The Effects of Processing Inhibitors of *N*-Linked Oligosaccharides on the Intracellular Migration of Glycoprotein E2 of Mouse Hepatitis Virus and the Maturation of Coronavirus Particles*

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We have studied the effects of tunicamycin and inhibitors of the processing of *N*-linked glycans including *N*-methyl-1-deoxynojirimycin, castanospermine, mannodeoxynojirimycin, and swainsonine on the transport of glycoprotein E2 and the intracellular maturation of the coronavirus mouse hepatitis virus A59. Indirect immunofluorescence staining with monoclonal antibodies revealed that glycoprotein E2 exhibits different antigenic properties depending on the presence and on the structure of the *N*-linked oligosaccharides and that efficient transport of glycoprotein E2 to the plasma membrane requires the removal of glucose residues. In the presence of tunicamycin in the nonglycosylated E2 apoprotein was synthesized in normal amounts and readily acylated throughout the infectious cycle. This E2-species could not be detected on the surface of mouse hepatitis virus A59-infected cells with indirect immunofluorescence staining or lactoperoxidase labeling. *N*-Methyl-1-deoxynojirimycin and castanospermine, both of which selectively inhibited the processing glucosidases, caused a drop in virion formation by two log steps and a drastic delay in the surface expression of glycoprotein E2. The E2 species synthesized under such conditions was acylated but accumulated intracellularly in a compartment distinct from the Golgi. Concomitantly, synthesis of the matrix glycoprotein E1 of mouse hepatitis virus A59 was drastically impaired. Mannodeoxynojirimycin and swainsonine, which block later stages of the processing pathway, had less or no effect on the transport of glycoprotein E2 and the formation of virus particles.

Glycoproteins of enveloped viruses have frequently been used as tools to study the synthesis, processing, and intracellular transport of membrane-associated polypeptides. For two reasons, MHV[1] A59, a member of the coronavirus family, is of particular interest in this respect: Unlike other viruses studied in detail, such as α-viruses, ortho- and paramyxoviruses, or rhabdoviruses, coronaviruses maturate intracellularly from membranes of the rough and smooth endoplasmic reticulum (1, 2). In addition, MHV A59 directs the synthesis of two entirely different membrane glycoproteins, designated E1 and E2, which differ in important structural and biosynthetic properties.

Glycoprotein E1 is a small matrix-like transmembrane protein ($M_r = 23$ K) (3–5) which is translated on membrane-associated ribosomes (6) but lacks a hydrophobic leader sequence at its amino-terminal end (4, 5). E1 is rapidly transported to transient elements, into which preferential virus particle formation occurs at the early stage of infection (2). E1 carries exclusively *O*-glycosidically linked carbohydrate side chains (7–10) which are added at a post-translational stage in the Golgi apparatus, presumably, when the virus particles are transported through this compartment (2, 10).

Glycoprotein E2 is a constituent of the viral spikes, which are responsible for attaching the virus to cell surface receptors and which carry the fusion capacity of the virus (3, 9, 11). E2 is translated and cotranslationally *N*-glycosylated as a $M_r = 180$ K species in the rough endoplasmic reticulum. It is post-translationally processed by acylation and proteolytic cleavage, yielding two $M_r = 90$ K species (7, 12). Like the *N*-glycosylated glycoproteins of other viruses mentioned above, E2 is transported to the plasma membrane of the infected cell where it induces fusion with neighboring cells and thus mediates spread of the virus (9, 12). Relatively little is known about the involvement of glycoprotein E2 in the intracellular maturation process and the secretion of MHV A59 particles. It has been reported previously that noninfectious E2-depleted virus particles are released from cells treated with tunicamycin, an inhibitor of *N*-glycosylation (7–9). In the work reported here, glycosylation and processing inhibitors of *N*-linked glycans have been used to study the requirements of carbohydrates attached to glycoprotein E2 for transport as well as for its ability to integrate properly into newly formed virus particles.

[1] The abbreviations used are: MHV, mouse hepatitis virus; VSV, vesicular stomatitis virus; MdN, *N*-methyl-1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol; DIM, 1,5-dideoxy-1,5-imino-D-mannitol; SDS, sodium dodecyl sulfate; endo-H, endo-*N*-acetylglucosaminidase H; PFU, plaque-forming units; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

MATERIALS AND METHODS AND RESULTS[2]

Effects of Tunicamycin and Processing Inhibitors of N-Linked Glycans on the Formation of MHV A59 Particles—It has been reported previously (7–9, 13) that tunicamycin and other inhibitors of N-glycosylation such as 2-deoxyglucose or 2-deoxy-2-fluoroglucose do not interfere with the intracellular formation and release of coronavirus particles, and it is generally accepted that particles produced under these conditions are noninfectious due to the lack of the peplomeric protein E2. We have studied the formation of virus particles in the presence of tunicamycin and inhibitors of the processing of N-linked glycans (Table 1). MdN and castanospermine are two compounds with dissimilar chemical structures known to inhibit the glucosidases I and II which are involved in the processing of N-linked oligosaccharides (14–16). DIM and swainsonine interfere with mannosidase I and II, respectively, which act at later stages of oligosaccharide processing on the products of the preceding glucosidase reactions (17–21). MdN was used instead of 1-deoxynojirimycin, since this compound did not interfere with the formation of lipid-linked oligosaccharides in 17Cl1 cells at concentrations of up to 6 mM MdN (data not shown; see also Refs. 15, 23).

The addition of MdN or castanospermine to the growth medium resulted in a reduction of virus titers by about two log steps (Table 1). It is noteworthy that the titers obtained after treatment of cells with 2, 4, or 8 mM MdN, respectively, differed only slightly. Furthermore, it was not possible to suppress viral growth completely with either inhibitor, even when the growth medium was replaced at various stages of the infectious cycle by medium containing fresh inhibitor (data not shown). In the presence of DIM the yield of infectious virus was reduced to about 20% of the untreated control, while swainsonine had no measurable effect. VSV (strain New Jersey) was grown as a control in 17Cl1 cells treated with the corresponding inhibitors to rule out cytotoxic effects. Table 1 shows that the replication and infectivity of VSV were not changed significantly in the presence of MdN. This finding is in agreement with previous reports which showed that the N-linked oligosaccharides of the G protein of this strain of VSV could be specifically modified by the addition of 1-deoxynojirimycin (24) or DIM (25) without affecting the infectivity of the virus.

An electron-microscopic inspection of thin sections of MHV A59-infected 17Cl1 sister cultures grown in the presence or absence of MdN revealed that 10 h after infection the intracellular maturation of MHV A59 was drastically retarded in cells treated with 4 mM MdN (data not shown).

Synthesis of MHV A59 Polypeptides in Cells Treated with Tunicamycin and Processing Inhibitors—To follow the synthesis and the intracellular distribution of E2 species made in the presence of tunicamycin or trimming inhibitors we used a series of monoclonal antibodies raised against the native antigen (26, 27). Previous studies have indicated that the E2 polypeptide exhibits at least six different epitopes (26). We have selected five out of a collection of 15 E2-specific hybridoma antibodies which reacted differently with individual E2 species depending on the degree of glycosylation (Table 2).

Two of these antibodies (E2-D13 and E2-F18) reacted specifically with the nonglycosylated E2 polypeptide synthesized in the presence of tunicamycin or with the intracellular forms of E2 synthesized in untreated or MdN-treated cells. However, these antibodies failed to bind to infected cells and had no virus neutralizing activity (26). In contrast, antibodies E2-L15 and E2-A4, which precipitated the mature forms of glycoprotein E2 (M_r = 180 K and 90 K) as well as E2 from MdN-treated cells (hereafter referred to as E2^{MdN}) showed no or only weak binding to the nonglycosylated apoprotein of E2.

Pulse-chase experiments performed in the presence of tunicamycin and trimming inhibitors indicated that there was no significant inhibition or delay of synthesis of the corresponding E2 species. The nonglycosylated E2 apoprotein was detectable as early as 5 h after infection. The molecular weights of the E2 species synthesized in the presence of trimming inhibitors varied slightly, indicating that processing of the oligosaccharides was inhibited at specific stages (Fig. 4). Glycoprotein E2^{MdN} was synthesized as a M_r = 190 K species carrying predominantly Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide side chains (85%), while the remaining 15% showed trimming of the mannose branches to Glc$_3$Man$_8$GlcNAc$_2$ and Glc$_3$Man$_7$GlcNAc$_2$ species (data not shown). Pulse-chase experiments performed in the presence of cycloheximide showed that E2^{MdN} had a prolonged half-life of 4 h (*versus* 2 h in nontreated cells), supporting the finding that E2^{MdN} was not chased efficiently into budding virus particles. Glycoprotein E2 isolated from virus particles from cells grown in the presence of DIM or swainsonine contained endo-N-acetylglucosaminidase H-sensitive oligosaccharides. Similar results have been obtained with several other glycoproteins, such as the G protein of VSV (25), the hemagglutinin of influenza virus (17, 25), IgM and IgD (28), or class I histocompatibility antigens (25, 28).

The synthesis of the E1 polypeptide was significantly impaired in cells treated with MdN or castanospermine, while DIM or swainsonine had less or no effect (Fig. 4). It is difficult to envisage a direct interaction between the E1 polypeptide, which carries exclusively O-glycosidically linked side chains composed of N-acetylgalactosamine, galactose, and neuraminic acid, and enzymes that are involved in the processing of N-linked glycans. It is likely, however, that the concentration of E1 becomes the limiting factor for virus formation in MdN-treated cells.

Intracellular Distribution of Glycoprotein E2 of MHV A59 in Cells Treated with Tunicamycin and Trimming Inhibitors—One of the biological functions of glycoprotein E2 of MHV A59 is reflected in its fusion capacity, which may play a role in the entry process of the particles into a host cell as well as in the spread of the disease by the fusion of these cells with neighboring tissues.

When monolayers of 171Cl1 cells (8×10^3 cells/cm^2) were infected at a multiplicity of 50 plaque-forming units/cell, fusion was normally observed after 5 h, and large syncytia with about 20 nuclei each had formed 9 h after infection. Cell fusion was complete after about 12 h when cells started to detach and to lyse.

Fig. 1 shows that 7 h after infection the E2 antigen was present only intracellularly in perinuclear regions and within the rough endoplasmic reticulum of tunicamycin-treated cells. It is interesting to note that this labeling pattern did not change at late stages of infection. Furthermore, surface labeling of tunicamycin-treated cells was negative at all stages (Fig. 1B). This finding was verified with other monoclonal antibodies (E2-L15 and E2-A4) which were capable of binding to the surface of nontreated infected 17Cl1 cells as listed in
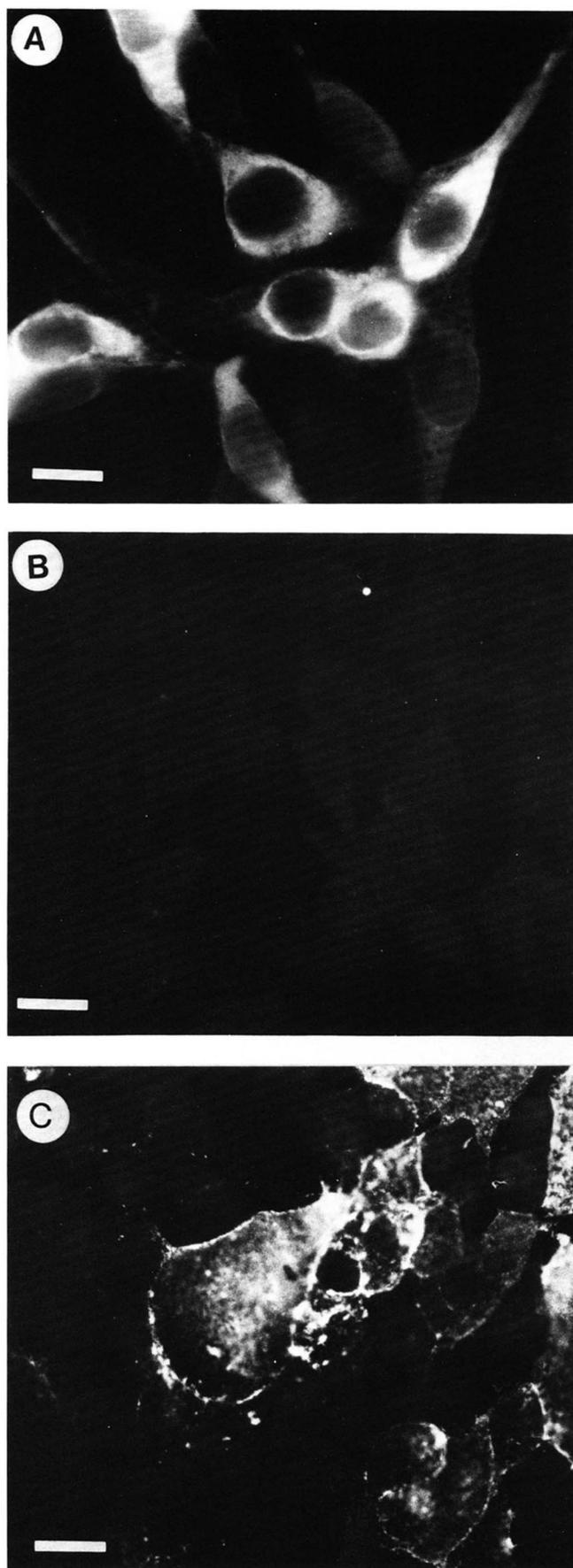
Table 2. In addition, we were unable to detect any E2 (M_r = 150 K) species by surface labeling with lactoperoxidase. The notion that the nonglycosylated E2 species was not transported to the plasma membrane is in agreement with the observation that MHC A59-infected cells did not fuse when the glycosylation inhibitor was present (7–9). Cell fractionation experiments indicated, however, that the E2 species synthesized in the presence of tunicamycin was transported into smooth membrane compartments (data not shown).

In infected sister cultures which were treated with 4 mM MdN or castanospermine after the 1-h adsorption period of the virus, cell fusion was drastically retarded and was first detected 9–10 h after infection, when occasionally fusion of two to three cells was observed. However, fusion of the monolayer was slowly progressing, and 24 h after infection the degree of fusion resembled that of nontreated cultures about 9 h post-infection. DIM had also some retarding effect when applied at a final concentration of 4 mM, while swainsonine did not influence this process at all.

To determine whether the MdN- and castanospermine-induced delay in fusion was caused by an inhibition of transport of glycoprotein E2 to the plasma membrane or by the presence of a biologically inactive E2 species, we studied the distribution of glycoprotein E2 in such cells by immunofluorescence.

Fig. 2A shows the results obtained by surface labeling of MHV A59-infected cells with monoclonal anti-E2-A4 9 h after infection. Glycoprotein E2 was present on the surface and predominantly along the border line of fused cells. In contrast, MdN-treated infected sister cultures did not express significant amounts of E2 on their surface at this time of infection (Fig. 2B). It should be noted that the absence of E2 was verified with polyclonal mouse and rabbit sera raised against native virus. Therefore, it can be excluded that the particular epitope recognized by the monoclonal E2 antibody was altered in the E2^{MdN} species (for further details, see Table 2).

Fig. 2C and D shows the differences in the intracellular distribution of glycoprotein E2 synthesized in the absence or presence of 4 mM MdN, respectively. As reported previously (29), the E2 antigen was dispersed throughout the cytoplasmic membrane system of the infected control cells. MdN-treated cells (Fig. 2D) did not show such a uniform distribution of E2. In some cells the E2 antigen accumulated apart from the nucleus within the distal cytoplasm (Fig. 2, D and E). Identical accumulations of glycoprotein E2 were also observed in cells treated with castanospermine. At present it remains unclear to which intracellular organelle these aggregates of E2 can be assigned. We performed double labeling of such cells using monoclonal anti-E2-F18 or anti-E2-A4 antibodies together with a Golgi-specific antiserum. These studies showed that the organelles with accumulations of E2 were not recognized by the Golgi antibody (Fig. 2, E and F).

Comparative cell fractionation experiments performed with MHV A59-infected cells grown in the presence or absence of 4 mM MdN indicated that the E2^{MdN} species was transported into smooth-membrane fractions (data not shown).

Acylation of Glycoprotein E2 in Tunicamycin- and MdN-treated Host Cells—We have shown previously (7) that gly-

FIG. 1. **Indirect immunofluorescence labeling of MHB A59-infected 17Cl1 cells grown in the presence of tunicamycin.** Cells were infected at a multiplicity of 20 plaque-forming units/ml and tunicamycin (2 μg/ml) was added after the 1-h adsorption period of the virus. *A*, labeling of fixed cells 7 h after infection using monoclonal E2-F18 antiserum; *B*, surface labeling of sister cultures of *A* with a mixture of monoclonal E2-A4 and E2-F18 antisera (mixed at a ratio of 1:1); *C*, surface labeling of nontreated control cells 7 h after infection using the same mixture of monoclonal antibodies as in *B*. *Bars*, 10 μm.
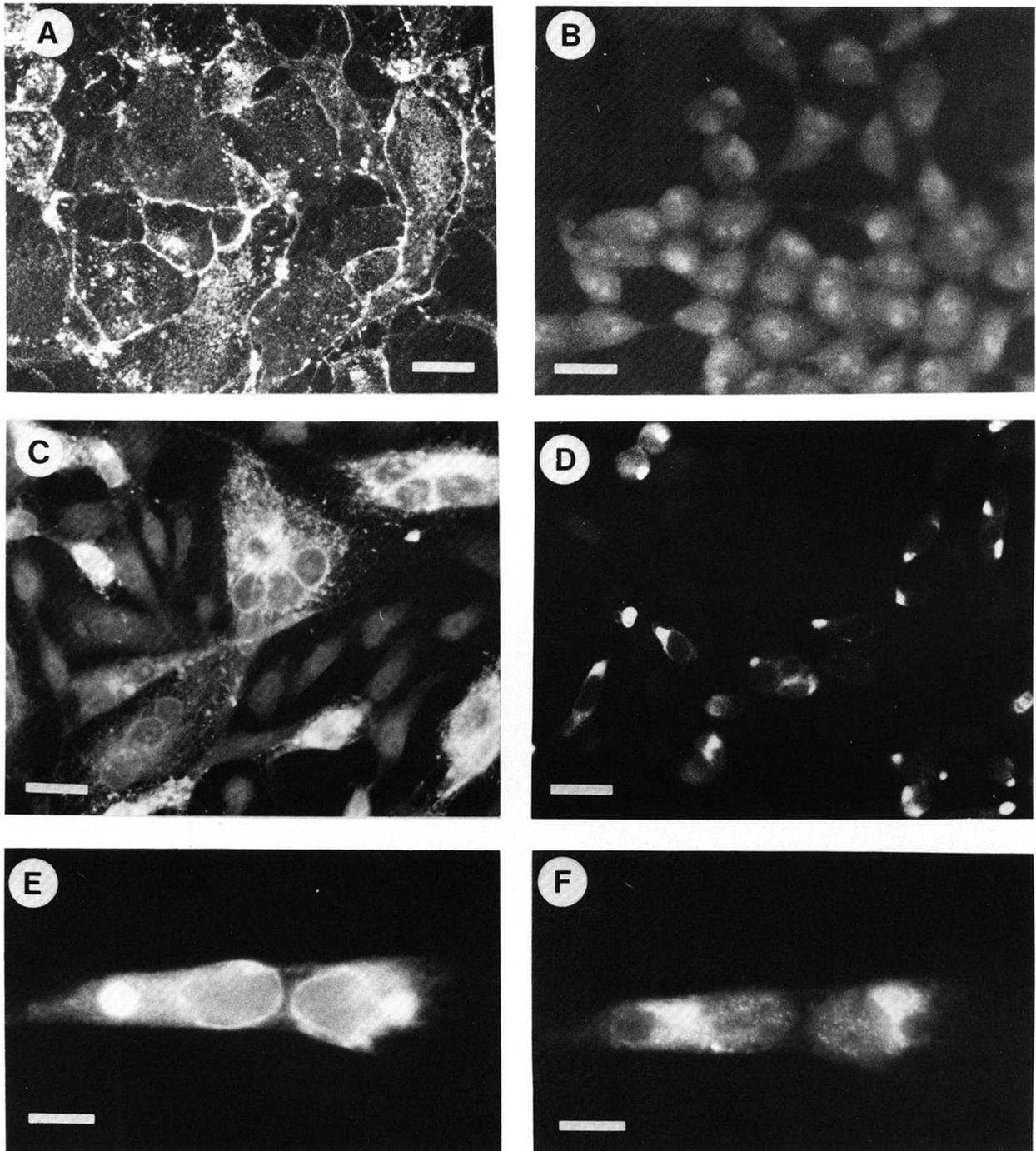
FIG. 2. **Effects of MdN on the migration of glycoprotein E2 in MHV A59-infected 17Cl1 cells.** Infected sister cultures were grown on cover slips in the presence or absence of 4 mM MdN and inspected 9 h after infection by indirect immunofluorescence labeling using monoclonal E2-L15 antiserum. *A,* surface labeling of cells grown in the absence of MdN; *B,* surface labeling of a culture grown in the presence of 4 mM MdN; *C,* MHV A59-infected cells labeled after fixation as described under "Materials and Methods"; *D,* the same as in *C,* but cells were grown in the presence of MdN; *E, F,* double labeling of MdN-treated cells after permeabilization using monoclonal E2-L15 (*E*) and a Golgi-specific rabbit serum (kindly provided by B. Burke, European Molecular Biology Laboratories, Heidelberg) (*F*). *Bars,* 20 μm in *A–D,* 10 μm in *E* and *F.*

coprotein E2 is post-translationally acylated, a feature observed with many viral glycoproteins, especially those carrying cell-fusion activity (30). Studies carried out with Sindbis virus-infected cells suggested that acylation occurred in the smooth endoplasmic reticulum or early Golgi complex (31, 32).

Fig. 3 shows that neither tunicamycin nor MdN interfered

with the acylation of the corresponding E2 polypeptides. At 8 h after infection, when no E2 could be detected on the surface of MdN-treated cells, the intracellular E2^{MdN} species was readily labeled with [^{3}H]palmitate. Although cell fractionation experiments carried out with palmitate-labeled infected cells grown in the presence of tunicamycin or MdN suggested that a considerable amount of label (30%) was incorporated
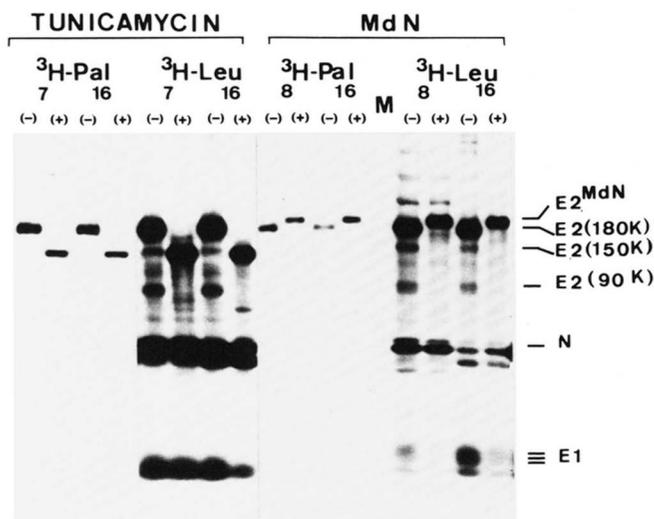
FIG. 3. **Acylation of the E2 polypeptide of MHV A59 in tunicamycin-treated cells or cells treated with 4 mM MdN.** MHV A59-infected sister cultures grown in the presence (+) or absence (−) of the corresponding inhibitor were labeled 4 h after infection with either [³H]palmitate (³H-Pal) or with [³H]leucine. Cytoplasmic lysates were prepared at the times indicated and immunoprecipitated using a polyclonal rabbit serum raised against virus (tunicamycin) or with a polyclonal mouse serum obtained from reconvalescent mice (MdN). Products were analyzed on a 5–15% sodium dodecyl sulfate-polyacrylamide gel. M, results from mock-infected cells.

already in the rough endoplasmic reticulum, most of the labeled material was found in the smooth-membrane fraction (data not shown). At this stage of infection the majority of E2^MdN molecules accumulated in an intracellular compartment clearly distinct from the Golgi complex (Fig. 2, E and F).

DISCUSSION

Our data show that treatment of MHV A59-infected cells with tunicamycin and trimming inhibitors of N-linked glycans influence the transport properties as well as the antigenic properties of the spike glycoprotein E2 and indirectly affect the growth of MHV A59.

The conclusion that these effects were due specifically to an altered structure of glycoprotein E2 is based on the following observations: (i) Monoclonal anti-E2 antibodies revealed different binding properties in immunoprecipitation reactions and immunofluorescence studies depending on the degree of glycosylation and the structures of the oligosaccharides. (ii) The same effects were obtained with two chemically dissimilar compounds which specifically inhibited glucosidase I: castanospermine and MdN. In contrast, inhibitors which interfere at later stages of the processing of N-linked glycans, DIM or swainsonine, had less or no effects, respectively.

Our finding that the nonglycosylated E2 apoprotein is synthesized with normal kinetics but is not transported to the cell surface is similar to that observed with the glycoproteins of Sindbis virus and the San Juan strain of VSV (33). In these systems treatment with tunicamycin results in the formation of apoproteins which exhibit a reduced solubility and are not transported to the plasma membrane.

The intracellular distribution of E2^MdN was distinct from that observed for the nonglycosylated E2 species. While infected cells grown in the presence of tunicamycin showed a diffuse distribution of E2 (Fig. 1A), the presence of MdN caused specifically localized accumulations of E2^MdN (Fig. 2D)

which could not be stained with a Golgi-specific antibody. We can only speculate about the cellular compartment harboring these accumulations. The E2^MdN species was readily labeled with [³H]palmitate (Fig. 3). It is likely, therefore, that the transport of E2^MdN is impaired in the smooth endoplasmic reticulum. However, further proof must await immunocytochemical labeling of frozen sections of MdN-treated cells.

There are other examples which show that the removal of glucose residues may be critical for the transport and the secretion of glycoproteins. Peyrieras *et al.* (28) demonstrated that the secretion of IgD, but not of IgM was blocked in the presence of 1-deoxynojirimycin, while swainsonine did not affect the release of either glycoprotein. Similar results were observed for the secretion of the 1-proteinase inhibitor. Again the presence of 1-deoxynojirimycin blocked secretion, whereas swainsonine had no effect (34, 35).

The mechanism by which MdN, castanospermine, and to a lesser extent DIM effect the growth of MHV A59 (whereas tunicamycin does not) is not yet fully understood. VSV (strain New Jersey), also a single-stranded RNA-containing virus, could be propagated to similar titers in the presence or absence of these drugs, indicating that the inhibition observed with MHV A59 cannot simply be assigned to cellular dysfunctions. We have shown that synthesis of the matrix glycoprotein E1 of MHV A59 was specifically reduced in the presence of MdN and castanospermine, while tunicamycin had no significant effects (Fig. 4).

It is also feasible to assume that E2^MdN exhibits a conformation which prevents its integration into virus particles. Recently, Schlesinger *et al.* (24) have demonstrated that the failure to remove the outermost glucose residue from the G protein of the San Juan strain of VSV results in a temperature-sensitive formation of virus particles. The authors concluded that the inability of the nonprocessed G protein to integrate into budding virus particles at the nonpermissive temperature was caused by an altered conformation of the G protein. Similar results were observed with Sindbis virus (23, 36). In this system castanospermine and deoxynojirimycin inhibited proteolytic processing of the viral glycoprotein precursor E2 and virus formation. In contrast, neither castanospermine nor deoxynojirimycin had any effect on the growth and infectivity of influenza virus (14, 15, 25). However, bromoconduritol, an inhibitor of oligosaccharide processing which interferes with the release of the innermost glucose residue, was shown to block the growth of influenza virus. This finding suggests that a functional structure of the hemagglutinin molecule may depend on a particular step in glucose removal. From these data it may be concluded that the establishment of functional conformation of a glycoprotein requires processing of its glycans to a specific stage of the trimming pathway and that the extent of trimming necessary is a variable of the individual glycoprotein.

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SUPPLEMENTARY MATERIAL TO

THE EFFECTS OF PROCESSING INHIBITORS OF N-LINKED OLIGOSACCHARIDES ON THE
INTRACELLULAR MIGRATION OF GLYCOPROTEIN E2 OF MOUSE HEPATITIS VIRUS AND
THE MATURATION OF CORONAVIRUS PARTICLES.

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MATERIALS AND METHODS

Viruses and cells.--- MHV A59 and VSV (strain New Jersey) were propagated from plaque-purified virus stocks in the spontaneously transformed 17C11 line of BALB/c 3T3 cells using Dulbecco's modified minimum essential medium (DMEM) supplemented with 10 % fetal calf serum. Plaque assays were performed in 17C11 cells with 0,8 % agar in the overlay medium.

Inhibitors.--- 1-Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol, Bay H5595) was a generous gift from Dr. E. Truscheit, Bayer AG. Wuppertal, West-Germany. The compound was N-methylated according to Murai et al. (22), to yield MdN. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was kindly provided by R.J. Molyneux Western Regional Research Center, USDA, Berkeley, and applied at 100 ug/ml. Swainsonine (1,2,8-trihydroxyoctahydroindolizine) was a gift from P.R. Dorling, Murdoch University, Western Australia, and used at a final concentration of 1 µg/ml. DIM (1,5-dideoxy-1,5-imino-D-mannitol) was a generous gift from Dr. G. Kinast, Bayer AG., Wuppertal, FRG. Tunicamycin was purchased from Eli Lilly (Indianapolis, Indiana/USA), and was used at a final concentration of 2 µg/ml. All inhibitors were added after the 1 h adsorption period of the virus and maintained throughout the experiment.

Labeling of cells and viruses.--- For pulse-chase labeling with (^{3}H)leucine MHV A59-infected 17C11 cells (Multiplicity of infection: 50 PFU/cell) grown on 4.5 cm Petri dishes were washed and starved in leucine depleted medium for 1 h. The cells were then labeled for 15 min with 50 µCi/ml (^{3}H)leucine (38 Ci/mM, Amersham-Buchler, Braunschweig), and were either lysed immediately or chased with nonradioactive medium containing 10x excess of leucine. Labeling of oligosaccharides was routinely performed in DMEM containing 10 mM fructose instead of glucose and 100 µCi/ml 2-(^{3}H)mannose (17 Ci/mmol). To study acylation of viral glycoproteins (9,10(n)^{3}H)palmitic acid was added to the growth medium at a final concentration of 100 µCi/ml.

Preparation and analysis of glycopeptides and oligosaccharides.--- The pronase digestion of glycoprotein E2 isolated from gels has been described previously (9). Glycopeptides were desalted by passage through a Biogel P2-column (35 x 1 cm), digested with endo-H and analyzed on a Biogel P4-column (150 x 1 cm) as described by Datema et al. (37).

Antisera.--- The preparation of a polyclonal MHV A59 antiserum in rabbits has been described previously (7). In addition, a polyclonal antiserum was obtained from reconvalescent mice, each of which was infected with 5 x 10^3 PFU of MHV A59. Monoclonal antibodies against glycoprotein E2 of MHV A59 were obtained by immunization of Balb/c-mice with glycoprotein E2 isolated under conditions as described by Sturman et al. (38). Alternatively mice were immunized by infection with MHV JHM, a virus related to MHV A59 (26). Fusion of spleen cells with X63-Sp8-653 or X63-Ag8 plasmacytoma cells and selection of antibody-producing hybrid cells were performed as described previously (39, 40, 26). The specificities of the monoclonal antibodies was determined by immunoprecipitation of infected cell lysates using rabbit anti mouse IgG (Sigma) for the precipitation of the immune complexes. Immunoglobulin isotypes were determined by double-diffusion tests.

Immunofluorescence.--- Cells grown on coverslips were washed for 10 min with solution A (DMEM containing 5 % fetal calf serum and 10 mM sodium azide) and incubated for 1 h with monoclonal anti-E1 or anti-E2 mouse sera in a high-humidity chamber. This incubation and the following steps were carried out at 4° C. Cells were washed twice with solution A and incubated for 1 h with FITC-conjugated rabbit-anti-mouse-IgG (Cappel, Dynatech, D-7310 Plochingen, FRG). After washing with solution A the cells were rinsed with solution B (phosphate buffered saline containing 3 % (w/v) bovine serum albumin and 10 mM sodium azide). After two washes with PBS (5 min each) and a brief wash with distilled water the coverslips were mounted on a microscope slide with Elvanol. Intracellular staining was performed according to Boschek et al.(41). Cells were fixed at 4°C with freshly prepared 2.5 % formaldehyde and 0.18 % Triton X-100 in PBS. For double label experiments a rabbit anti-Golgi serum (kindly provided by B. Burke, EMBL, D-6900 Heidelberg, FRG) was used in combination with rhodamine-conjugated goat anti-rabbit-IgG (Cappel). The stained preparations were viewed and photographed with a Zeiss-Universal microscope simultaneously equipped with epifluorescent illuminator (Zeiss FITC-selective filter set No. 10H or rhodamine-selective filter set Nr. 14H) and Nomarsky interference contrast optics.

Immunoprecipitation, gel electrophoresis and fluorography.--- The lysis of infected cells, immunoprecipitation with a polyclonal anti-MHV A59 rabbit serum and the analysis of the immunoprecipitates on 5 to 15 % SDS-polyacrylamide slab gels has been described previously (7).

RESULTS

TABLE 1: EFFECTS OF VARIOUS PROCESSING INHIBITORS ON THE RELEASE OF INFECTIOUS MHV A59 OR VSV FROM 17C11-CELLS.

| INHIBITOR[A] | MHV A59 (PFU/ml)[B] | | | | VSV (PFU/ml)[C] | | |
	6 h p.i.	9 h p.i.	16 h p.i.	30 h p.i.	3 h p.i.	8 h p.i.	24 h p.i.
no inhibitor	4.9×10^4	6.2×10^5	4.2×10^6	1.5×10^7	5.6×10^3	6.8×10^7	1.8×10^8
MdN (2 mM)		3.0×10^3				2.5×10^7	
MdN (4 mM)	2.6×10^2	2.5×10^3	7.0×10^4	1.6×10^5	4.2×10^3	2.8×10^7	6.8×10^7
MdN (8 mM)		1.8×10^3					
Castanospermine (100 μg/ml)		2.0×10^3					
DIM (4 mM)		4.4×10^4					
Swainsonine (1 μg/ml)		2.6×10^5					
Tunicamycin (2 μg/ml)	1.2×10^2	2.0×10^2	4.0×10^2	3.8×10^2			

A) all inhibitors (final concentration) were added after the 1 h adsorption period of the virus.
B) multiplicity of infection: 20 plaque forming units/ml.
C) strain New Jersey, multiplicity of infection: 10 plaque forming units/ml.

TABLE 2: CHARACTERIZATION OF HYBRIDOMA ANTIBODIES DIRECTED AGAINST GLYCOPROTEIN E2 OF MHV A59.

| DESIGNATION (EPITOPE CLASS[A]) | ISOTYPE[B] | SPECIFICITY IN IMMUNO-PRECIPITATION | IMMUNOFLUORESCENCE OF MHV A59-INFECTED CELLS | | | | | |
| | | | NONTREATED CONTROL | | TUNICAMYCIN | | MdN | |
			SURFACE	INTRACELL.	SURFACE	INTRACELL.	SURFACE	INTRACELL.
E2-L15 (A)	IgG-I	gpE2(180K)[C], gpE2(90K)[D], gpE2[MdN]	+++[E]	+++	-	-	++[F]	+++
E2-A4 (A)	IgG-2A	gpE2(180K)[C], gpE2(90K)[D], gpE2[MdN]	+++	+++	-	-	++[F]	+++
E2-C8 (C)	IgG-2A	gpE2(180K)[G], gpE2[MdN]	(+)	+++	-	(+)	++[F]	+++
E2-D13 (D)	IgG-2A	E2(150K), gpE2[MdN], gpE2(180K)[G]	(+)	+++	-	+++	++[F]	+++
E2-F18 (F)	IgG-2A	E2(150K), gpE2[MdN] gpE2(180K)[G]	+	+++	-	+++	++[F]	+++

A) according to Wege et al.(26).
B) determined by Ouchterlony double diffusion tests.
C) isolated from mature virus particles containing 75% endo H-resistant side chains.
D) we did not determine which of the E2(90K)-fragments was precipitated.
E) the rating was: +++ (brilliant fluorescence), ++ (strong), + significant, (+) (barely above background).
F) surface expression was drastically delayed in comparison to nontreated cells.
G) intracellular E2(180K) with endo-H-sensitive oligosaccharides.
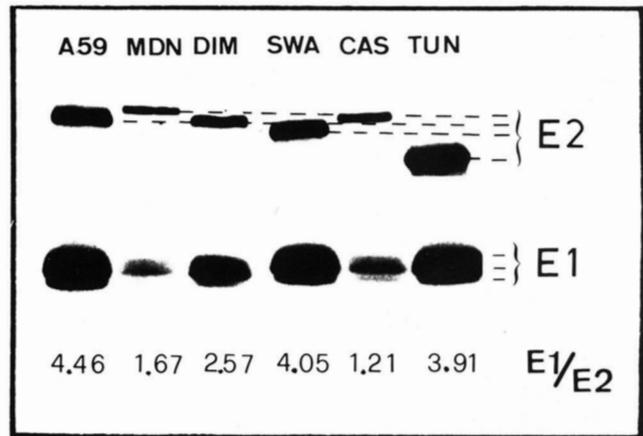
Fig. 4. Effects of tunicamycin and of processing inhibitors of N-linked oligosaccharides on the synthesis of glycoproteins E1 and E2 of MHV A59. MHV A59-infected sister cultures (multiplicity of infection: 50 PFU/cell) were grown in the presence or absence of the inhibitors as indicated. 7 h after infection the cells were pulse-labeled for 10 min with (^{3}H)leucine and chased for 1 h in the presence of the corresponding drug. Cell lysates were immunoprecipitated using a polyclonal antiserum obtained from MHV A59-infected reconvalescent mice. The products were analyzed on an SDS-polyacrylamide gel. After fluorography, bands corresponding to the positions of E1 and E2 were excised from the dried gel, reconstituted with water and assayed by liquid scintillation counting as described previously (7).