, BFA has been effectively used to functionally dissect the compartments of the Golgi complex proximal to the TGN from the TGN itself.

The M (previously called E_1) protein of mouse hepatitis virus strain A59 (MHV-A59), a triple-spanning membrane glycoprotein, contains only *O*-linked sugars (Holmes *et al.*, 1981; Niemann *et al.*, 1984; Rottier *et al.*, 1981a). It therefore provides a useful model for studying *O*-linked glycosylation. The structure of its major oligosaccharide side chains have been characterized in infected cells. They consist of S/T-GalNAc-Gal-SA, and, in addition, a subpopulation of these oligosaccharides possess an extra SA bound to the GalNAc moiety (Niemann *et al.*, 1984). The biosynthesis of the sugar chains can easily be followed as each maturation step is represented by a distinct mobility shift in SDS-PAGE (Tooze *et al.*, 1988).

Mouse hepatitis virus has been shown to bud into a region between the RER and the Golgi complex (Tooze *et al.*, 1984, 1988) before being released via the normal exocytotic route. The Golgi maturation steps of the M protein thus occur after the protein has already been packaged into virions. When the M protein is expressed independently, it shows the same glycosylation pattern as in infected cells and appears to localize to the Golgi region as judged by indirect immunofluorescence (Rottier and Rose, 1987).

In this study we made a detailed analysis of *O*-glycosylation in the MHV-A59 M protein expressed using a recombinant vaccinia system. We also looked at the effects of BFA on glycosylation as well as on a membrane protein containing *N*-linked oligosaccharides, the VSV G protein. We provide evidence for the differential localization of sialyltransferases for *O*- and *N*-linked glycosylation. In addition, we show both by EM immunocytochemistry and biochemically that the M protein, when expressed, localizes to *trans*-Golgi/*trans*-Golgi network.

MATERIALS AND METHODS

Viruses, Cells, and Antisera—Sac(−), COS-1, and Ratec (rat embryonic cells) cells were maintained in Dulbecco's minimal essential medium containing 5% fetal calf serum, penicillin, and streptomycin (DMEM/5%). MHV-A59 was propagated in Sac(−) cells as described (Spaan *et al.*, 1981). The preparation of VMM (recombinant vaccinia virus expressing the MHV-A59 M protein) will be described.[2] Vesicular stomatitis virus strain San Juan (VSV) was propagated in BHK-21 cells and plaque-titrated on L cells. The production of the polyclonal MHV-A59 antiserum has been described (Rottier *et al.*, 1981b) and the generation of the rabbit antiserum raised to a peptide with the sequence of the COOH-terminal 18 amino acids of the M protein will be described.[2] The VSV antiserum was a kind gift of Dr. J. K. Rose (Yale University, New Haven, CT).

[^{35}S]Methionine, [^{3}H]Galactose, [^{3}H]Glucosamine, and [^{3}H]Fucose Labeling of MHV-A59, VMM, and VSV—For the pulse-chase experiments, confluent monolayers of COS-1 or Sac(−) cells in 16-mm or 35-mm dishes were infected with MHV-A59 or VSV in PBSCM/DEAE (PBS containing 5 mM $CaCl_2$, 8 mM $MgCl_2$, and 50 mg/liter DEAE-dextran) for 1 h at 37 °C or with VMM in PBSCM for 45 min at room temperature at a multiplicity of infection of 10. Starting at 5.5 h (Sac(−) cells) or 4.5 h (COS-1 cells) postinfection, cells were starved for 30 min in MEM/2% FCS (GIBCO) without methionine and cysteine. When indicated, Brefeldin A (BFA; a generous gift from Sandoz Ltd., Basel, Switzerland) was added to a concentration of 6 μg/ml at the start of the starvation period. Cells were pulse-labeled for 5 or 15 min with 25–200 μCi of Tran^{35}S-label (ICN Biomedicals), rinsed once with PBSCM, and chased for various times in DMEM/5% FCS supplemented with 2 mM methionine and cysteine. Labeled cells were solubilized in lysis buffer (50 mM Tris-Cl, pH 8.0, 62.5 mM EDTA, 1% Triton X-114) containing 2 mM phenylmethylsulfonyl fluoride, 40 μg/ml aprotinin (Sigma), and 1 μg/ml leupeptin (Sigma). When the reversibility of the effect of BFA was tested, cells previously labeled in the presence of the drug were rinsed four times with PBSCM before DMEM/5% without BFA was added. For the sugar labeling, confluent monolayers of Sac(−) cells grown in 35-mm dishes in glucose-free RPMI 1640 (Flow Laboratories) supplemented with 2 mg/ml D-glucose, 2 mM glutamine, and 5% FCS were infected with MHV-A59 or VSV as above. After the 1-h infection period, incubation at 37 °C was continued in 0.6 ml of RPMI 1640 containing 2% dialyzed FCS, 2 mM glutamine, and 10 μg/ml D-glucose. Cells were labeled from 5 to 8 h postinfection with 50 μCi of [3H]galactose, [3H]glucosamine, or [3H]fucose (Amersham International plc, Buckinghamshire, UK). The cells were lysed by adding to the medium 0.15 ml of a 5 times concentrated detergent solution (50 mM Tris-Cl, pH 8.0, 62.5 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40, final concentrations) containing 5 times the amount of the above-mentioned protease inhibitors. Lysates were centrifuged at 10,000 × *g* and 4 °C, and proteins were immunoprecipitated as described[2] using the MHV-A59 antiserum, the peptide antiserum (when immunoprecipition was followed by neuraminidase treatment), or the VSV antiserum.

Neuraminidase Digestions—Neuraminidase treatment of immunoprecipitated proteins was performed essentially as described by Tooze *et al.* (1988). Immune complexes were collected by centrifugation after adsorption to Pansorbin (Calbiochem) for 30 min at room temperature and washed three times with RIPA buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Nonidet P-40). Samples were resuspended in 0.1 ml of sodium acetate buffer (10 mM NaAc, 1 mM $CaCl_2$, pH 5.6) aliquoted in two equal samples to one of which 40 milliunits/ml neuraminidase from *Arthrobacter ureafaciens* (Boehringer GmbH, Mannheim, Germany) was added. All samples were incubated overnight at 37 °C, then washed once with 10 mM Tris-Cl, pH 7.4, containing 2 mM phenylmethylsulfonyl fluoride, resuspended in Laemmli sample buffer, and analyzed in a 10% (VSV G protein) or 15% (M protein) polyacrylamide gel.

Indirect Immunofluorescence—Subconfluent monolayers of COS-1 cells grown on coverslips were infected at a multiplicity of infection of 10 with VMM, incubated in the absence or in the presence of BFA added at 3 h postinfection, and fixed at 6 h postinfection with 3% paraformaldehyde. Indirect immunofluorescence using the polyclonal MHV-A59 antiserum (1:150) and an affinty-purified fluorescein-conjugated goat anti-rabbit IgG (1:150; Protos Immunoresearch, San Fransisco, CA) was performed as described (den Boon *et al.*, 1991).

Electron Microscopy—Ratec (rat embryonic) cells grown in 60-mm dishes, in which a 12-mm coverslip was included to check the expression of the M protein separately by indirect immunofluorescence, were infected with VMM at a multiplicity of infection of 10. At 6 h postinfection, after removal of the coverslip, cells were prepared for EM. Monolayers were washed twice with ice cold 0.25 M Hepes-KOH, pH 7.4, and treated on ice with 1 ml of Hepes buffer containing 25 μg/ml proteinase K until cells came off the dish. Detached cells were gently resuspended, and 0.2 ml of 1% glutaraldehyde (EM grade, Agar Aids, Essex, UK) was added. Cells were spun down at 2,000 × *g* for 30 s, supernatant was removed, and cells were postfixed in 1% glutaraldehyde in Hepes-KOH for 30 min at room temperature. The fixed cell pellet was cryo-sectioned, and the sections were labeled with the polyclonal MHV antiserum followed by protein A gold, as described (Griffiths *et al.*, 1984). The TGN 38 antiserum was kindly provided by Dr. Paul Luzio (Luzio *et al.*, 1990), and double-labeling was carried out according to Geuze *et al.* (1981). Labeling with *Limax flavus* lectin was carried out as described (Roth *et al.*, 1984). *L. flavus* lectin was applied to the sections at 12 μg/ml in 1% fish skin gelatin (Sigma) for 30 min. Sections were rinsed and fetuin (Sigma), conjugated to colloidal gold at 10 μg/ml, pH 6.5, was applied.

RESULTS

Glycosylation of the Expressed MHV-A59 M Protein—Several forms of the M protein can be distinguished after labeling of MHV-A59-infected Sac(−) cells (Rottier *et al.*, 1981a; Rot-

[2] J. Krÿnse Locker, J. K. Rose, M. C. Horzinek, and P. J. M. Rottier, submitted for publication.

tier and Rose, 1987; Tooze *et al.*, 1988). The biogenesis and *O*-linked sugar composition of the major forms has been determined by Tooze *et al.* (1988). It was shown that the unglycosylated protein designated E_{1u} by those authors is post-translationally converted to an intermediate form (E_{1i}) that migrates slightly slower than E_{1u} in SDS gels and contains *N*-acetylgalactosamine (GalNAc). Subsequently, three forms (E_{1m}) of higher molecular weight appear simultaneously. Studies using neuraminidase treatment and labeling with sugar precursors have shown that these forms contain galactose (Gal) and sialic acid (SA) in addition to GalNAc. Glycosylation of the M protein in MHV-A59-infected Sac(−) cells is never complete, and usually all five forms, including E_{1u}, remain detectable even after a long chase period (Tooze *et al.*, 1988). The site of addition of GalNAc to M appears to coincide with the initial location of budding of MHV, the intermediate compartment (Tooze *et al.*, 1984, 1988).

To characterize the glycosylation of the expressed MHV-A59 M protein, Sac(−) cells infected with a recombinant vaccinia virus vector expressing the M protein (VMM) were pulse-labeled for 5 min at 6 h postinfection and then chased for up to 60 min (Fig. 1). For comparison, MHV-A59-infected Sac(−) cells were pulse-labeled and chased for 60 min under identical conditions. Consistent with the above-mentioned studies, the well known set of M species was identified in the coronavirus-infected cells (Fig. 1); the unglycosylated protein synthesized during the pulse, the intermediate form, and the three more mature forms. Each of the latter forms appeared to be sialylated to the same extent as was demonstrated by neuraminidase treatment. For simplicity, we shall refer to the forms as M_0 to M_5. M_0 represents the unglycosylated protein, M_1 the GalNAc-containing species, M_3 to M_5 the three mature forms containing SA, while M_2 refers to the form derived from M_3 after removal of SA. When expressing the M protein from VMM, the same 5 species appeared but with slightly different kinetics and relative ratios. The M_1 form was already detectable after the 5-min pulse. In addition, the protein was completely converted to the M_3, M_4, and M_5 forms after 60 min of chase. In contrast, in MHV-infected cells the protein was never completely converted to these three mature forms. Longer chase times did not significantly change the pattern of glycosylation of the expressed protein (not shown). The broad host range of the vaccinia virus allowed us to express the protein in different cells, and we found that identical forms of M were made in all the cell types tested but at different proportions. In COS-1 and HeLa cells, for example, glycosylation was never complete, since a substantial fraction of the protein remained unglycosylated after long chase times (see Fig. 2), whereas in Sac(−) cells all the expressed protein became glycosylated (Fig. 1). In AtT-20 and HepG2 cells, M was almost quantitatively converted to M_3, while in HeLa cells M_4 is a major species with little M_3 and M_5 (data not shown).

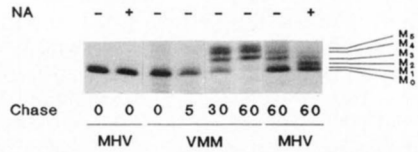

FIG. 1. **Glycosylation of the M protein expressed from a recombinant vaccinia vector (VMM).** VMM-infected Sac(−) cells were pulse-labeled with [^{35}S]methionine at 6 h postinfection for 5 min and either harvested directly or chased for 5 min, 30 min, or 60 min. M protein was immunoprecipitated with a polyclonal MHV antiserum. In parallel, MHV-A59-infected Sac(−) cells were pulse-labeled for 5 min, lysates were prepared directly or after a 60-min chase, and the M protein was precipitated with a COOH terminus-specific peptide antiserum. The dissolved immunoprecipitates were split into two equal aliquots, one of which was treated with neuraminidase (NA) (+), while the other one was incubated without the enzyme (−). Radiolabeled proteins were analyzed in a 15% SDS-PAGE. M_0 through M_5 designate the unglycosylated form and the glycosylated forms of M, respectively (see text).

BFA Blocks the Terminal Glycosylation of M but Not Its Sialylation—As BFA inhibits membrane protein transport to the TGN, it can be used to localize glycosylation enzymes which reside in this compartment. We therefore tested its effect on the glycosylation of the M protein. COS-1 cells were used predominantly in these experiments because the level of M expression in these cells appeared to be higher; results obtained in Sac(−) cells were, however, essentially identical (see below). The cells were infected with VMM, pulse-labeled at 5 h postinfection for 5 min, and then chased for up to 60 min. To one set of cells, BFA was added 30 min before the labeling and maintained in all chase media. Immunoprecipitates of the M protein were prepared, subjected to the neuraminidase assay to monitor the acquisition of SA by PAGE. Samples obtained from cells which were pulse-labeled or chased for 60 min in the absence of BFA were included for reference. As is shown in Fig. 2, BFA clearly affects the kinetics and extent of oligosaccharide processing. Already during the 5-min pulse-labeling about half of the protein was converted to a form co-migrating with M_2, which contained GalNAc and Gal but no SA (see below). This conversion occurred without significant accumulation of M_1 and was rapid as it was almost completed after 5 min of chase. At this time, the M_3 species started to appear. In the presence of BFA, the M protein was quantitatively converted to the M_3 form. This form contained SA as it was trimmed to the M_2 form by neuraminidase treatment. No further maturation of the sugars occurred in the presence of BFA, even after chasing for up to 3 h (see below). These data strongly suggest that the major steps in *O*-glycosylation including sialylation occur in compartments preceding the TGN.

Since Golgi enzymes relocate to the ER following BFA treatment, it is interesting to note the difference in the kinetics of the conversion of the different forms of M in the presence of BFA. The conversion of the unglycosylated form (M_0) to the M_2 form is significantly more rapid; it is so rapid, in fact, that the M_1 form is hardly detected. In contrast, whereas the M_2 form is never detected in untreated cells (it is evidently directly sialylated to form M_3), it becomes a relatively stable species after BFA treatment, with a half-life of about 15 min. While the reasons for these changes are far from clear, these data suggest that following BFA treatment significant alterations have occurred in the manner in which the M protein meets the various glycosyltransferases.

Effect of BFA on Sialylation in O- and N-Glycosylation—As BFA has been reported to inhibit SA addition to *N*-glycosylated proteins (Lippincott-Schwartz *et al.*, 1989; Doms *et al.*,

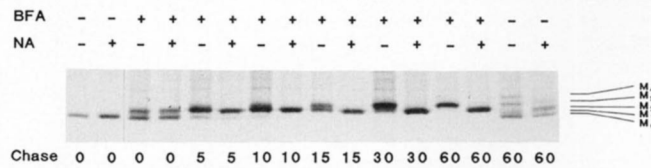

FIG. 2. **Glycosylation of the M protein in the presence of Brefeldin A.** VMM-infected COS-1 cells were pretreated for 30 min with BFA from 4.5 h postinfection, pulse-labeled for 5 min at 5 h postinfection, and chased for 0, 5, 10, 15, 30, and 60 min all in the presence of the drug. In parallel, cells were pulse-labeled for 5 min at 5 h postinfection and chased for 60 min without BFA. The M protein was immunoprecipitated with the COOH terminus-specific antiserum, and half of each sample was treated with neuraminidase.

1989; Nuchtern *et al.*, 1989; Shite *et al.*, 1990), our data showing sialylation of the M protein in the presence of the drug initially came as a surprise. To test the generality of these findings, two experiments were done. First, the effect of BFA on the maturation of the M protein was studied in another cell type. M was expressed by VMM in COS-1 and Sac(−) cells and analyzed after pulse-labeling for 15 min followed by a 60-min chase both in the presence and in the absence of the drug. Acquisition of SA was again monitored by neuraminidase treatment. Fig. 3*A* shows that the effect of BFA in the two cell lines was identical, showing maturation up to the sialylated M_3 form in both cases. Secondly, we checked the pattern of *N*-glycosylation of the VSV G protein, a protein containing only *N*-linked oligosaccharides, in Sac(−) cells during BFA treatment. Cells were infected with VSV, and the VSV G protein was analyzed in a similar pulse-chase experiment. Consistent with the experiments of Doms *et al.* (1989), the G protein apparently did not acquire SA during the 60-min chase period in the presence of BFA, but was neuraminidase-sensitive in its absence (Fig. 3*B*). The same results were obtained when the G protein was expressed in COS-1 cells from a recombinant vaccinia virus vector (VVG; not shown).

The Oligosaccharide Structures of the M and G Protein Synthesized in the Presence of BFA—Until now we assumed that the oligosaccharide composition of the M species synthesized in the presence of BFA was identical with the corresponding forms of M made in untreated cells. To confirm this assumption and exclude that BFA would lead to aberrant glycosylation, Sac(−) cells were infected with MHV-A59 and labeled from 5 to 8 h postinfection either with [^{3}H]galactose or with [^{3}H]glucosamine, which is metabolized in cells to GlcNAc, GalNAc, and SA. We also included in this analysis virions that had been released from infected cells into the extracellular medium. For this purpose, total lysates were prepared by adding concentrated lysis buffer directly to the culture medium. Consistent with results by Tooze *et al.* (1988) M_3, M_4, and M_5 made under drug-free conditions incorporated radioactivity in the presence of [^{3}H]galactose and [^{3}H]glucosamine and were reduced in apparent molecular mass after neuraminidase treatment (Fig. 4*A*). When BFA was present, [^{3}H]glucosamine labeling revealed two species of M. The major form co-migrated with M_3. Since this form could also be labeled with [^{3}H]galactose and was sensitive to neuraminidase, it is apparently identical with the M_3 species labeled in the absence of BFA. The minor form co-migrated with M_1 and contained only GalNAc, since it incorporated [^{3}H]glucosamine but not [^{3}H]galactose. Since this form migrated slightly slower than the unglycosylated protein, it appears to represent the intermediate form, M_1. The M_2 species generated by neuraminidase treatment of M_3 evidently contains GalNAc and Gal since it was labeled by both ^{3}H precursors. This glycoprotein was also transiently detectable in a pulse-chase experiment in the presence of BFA (see Fig. 2). These data confirm that during *O*-glycosylation GalNAc, Gal, and SA are sequentially added in pre-TGN compartments.

The overall incorporation of ^{3}H-labeled oligosaccharide precursors into the glycosylated M protein appeared to be reduced when BFA was present (Fig. 4*A*). This might indicate that these sugars can be incorporated both before and after the BFA block. However, the same effect of BFA was also observed with [^{3}H]galactose and [^{3}H]fucose when looking at *N*-glycosylation (see below). It is therefore more likely that these findings are due to some nonspecific effect of BFA such as reduced activity of the enzymes in the fused ER compartment or a reduction in the mobilization of the precursors.

Interestingly, these experiments also provided additional evidence for the different sites of sialylation of *N*- and *O*-

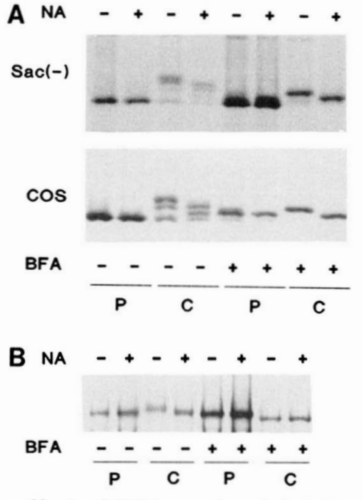

FIG. 3. **The effect of BFA on the sialylation of *O*- and *N*-linked oligosaccharides.** *A*, Sac(−) and COS-1 cells infected with VMM were pulse-labeled for 15 min at 6 h postinfection and harvested (*P*) or chased (*C*) for 60 min. BFA was either present from 30 min before labeling or not present. Samples were processed for treatment with neuraminidase as described for Fig. 2. *B*, VSV-infected Sac(−) cells were labeled and processed as described in *A* using a VSV antiserum.

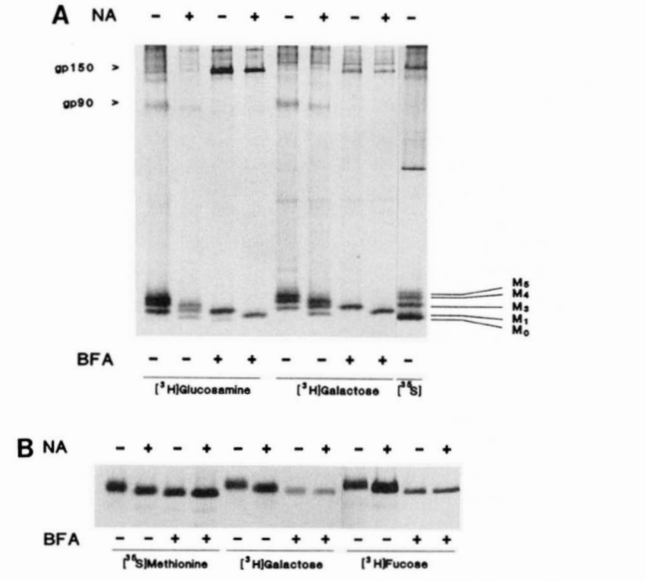

FIG. 4. **Oligosaccharide structures of *O*- and *N*-glycosylated proteins made in the presence of BFA.** *A*, sugar labeling of the M protein. MHV-A59-infected Sac(−) cells were labeled from 5 to 8 h postinfection with either [^{3}H]glucosamine or [^{3}H]galactose in RPMI containing 10 μg/ml D-glucose or with [^{35}S]methionine in methionine-free MEM. BFA was added at 3 h postinfection. For immunoprecipitation with the polyclonal MHV antiserum, total lysates of cells and culture medium were prepared by adding concentrated lysis buffer directly to the medium. Immunoprecipitates were processed for neuraminidase treatment followed by analysis in a 15% SDS-PAGE. *Arrows* indicate the positions of the precursor (gp150) and mature (gp90) forms of the spike protein of MHV. Fluorographs were obtained after exposure for 3 days ([^{35}S]methionine) and 7 weeks ([^{3}H]glucosamine and [^{3}H]galactose). *B*, sugar labeling of the VSV G protein. VSV-infected Sac(−) cells were labeled as described for *A* with [^{3}H]galactose, [^{3}H]fucose, and [^{35}S]methionine and processed using a polyclonal VSV antiserum. Samples were analyzed by SDS-PAGE in a 10% gel. Fluorographs show exposures of 2 days ([^{35}S]methionine and [^{3}H]galactose) and 9.5 months ([^{3}H]fucose).

linked glycoproteins. The spike (S) protein of MHV is synthesized as an Endo H-sensitive 150-kDa precursor, bearing only *N*-linked oligosaccharides. After its incorporation into virions, it is processed to an Endo H-resistant, 180-kDa form, which is cleaved shortly before release from the cell to yield two 90-kDa species (Ricard and Sturman, 1985; Sturman *et al.*, 1985). As expected, the 90-kDa proteins synthesized in the absence of BFA could be labeled both by [^{3}H]galactose and by [^{3}H]glucosamine and were decreased in molecular size after neuraminidase treatment (Fig. 4*A*). In the presence of BFA, only the 150-kDa immature, uncleaved precursor was formed which was not sensitive to neuraminidase. To extend these observations to our *N*-glycosylated reference protein, VSV-infected Sac(−) cells were labeled with either [^{3}H]galactose, [^{3}H]fucose, or [^{35}S]methionine, both in the absence and in the presence of BFA. The results shown in Fig. 4*B* indeed demonstrate that the G protein incorporated [^{3}H]Gal and [^{3}H]fucose under both conditions, but that no SA was added during the 3-h labeling period in the presence of BFA. In addition, we observed in this experiment that in control cells the G protein, after removal of SA with neuraminidase, migrated slightly slower than the protein made under BFA treatment (Fig. 4*B*). This suggests that other components may be attached to *N*-linked oligosaccharide side chains in the TGN in addition to SA. We expected fucose to be such a component, but the labeling with the ^{3}H-labeled precursor demonstrated that this sugar is incorporated in an earlier compartment.

Reversibility of the BFA Effect—In several studies, the effects of BFA have been shown to be reversible (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Ulmer and Palade, 1989). Morphologically, the Golgi complex appeared to reassemble quite rapidly but the functional recovery took much more time. For instance, the VSV G protein was completely sialylated only about 45 min after removal of BFA (Doms *et al.*, 1989). Recently, we have been able to demonstrate that during BFA treatment of MHV-A59-infected Sac(−) cells, fully formed virions can bud and are retained intracellularly.[3] The block of virus release appeared to be fully reversible; after washing out, the drug-infectious virus was released within 120 min. This extracellular virus apparently passes the normal exocytotic route since it contains the same Endo H-resistant, sialylated, and cleaved spike proteins that are made under drug-free conditions.

We reasoned that if the M_4 and M_5 forms do indeed represent TGN modifications, these forms, which are not made in the presence of BFA, would reappear after removal of the drug. To test this, the following experiment was done: Sac(−) cells infected with MHV-A59 and VMM were pretreated with BFA for 30 min before a 15-min pulse-labeling in the presence of BFA. Cells were then chased in BFA-free medium for 60 min and 180 min. In parallel, the same pulse-chase experiments were done with MHV- and VMM-infected cells in the absence of BFA and with VMM-infected cells continuously treated with BFA. Total lysates including both the medium and the cells were prepared, and the M protein was precipitated with the polyclonal MHV antiserum. The results show that the late modifications of the M protein do reappear, but with slow kinetics. Both in MHV- and in VMM-infected cells, the M_4 form appeared after 1 h of chase, while the M_5 form was detectable only after the 3-h chase (Fig. 5). Neuraminidase treatment showed that both forms underwent the expected electrophoretic mobility shift (not shown). It is unclear whether the observed slow recovery from the BFA block is specific for the M modifications (*i.e.* the enzymes involved) or whether it relates to properties of the M protein itself. In agreement with earlier published data (Doms *et al.*, 1989), the addition of SA to the VSV G protein was already complete 1 h after removal of BFA, both in Sac(−) and COS-1 cells (not shown).

[3] J. Meertens, J. Krÿnse Locker, G. Griffiths, M. C. Horzinek, and P. J. M. Rottier, manuscript in preparation.

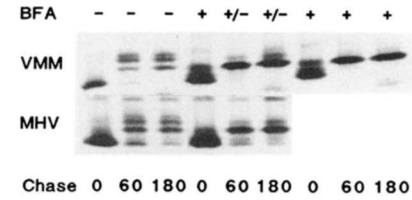

FIG. 5. **Kinetics of appearance of the M_4 and M_5 forms after removal of BFA.** MHV-A59- or VMM-infected Sac(−) cells were pulse-labeled with [^{35}S]methionine for 15 min at 6 h postinfection and chased for 0, 60, and 180 min. When BFA treatments were done, they started 30 min before the labeling and continued during the labeling period after which the drug was either omitted from the chase media (+/−) or maintained (+). Cell lysates including the culture medium were prepared as described under "Materials and Methods," and the M protein was immunoprecipitated and analyzed in a 15% SDS-PAGE. Exposure times: VMM, 7 days (*upper panel*); MHV, 3 days (*lower panel*).

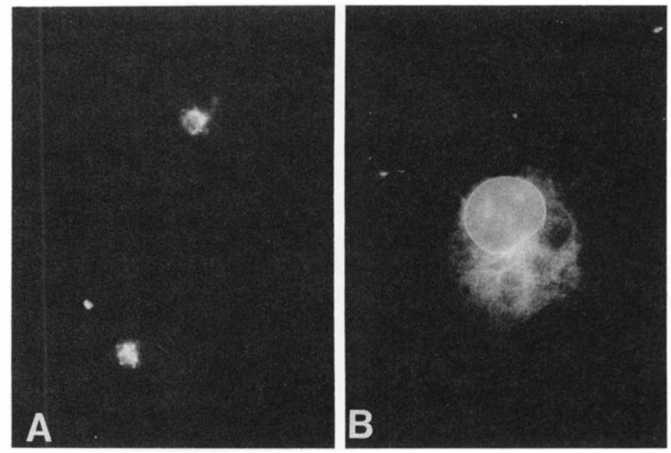

FIG. 6. **Localization by indirect immunofluorescence of the M protein expressed from VMM.** VMM-infected COS-1 cells incubated in the absence (*A*) or presence (*B*) of BFA added at 3 h postinfection were fixed at 6 h postinfection and stained after permeabilization using the polyclonal MHV antiserum.

Intracellular Localization of the M Protein—When expressed in COS-1 cells using a SV40-based vector, the MHV-A59 M protein has been shown to co-localize with a Golgi marker (Rottier and Rose, 1987). To ascertain that the M protein expressed by a recombinant vaccinia virus localizes to the same location, COS-1 cells were infected with VMM and fixed for indirect immunofluorescence at 6 h postinfection. To a parallel culture, BFA was added at 3 h postinfection. As Fig. 6*A* illustrates, the M protein in the absence of BFA as expected showed a typical Golgi staining pattern. A similar localization was observed when the protein was expressed in several other cell types (data not shown). In contrast, upon addition of BFA, the immunofluorescence pattern changed to a reticular appearance, consistent with published data (Lippincott-Schwartz *et al.*, 1989). The M glycoprotein of another coronavirus, infectious bronchitis virus (IBV), has also been found to accumulate in the Golgi apparatus, more specifically in the *cis*-Golgi compartment (Machamer *et al.*, 1990). Our biochemical observations, however, suggest that the MHV M protein is transported beyond this compartment.

To clarify this apparent discrepancy between the two proteins we next used immunoelectron microscopy in order to localize the M protein at the ultrastructural level. When thawed cryosections of Ratec cells expressing the M protein were labeled with the MHV antiserum and protein A gold, the labeling was predominantly associated with the Golgi complex. The pattern within the Golgi showed a clear polarization, label being predominantly found on cisternae on one side of the Golgi stack. In order to ascertain which pole of the stack was labeled, we used two different Golgi markers in association with the M protein in double localization studies. The first was the sialic acid lectin *L. flavus*, which would be expected to label the *trans* side of the Golgi stack (Roth *et al.*, 1984). For this, a two-step protocol was used in which the lectin was first applied on the section and in a second step visualized with fetuin, a highly sialylated glycoprotein, conjugated to colloidal gold. This procedure takes advantage of the fact that the *L. flavus* lectin has multiple binding sites for sialic acid and can effectively bind to sialic acid sites present both on the section as well as on the fetuin. This lectin has been shown to localize sialic acid moieties in the *trans* side of the Golgi (Roth *et al.*, 1984). When this procedure was combined in a double-labeling immunogold protocol, the lectin-binding sites and the M protein clearly co-localized in cisternae and tubulocisternal elements on the *trans* side of the Golgi stack (Fig. 7*C*). An essentially identical result was obtained when a TGN 38 antiserum was used as a compartment marker (Fig. 7, *A* and *B*). These results show clearly that the bulk of the M protein is present in the cisternae on the *trans* aspect of the Golgi stack and, at least in part, co-localizes with the TGN marker, TGN 38. However, the results do not enable us to determine whether and how much of the M protein is also present in Golgi compartments proximal to the TGN.

DISCUSSION

Mouse hepatitis virus has been shown to bud into the compartment that lies at the boundary of the ER and the *cis*-Golgi complex (Tooze *et al.*, 1984, 1988). The M protein probably plays a major role in this budding event. In the case of infectious bronchitis virus (IBV), the M protein by itself localizes to the budding region when expressed independently using a recombinant vaccinia virus vector (Machamer *et al.*, 1990). Treatment of MHV-infected cells with tunicamycin leads to the release of virions containing M protein but apparently lacking the spike (S) (Holmes *et al.*, 1981; Rottier *et al.*, 1981a; Niemann and Klenk, 1981; Stern and Sefton, 1982). Although such experiments have led to believe that the M protein is the sole determinant of budding, in those experiments it was not formally ruled out that these virions lacking spikes contained residual membrane anchors and cytoplasmic tails as was shown for spikeless vesicular stomatitis virus (Chen *et al.*, 1988; Metsikkö and Simons, 1986). Also, the spike protein of MHV, when expressed independently using a vaccinia virus expression system, appears to localize to the intermediate compartment,[4] suggesting some role for this protein in the assembly as well. Moreover, the kinetics of maturation of both membrane proteins argue for a cooperative interaction in defining the process and the site of budding (Vennema *et al.*, 1990).[4]

While their significance with respect to viral budding is not clear, our data here show clearly that the M protein of MHV, when expressed by itself, has information that targets it to the *trans*-Golgi/TGN. This was shown by partial co-localization both with an established marker of the TGN, TGN 38 (Luzio *et al.*, 1990), and with a lectin that binds SA residues. These data are consistent with the analysis of the *O*-linked sugars of the protein. In a pulse-chase analysis, we showed the acquisition of Gal and SA, the addition of which generally are considered to be late Golgi events (Elhammer and Kornfeld, 1984; Cummings *et al.*, 1983).

We also studied the effect of BFA on the maturation of the M protein in the hope of pinpointing more precisely where in the biosynthetic pathway the sugar modifications occur. While the precise molecular mechanism of BFA is still unclear, its main effects are now well established. It blocks secretion of newly synthesized proteins out of the ER (Fujiwara *et al.*, 1988; Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1989). This block is accompanied by the disappearance of the typical organization of the Golgi stack and the appearance in the ER region of marker enzymes that normally localize to the Golgi region (Lippincott-Schwartz *et al.*, 1990). These enzymes can moreover function in this region as witnessed, for example, by the acquisition of galactose and Endo H resistance by newly synthesized proteins localized to the ER (Doms *et al.*, 1989; Chege and Pfeffer, 1990; Lippincott-Schwartz *et al.*, 1989). In contrast, TGN modifications do not appear to occur. Collectively, the data argue for a movement back to the ER region of Golgi proteins normally residing in compartments proximal to the TGN (Chege and Pfeffer, 1990). Our results are consistent with this model. Specifically, in the presence of BFA, the G protein of VSV acquires galactose but does not appear to acquire terminal sialic acid on its *N*-linked oligosaccharides. This agrees with the results of Doms *et al.* (1989), as well as with the acquisition of Gal by newly synthesized mannose 6-phosphate receptors (Chege and Pfeffer, 1990). Under the same conditions as the experiments with the G protein, the M protein of MHV acquired three of the five post-translational modifications we could detect in the absence of BFA. By comparing the effects of the drug on the glycosylation of the G protein to the M protein, we have inferred that the M_4 and M_5 forms are TGN modifications.

In addition to the acquisition of GalNAc, believed to be added in the intermediate compartment (Tooze *et al.*, 1988) and Gal, a late Golgi modification, the M protein also acquired SA in the presence of BFA. This strongly suggests that the addition of SA on *O*-linked oligosaccharides occurs in a pre-TGN compartment. Since it is now accepted that the *N*-linked sialic acids are added in the TGN (Roth *et al.*, 1985), it may be initially surprising that this sugar would be added to *O*-linked oligosaccharides in an earlier Golgi compartment; this suggests, for example, that the CMP-sialic acid transporter would need to be present in at least two different Golgi compartments. Nevertheless, our data are consistent with earlier reports. Thus, Ulmer and Palade (1989) and Shite *et al.* (1990) also found sialylation of *O*-linked oligosaccharides in the presence of BFA. Since they assumed that this modification was normally a TGN event, they favored the idea that the TGN, or a part thereof, had also fused with the ER in the presence of BFA. Our data are clearly inconsistent with this notion: following BFA treatment, the G protein of VSV acquired galactose but did not acquire any detectable sialic acid. Under identical conditions, the M protein became fully sialylated. The simplest interpretation of our data is that sialylation of *O*-linked oligosaccharides occurs in a Golgi compartment proximal to the TGN. Our observations are consistent with the notion that each sugar is added by a single enzyme with unique localization. We cannot, however, rule out the possibility that some sugars are added in multiple

[4] J. Krÿnse Locker, G. Griffiths, and P. J. M. Rottier, unpublished observations.

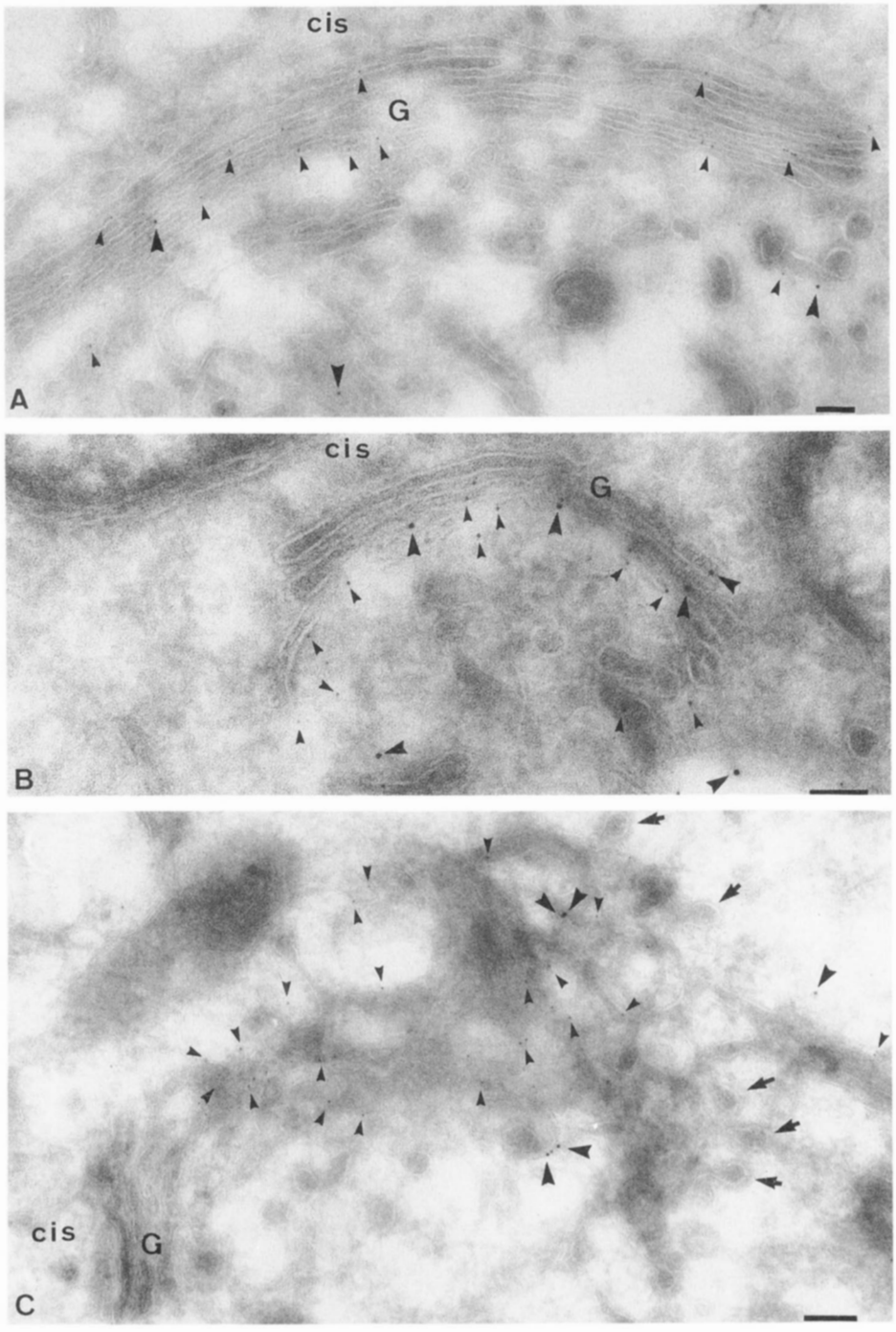

FIG. 7. **Localization of the M protein in double-labeling studies.** Thawed cryosections of glutaraldehyde-fixed Ratec cells double-labeled with two sizes of gold. *A* and *B* show localization of the M protein (small gold, *small arrowheads*) with TGN 38 (large gold, *large arrowheads*). Note that the bulk of the M labeling is on the *trans* side of the stack. In *C*, an extended region of the TGN is seen in a section double-labeled for M (small gold, *small arrowheads*) and *L. flavus* lectin, visualized with fetuin gold (large gold, *large arrowheads*). The network appearance of the TGN is especially evident in this image as are the typical coated bud structures (*arrows*). *Bars*, 100 nm.

locations, possibly by different enzymes, as is the case for glycosphingolipid synthesis (Young *et al.*, 1990; van Echten *et al.*, 1990).

We were not successful in identifying the terminal modifications leading to M_4 and M_5 forms occurring in a late, presumably TGN, compartment. Several options were, however, ruled out. Sulfation and fucosylation are probably not involved since we were unable to radiolabel the M protein with the respective precursors (not shown). Attachment of an *O*-acetylated derivative of SA is also considered unlikely since this modification should be rendered neuraminidase-sensitive after treatment with alkali (Varki and Diaz, 1984; Corfield *et al.*, 1986), a condition which was not fulfilled (not shown). Possible options remaining are the addition of GlcNAc and GalNAc to *O*-linked sugars, but the identification of such sugars will have to await chemical analysis as they cannot be specifically labeled *in vivo*.

We showed that in the presence of BFA the G protein also acquired fucose. Fucosyltransferase has not yet been localized by immunocytochemistry although fucosyl lectins have been shown to bind preferentially to cisternae on the *trans* side of the Golgi stack (Ellinger and Pavelka, 1988). The kinetic and fractionation data of Goldberg and Kornfeld (1983) suggested that this modification occurs in the medial Golgi. Our data are fully consistent with these results and argue strongly that fucosyltransferase is present in a compartment proximal to the TGN.

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