

Tunicamycin Resistant Glycosylation of a Coronavirus Glycoprotein: Demonstration of a Novel Type of Viral Glycoprotein

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Tunicamycin has different effects on the glycosylation of the two envelope glycoproteins of mouse hepatitis virus (MHV), a coronavirus. Unlike envelope glycoproteins of other viruses, the transmembrane glycoprotein E1 is glycosylated normally in the presence of tunicamycin. This suggests that glycosylation of E1 does not involve transfer of core oligosaccharides from dolichol pyrophosphate intermediates to asparagine residues, but may occur by O-linked glycosylation of serine or threonine residues. Synthesis of the peplomeric glycoprotein E2 is not readily detectable in the presence of tunicamycin. Inhibition of N-linked glycosylation of E2 by tunicamycin either prevents synthesis or facilitates degradation of the protein moiety of E2. Radiolabeling with carbohydrate precursors and borate gel electrophoresis of glycopeptides show that different oligosaccharide side chains are attached to E1 and E2. The two coronavirus envelope glycoproteins thus appear to be glycosylated by different mechanisms. In tunicamycin-treated cells, noninfectious virions lacking peplomers are formed at intracytoplasmic membranes and released from the cells. These virions contain normal amounts of nucleocapsid protein and glycosylated E1, but lack E2. Thus the transmembrane glycoprotein E1 is the only viral glycoprotein required for the formation of the viral envelope or for virus maturation and release. The peplomeric glycoprotein E2 appears to be required for attachment to virus receptors on the plasma membrane. The coronavirus envelope glycoprotein E1 appears to be a novel type of viral glycoprotein which is post-translationally glycosylated by a tunicamycin-resistant process that yields oligosaccharide side chains different from those of N-linked glycoproteins. These findings suggest that E1 may be particularly useful as a model for studying the biosynthesis, glycosylation, and intracellular transport of O-linked glycoproteins.

INTRODUCTION

Elucidation of the synthesis, glycosylation, and intracellular transport of glycoproteins is essential to understanding the structure and function of cell membranes and the role of oligosaccharides in glycoprotein processing and secretion. Because glycosylation and transport of viral envelope glycoproteins depend upon cellular processes, the G glycoprotein of vesicular stomatitis virus has been used as an excellent model for glycosylation and transport of N-linked glycoproteins (Roth-

man and Lodish, 1977; Rothman *et al.*, 1978; Morrison *et al.*, 1978; Gibson *et al.*, 1979). Oligosaccharides may also be O-linked to serine or threonine residues of the polypeptide chain by a process which is less well understood (Sharon and Lis, 1981). O-Linked oligosaccharides are predominant in many cell surface glycoproteins such as glycophorin (Tomita and Marchesi, 1975) and in secreted glycoproteins such as submaxillary mucins (Slomiany and Slomiany, 1978).

Although tunicamycin inhibits glycosylation of N-linked glycoproteins (Takatsuki *et al.*, 1971; Lehle and Tanner, 1976; Schwarz *et al.*, 1976; Schwarz *et al.*, 1979;

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Elbein, 1979; Schwarz and Datema, 1980), no drug to inhibit O-linked glycosylation has yet been identified (Schwarz *et al.*, 1979; Sharon and Lis, 1981). All viral envelope glycoproteins studied to date have been of the N-linked type of glycoproteins (Leavitt *et al.*, 1977; Morrison *et al.*, 1978; Schwarz *et al.*, 1979; Nakamura and Compans, 1978a; Cash *et al.*, 1980; Pizer *et al.*, 1980; Klenk and Rott, 1980; Choppin and Scheid, 1980; Ghosh, 1980; Stallcup and Fields, 1981). We now present evidence that a coronavirus glycoprotein may be glycosylated by a different mechanism. This glycoprotein may serve as a useful model for the study of O-linked glycoproteins.

Coronaviruses are enveloped viruses containing $\sim 5.8 \times 10^6$ daltons of positive sense, single-stranded polyadenylated RNA (Tyrrell *et al.*, 1978; Wege *et al.*, 1978; Lai and Stohman, 1978; Macnaughton *et al.*, 1978). These viruses cause a variety of respiratory, enteric, or neurological diseases in animals and man (Andrewes *et al.*, 1978). The virions of the A59 strain of mouse hepatitis virus contain three structural polypeptides: a phosphorylated nucleocapsid protein N, and two glycoproteins E1 and E2 which have several interesting properties (Sturman, 1977; Sturman and Holmes, 1977; Sturman *et al.*, 1980; Sturman, 1981). The glycoprotein E2 forms the large petal-shaped peplomers characteristic of the coronavirus envelope. E2 is a 180K-dalton glycoprotein which can be cleaved by trypsin to yield two 90K components. The glycoprotein E1 appears to be a transmembrane molecule with three domains: A glycosylated domain projects from the envelope, a second domain lies within the membrane, and a third domain appears to interact with the nucleocapsid inside the viral envelope. Unlike most proteins, when E1 is boiled in the presence of SDS and mercaptoethanol it aggregates into dimers, trimers, and tetramers. The intracellular distribution of E1 is also unusual. Labeling with monospecific fluorescent antibody against isolated E1 or E2 (Sturman *et al.*, 1980) showed that E1 remains restricted to the perinuclear area of the cell while E2,

like most other viral glycoproteins, migrates rapidly via intracellular membranes to the plasma membrane (Doller and Holmes, 1980).

In the present study we have used the antibiotic tunicamycin to study the synthesis and glycosylation of the coronavirus MHV. Tunicamycin, an analog of UDP-N-acetylglucosamine, interferes with the formation of dolichol pyrophosphate-N-acetylglucosamine which acts as a carrier for N-glycosidic linkage of core oligosaccharides to asparagine residues on glycoproteins. Tunicamycin interferes with the cotranslational glycosylation of glycoproteins (Takatsuki *et al.*, 1971; Lehle and Tanner, 1976; Schwarz *et al.*, 1979). We have demonstrated that tunicamycin inhibits formation of the E2 glycoprotein of MHV, but does not prevent synthesis or glycosylation of the transmembrane glycoprotein E1, formation of virions, or release of virions from cells. Our evidence indicates that the unique E1 glycoprotein of the coronavirus may be an O-linked glycoprotein, and thus could be a particularly useful model for studying the synthesis, glycosylation, and intracellular transport of O-linked glycoproteins in mammalian cells.

MATERIALS AND METHODS

Virus propagation and purification. The A59 strain of mouse hepatitis virus (MHV) was grown in the spontaneously transformed 17 Cl-1 line of BALB/c 3T3 mouse fibroblasts as previously described (Sturman and Takemoto, 1972; Sturman, 1977) and assayed by plaque titration in 17 Cl 1 cells. To prepare radiolabeled virus, cells in 150-mm² plastic flasks were inoculated with 1.0 ml of MHV at a multiplicity of 0.3 to 3 PFU/cell in Dulbecco's modified Eagles' minimal essential medium, high glucose (DMEM; Gibco, Grand Island, N. Y.), and incubated for 1 hr at 37°. The inoculum was removed and cells were refed with 30 ml EMEM + 10% dialyzed fetal bovine serum (dFBS) containing 20 μ Ci/ml of L-[³H]amino acid mixture (New England Nuclear) and incubated for 24 hr. Virus released into the supernatant medium was harvested and purified by a

modification of the method described previously (Sturman *et al.*, 1980), using discontinuous and continuous sucrose density gradients in TMS buffer (containing 0.05 *M* Tris-maleate and 0.1 *M* NaCl, pH 6.0) and omitting the polyethylene glycol precipitation step.

Labeling and electrophoresis of intracellular viral polypeptides. To study synthesis, processing, and release of viral specific polypeptides, pulse-chase experiments were performed. Confluent monolayers of 17 Cl 1 cells in 60-mm petri dishes (Falcon, Inc.) were preincubated for 18 hr at 37° in L-leucine deficient EMEM (leu-def MEM) with 10% dFBS and either mock infected with 0.5 ml/plate of leu-def MEM with 10% dFBS or inoculated with 3 to 5 PFU of MHV/cell in 0.5 ml/plate of leu-def MEM with 10% dFBS. After incubation for 1 hr at 37°, the inocula were removed, the cells were refed with leu-def MEM with 10% dFBS, with or without 0.5 µg/ml of tunicamycin (Eli Lilly, Indianapolis, Ind.), and the cultures were held at 37°. Four hours prior to pulse labeling, 5 µg/ml of actinomycin D was added to the medium. At 8 and 10 hr after virus inoculation infected and control cells were pulse labeled for 15 min with 20 or 40 µCi/ml of [³H]L-3, 4, 5-leucine (New England Nuclear, Inc.). The labeled medium was removed, cells were washed and refed with DMEM containing a 10-fold excess of unlabeled L-leucine, 10% FBS, 0.5 µg/ml tunicamycin. At intervals after the pulse, labeled intracellular polypeptides were extracted. Cells were washed twice in PBS and solubilized with 1.0 ml/plate of 1% Nonidet P40 (NP40, Accurate Chemical Corp.) in PBS. Nuclei and debris were removed by centrifugation at 1800 *g* for 10 min at 4°. Radiolabeled polypeptides were analyzed directly by polyacrylamide gel electrophoresis (PAGE) or were immunoprecipitated with rabbit antiserum against purified, detergent-disrupted virions in the presence of staphylococcal protein A prior to analysis by PAGE (Sturman *et al.*, 1980).

Samples for SDS-PAGE slab gels were heated at 37° for 30 min with an equal volume of sample treatment mixture composed of 6 *M* urea, 4% SDS, and 0.05%

bromphenol blue in 0.0625 *M* Tris-chloride, pH 6.7. SDS-PAGE in cylindrical gels was performed using a high pH discontinuous buffer system and fractionation of gels with a Gilson automatic linear gel fractionator as previously described (Sturman and Holmes, 1977). Five to twenty percent polyacrylamide gradient slab gels were prepared and fluorographed as previously described (Sturman *et al.* 1980).

Analysis of glycopeptides. The glycoproteins E1 and E2 were isolated from SDS-PAGE of gradient-purified MHV which had been grown for 24 hr in medium containing 3 µCi/ml [³H]glucosamine (New England Nuclear, Inc.). Isolated E1 and E2 were eluted from the gels, digested with 15 µg of self-digested Pronase (Sigma) per milliliter for 30 hr at 60°, concentrated by lyophilization, and analyzed as borate esters by PAGE in Tris-borate buffer at pH 8.3 according to the method of Weitzman *et al.* (1979).

Electron microscopy. Concentrated, gradient-purified virions were prepared for electron microscopy using 2% phosphotungstic acid (PTA) at pH 7.2 on carbon-coated, Formvar-covered, 400-mesh copper grids. Electron microscopy of cells was done by fixation with 1% glutaraldehyde followed by postfixation with 1% osmium tetroxide, dehydration in a graded series of ethyl alcohol solutions and propylene oxide, and embedding in Epon 812 resin as previously described (Compans *et al.*, 1966). Sections were stained with lead citrate and uranyl acetate and examined in a JEOL 100CX electron microscope.

RESULTS

Effects of Tunicamycin on the Synthesis of Coronavirus Structural Polypeptides

The synthesis and processing of coronavirus-specific polypeptides were analyzed by pulse-labeling techniques in cells treated for 4 hr with actinomycin D to reduce cellular protein synthesis. Without tunicamycin, a 15-min pulse label with [³H]leucine 8 hr after virus inoculation showed synthesis of the three structural

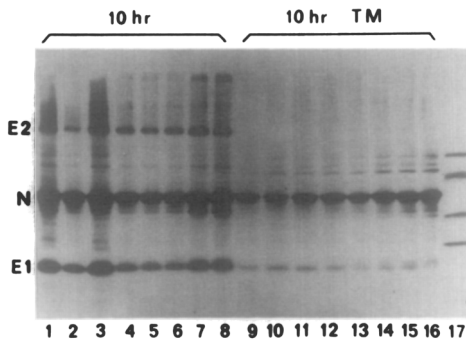


FIG. 1. Effects of tunicamycin on the synthesis and glycosylation of MHV polypeptides. 17 Cl 1 cells were infected with A59 strain of MHV in the absence or presence of tunicamycin (0.5 $\mu\text{g}/\text{ml}$). At 10 hr post-infection, the cells were pulse labeled with 40 $\mu\text{Ci}/\text{ml}$ of [^3H]leucine for 15 min. The label was removed and the cells were washed and refed with medium containing $10\times$ leucine. At intervals after the pulse labeling, cell extracts were prepared with 1% NP40. Samples were analyzed by fluorography after PAGE in 5 to 20% gradient slab gels. Channels 1-8 show extracts of MHV-infected cells without tunicamycin at 0 (Ch. 1 and 2, samples from duplicate plates), 15 (Ch. 3), 30 (Ch. 4), 45 (Ch. 5), 60 (Ch. 6), 75 (Ch. 7), and 90 min (Ch. 8) after the end of the labeling period. Channels 9-16 show extracts of MHV-infected cells treated with tunicamycin at 0 (Ch. 9 and 10, samples from duplicate plates), 15 (Ch. 11), 30 (Ch. 12), 45 (Ch. 13), 60 (Ch. 14), 75 (Ch. 15), and 90 min (Ch. 16) after the end of the labeling period. ^{14}C -labeled molecular weight standards (30K, 46K, 69K, and 92K) are shown in channel 17.

polypeptides E1, N, and E2 (Fig. 1, channels 1-8). No high-molecular-weight polypeptides were detected. Thus each structural polypeptide appears to be translated independently, as also shown by *in vitro* translation studies with isolated MHV mRNAs (Siddell *et al.*, 1980; Rottier *et al.*, 1981). During successive chase periods, no shift in the molecular weight of the nucleocapsid protein N or the peplomeric glycoprotein E2 was observed. Since the E2 glycoprotein is known to be extensively glycosylated (Sturman, 1977; Sturman and Holmes, 1977), this suggests either that glycosylation of E2 is a cotranslational event and/or that the glycosylated and nonglycosylated E2 are not resolved in this region of the gradient slab gel. Pulse-chase experiments with labeling at 6, 8, or 10 hr after infection show that E1 is synthesized as a 20K species and then chased

up to a broad band of up to 23K (not apparent in Fig. 1, channels 7 and 8 due to overexposure). Double labeling studies of intracellular viral polypeptides demonstrated that the 20K form of E1 is not glycosylated whereas the 23K species can be labeled with [^3H]glucosamine (data not shown). Antibody against E1 purified from NP40-disrupted virions by sucrose density gradient sedimentation immunoprecipitates the broad band of E1 from 20K to 23K (Sturman *et al.*, 1980).

Similar pulse-chase radiolabeling studies were performed on cells infected with MHV, treated with 0.5 $\mu\text{g}/\text{ml}$ of tunicamycin, and labeled with [^3H]leucine (Fig. 1, channels 9-16). Synthesis of the nonglycosylated nucleocapsid protein N was not affected by tunicamycin. Synthesis of the 180K E2 glycoprotein was not detectable in the presence of tunicamycin. Thus, tunicamycin either inhibits synthesis of E2 or facilitates rapid degradation of newly synthesized E2. Tunicamycin has been shown to interfere with synthesis or detection of several other glycoproteins which are cotranslationally glycosylated via N-linked glycosidic bonds (Lehle and Tanner, 1976; Leavitt *et al.* 1977; Olden *et al.* 1978; Gibson *et al.*, 1979; Stallcup and Fields, 1981). The three polypeptides of molecular weight 70 to 90K seen in channels 9-16 are cellular polypeptides since they also appear with equal intensity and kinetics in uninfected cells treated with tunicamycin.

In the presence of tunicamycin the rate of synthesis of the membrane glycoprotein E1 is reduced (Fig. 1, channels 9 and 10) in comparison to the control (Fig. 1, channels 1 and 2). However, it is clear that E1 is synthesized as a 20K polypeptide (Fig. 1, channels 9 and 10) which is chased into the glycosylated 23K form (Fig. 1, channels 13-16). Thus glycosylation of E1, unlike other viral structural polypeptides, appears to be resistant to inhibition by tunicamycin.

Effects of Tunicamycin on Virus Maturation

The maturation of coronaviruses in the absence of tunicamycin is shown in Fig.

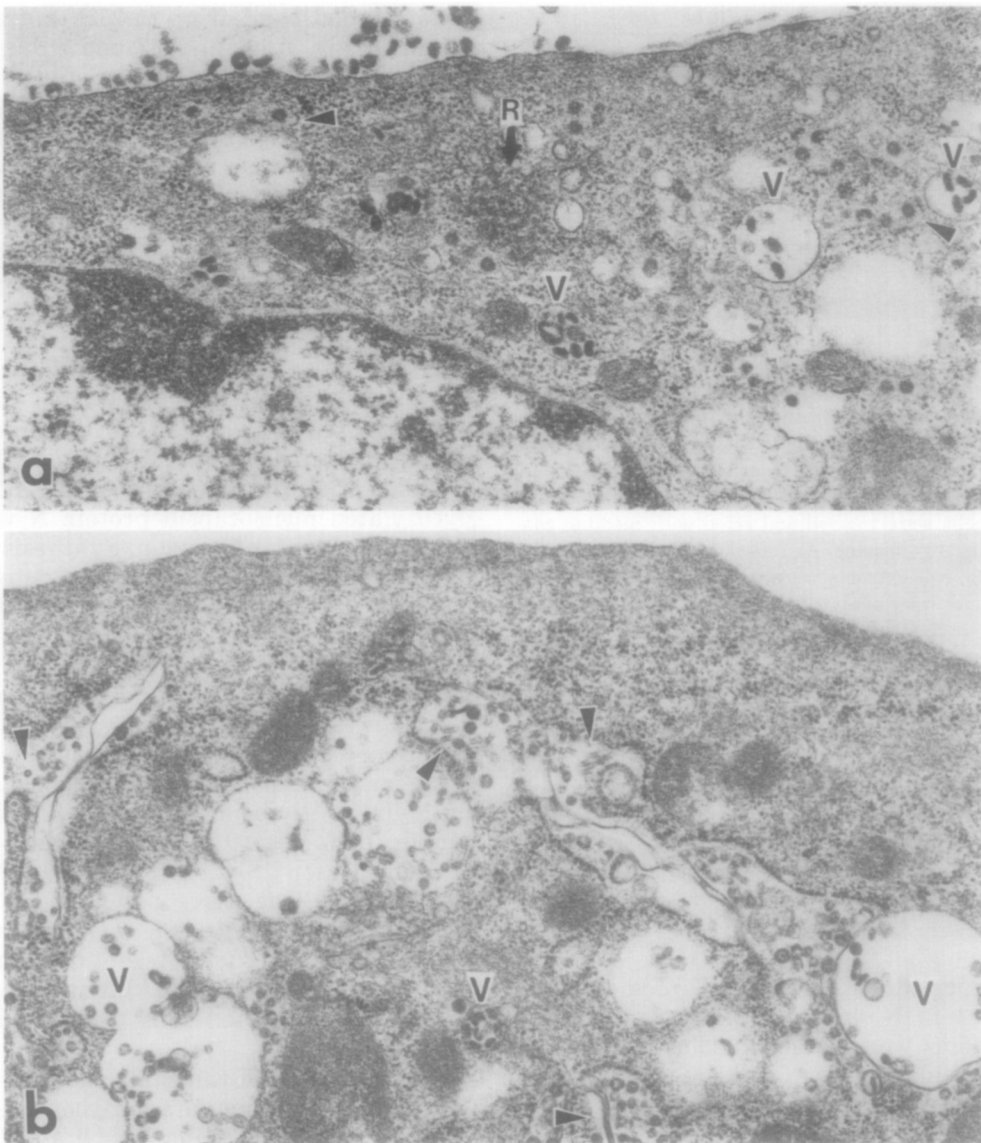


FIG. 2. Effects of tunicamycin on coronavirus maturation. (a) 17 Clone 1 cells infected with MHV A59 for 24 hr showed virions in the rough endoplasmic reticulum, in smooth-walled vesicles (v) and adsorbed to the plasma membrane. (b) Infected 17 Clone 1 cells treated with tunicamycin from the end of the adsorption period until harvesting at 24 hr showed virions in the RER and in smooth-walled vesicles. No virions adsorbed to the plasma membrane even though released virions were present in the medium. Arrows indicate tubules within membranes. R indicates a reticular inclusion. Magnification: 23,000 \times

2a. Coronaviruses mature by budding from intracellular membranes of the rough endoplasmic reticulum (RER) or Golgi apparatus. Virions migrate through the Golgi apparatus into large, smooth-walled ves-

icles. When these vesicles fuse with the plasma membrane, virions are released from intact cells. Although large numbers of virions are commonly seen adsorbed to the plasma membrane of infected cells,

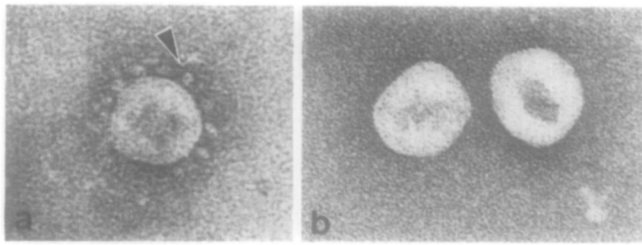


FIG. 3. Effect of tunicamycin on coronavirus structure. Released virions purified from the medium over 17 Cl 1 cells infected with MHV were covered with peplomers (arrow) which are composed of the glycoprotein E2 (a). Released virions from tunicamycin-treated infected cells had no peplomers projecting from the surface of the virions (b). Magnification: 110,000 \times .

virions have not been observed budding from the plasma membrane. Formation of virions was not inhibited by 0.5 $\mu\text{g}/\text{ml}$ of tunicamycin (Fig. 2b). Numerous virions were observed in dilated cisternae of the RER and in smooth-walled vesicles of tunicamycin-treated cells. Although no virions were adsorbed to the plasma membrane (Fig. 2b), virions were released from tunicamycin-treated cells. Although 0.5 $\mu\text{g}/\text{ml}$ of tunicamycin reduced the 24-hr yield of infectious virus 1000-fold, large quantities of virions could be purified from the medium by sucrose density gradient ultracentrifugation. The number of virions released from tunicamycin-treated cultures was only about 5-fold less than from untreated cultures, as estimated by electron microscopy. The virions from tunicamycin-treated cells lacked the characteristic large peplomers of coronaviruses (Fig. 3). The absence of peplomers correlated with the inability of the virions to attach to receptors on the cell surface (Fig. 2b) or to initiate infection.

The structural proteins of virions purified from tunicamycin-treated or control cultures were compared (Fig. 4). The normal virus contained E1, N, and three forms of the E2 glycoprotein: the native 180K form, the 90K cleavage products, and a high-molecular-weight aggregated form (Fig. 4A). In contrast, virus grown with tunicamycin contained E1 and N in normal amounts but completely lacked the peplomeric glycoprotein E2 (Fig. 4B). These biochemical data thus confirm the ultrastructural observations on the absence of

peplomers on virions from tunicamycin-treated cells.

The E1 membrane glycoprotein in the virus grown with tunicamycin appeared to be glycosylated normally, as shown by the ratio of glucosamine to methionine labels in the E1 peaks in Fig. 4A and B. This confirms the observation made in the pulse-chase experiments (Fig. 1) that glycosylation of E1 is resistant to inhibition by tunicamycin. In Fig. 4 it is also apparent that the ratio of glucosamine to methionine label was not constant across the broad peak of E1. More extensive glycosylation corresponded with decreased electrophoretic mobility.

The Glycopeptides of E1 and E2

The oligosaccharide chains of N- and O-linked glycoproteins differ markedly in size, diversity, and carbohydrate composition (Sharon and Lis, 1981). Useful information about the oligosaccharides can be obtained by analyzing the glycopeptides isolated from glycoproteins by PAGE in Tris-borate buffer (Weitzman *et al.*, 1979). At alkaline pH, neutral sugars form negatively charged complexes with borate. The number of borate ions which react with glycopeptides is a function of the composition, sequence, and linkages of the carbohydrates. Glycopeptides of dissimilar carbohydrate composition and length exhibit different electrophoretic mobilities. Although this separation of glycopeptides is not based on their molecular weights, in general, larger oligosaccha-

rides bind more borate than smaller ones, and therefore migrate faster in the gel. Differences in the peptide components appear to have little, if any, effect on the electrophoretic mobilities of the glycopeptides. Thus glycopeptides from two different N-linked glycoproteins would be expected to migrate rather similarly whereas glycopeptides from N- and O-linked glycoproteins would differ markedly in electrophoretic mobility and distribution.

We have used this technique to compare the glycopeptides derived by Pronase digestion of isolated E1 and E2. Figure 5 shows that the borate complexes of E1 and E2 glycopeptides exhibited markedly different electrophoretic patterns. The glycopeptides of E1 exhibited significantly greater mobility than those of E2. Therefore, the borate-glycopeptide complexes of

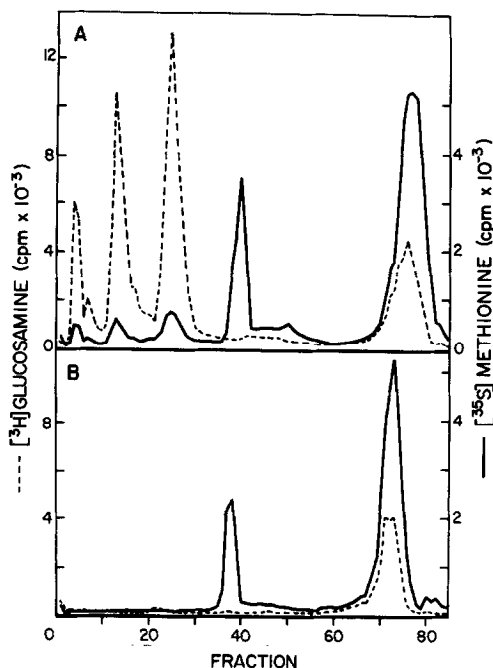


FIG. 4. Effects of tunicamycin on the polypeptide composition of virions. Cells infected with MHV were radiolabeled with [^3H]glucosamine and [^{35}S]methionine in the presence or absence of tunicamycin. Released virions were harvested at 24 hr, gradient purified, pelleted, and analyzed by SDS-PAGE. Profiles of radiolabeled polypeptides from virions produced in untreated cultures (A), and from tunicamycin ($1\text{ }\mu\text{g/ml}$)-treated cultures (B) are shown.

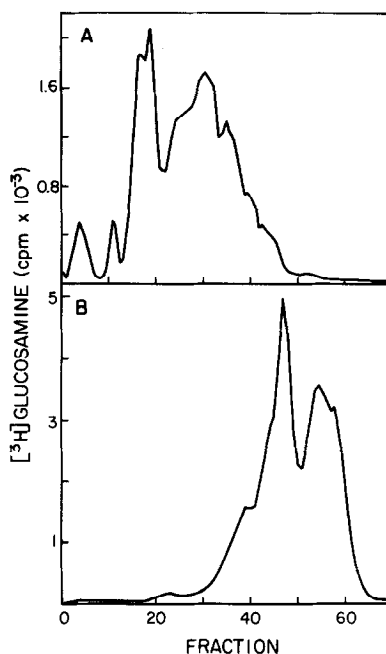


FIG. 5. Comparison of polyacrylamide gel electrophoretic profiles of E1 and E2 glycopeptides. [^3H]glucosamine-labeled glycoproteins E1 and E2 of MHV were separated by SDS-PAGE, eluted from the gels, and digested with Pronase for 30 hr at 60° . The glycopeptides were lyophilized and analyzed by electrophoresis on 10% polyacrylamide gels in Tris-borate buffer, pH 8.3. Profiles of [^3H]glucosamine-labeled glycopeptides from E2 (A) and E1 (B) are shown. The anode is to the right.

E1 were significantly more negatively charged than those of E2. Figure 5 also shows that fewer glycopeptide components were resolved from E1 than from E2. This suggests that E1 may have less diversity of oligosaccharide side chains than E2. These data suggest that the oligosaccharides of E1 differ markedly from those of E2, and support the hypothesis that the oligosaccharides of E1 and E2 may be derived by different mechanisms of glycosylation.

DISCUSSION

Tunicamycin inhibits glycosylation of the structural glycoproteins of alphaviruses (Leavitt *et al.*, 1977), bunyaviruses (Cash *et al.*, 1980), herpes viruses (Pizer *et al.*, 1980), myxoviruses (Nakamura and Compans, 1978a; Klenk and Rott, 1980),

paramyxoviruses (Stallcup and Fields, 1981), rhabdoviruses (Morrison *et al.*, 1978; Gibson *et al.*, 1979; Klenk and Rott, 1980), and retroviruses (Schwarz *et al.*, 1976; Diggelman, 1979), suggesting that all of these viruses contain N-linked glycoproteins which are glycosylated by the transfer of core oligosaccharides from a dolichol pyrophosphate carrier to asparagine residues on the polypeptide. Use of tunicamycin has often permitted the identification of the nonglycosylated protein moiety of a viral glycoprotein (Morrison *et al.*, 1978; Gibson *et al.*, 1979; Nakamura and Compans, 1978a; Diggelman, 1979). In some virus strains, however, synthesis of the nonglycosylated polypeptide in the presence of tunicamycin is difficult to detect because complete translation of the glycoprotein mRNA may be dependent on cotranslational addition of N-linked oligosaccharide chains, or because the nonglycosylated polypeptide may be highly susceptible to degradation by host cell proteases, or because the nonglycosylated polypeptide may be insoluble (Schwarz *et al.*, 1976; Leavitt *et al.*, 1977; Gibson *et al.*, 1979; Diggelman, 1979; Pizer *et al.*, 1980; Stallcup and Fields, 1981).

The coronavirus MHV contains two structural glycoproteins which have been isolated and partially characterized (Sturman *et al.*, 1980). In 17 Cl 1 cells infected with the A59 strain of MHV, tunicamycin specifically interfered with the synthesis of the peplomeric glycoprotein E2 (Fig. 1). Neither glycosylated nor nonglycosylated forms of E2 were detected either directly or by immunoprecipitation. This suggests that E2, like other viral structural glycoproteins, may be an N-linked glycoprotein.

The transmembrane glycoprotein E1 or MHV is so far unique among viral structural glycoproteins in that it is glycosylated in the presence of tunicamycin. Glycosylation of E1 appears to be a post-translational event (Fig. 1) and the shift from the nonglycosylated 20K form to the glycosylated 23K form is not inhibited by tunicamycin. This provides indirect evidence that E1 is not an N-linked glycoprotein but may be an O-linked glycoprotein. This hypothesis is also supported by

direct evidence concerning the carbohydrate composition and the oligosaccharide side chains of E1. Early studies on the incorporation of radiolabeled sugars into E1 and E2 showed that both E1 and E2 were labeled with [3 H]glucosamine, but only E2 was labeled with [3 H]fucose (Sturman and Holmes, 1977). Recent studies by Niemann and Klenk (1981) have identified additional differences between the carbohydrate composition of E1 and E2. E1, like cellular O-linked glycoproteins (Thomas and Winzler, 1969; Spiro and Bhoyroo, 1974; Slomiany and Slomiany, 1978; Sharon and Lis, 1981), contains little mannose, no fucose, and possesses a high proportion of *N*-acetyl galactosamine; whereas E2, like many other N-linked glycoproteins (Nakamura and Compans, 1978b; Prehm *et al.*, 1979; Weitzman *et al.*, 1978; Burke and Keegstra, 1979), contains both mannose and fucose but no *N*-acetyl galactosamine. Oligosaccharides of E1 but not E2 are removed by β -elimination (H. Niemann and H.-D. Klenk, personal communication). Although the size, linkages, and sequences of sugars of the individual oligosaccharide chains of the two MHV glycoproteins have not yet been determined, analysis of the oligopeptides of E1 and E2 by Tris-borate PAGE has shown that the two glycoproteins have distinct oligosaccharide side chains (Fig. 5). This technique affords resolution comparable to or better than Bio-Gel P6 gel filtration columns. The electrophoretic mobilities of the glycopeptides of E2 were similar to those of glycopeptides from N-linked glycoproteins of Sindbis virus or ovalbumin which have been previously described (Weitzman *et al.*, 1979). In contrast, the glycopeptides of E1 were more negatively charged than those of E2. Thus, we have shown that E1 and E2 differ in carbohydrate composition, electrophoretic patterns of glycopeptides, and response to tunicamycin. These data suggest that E2 is an N-linked glycoprotein and E1 may be an O-linked glycoprotein.

The amino acid sequence and the locations and number of oligosaccharide side chains on the MHV glycoproteins are not yet known. However, all of the oligosaccharide moieties of E1 appear to be located

near one end of the polypeptide chain. Treatment of MHV virions with bromelain or Pronase removed E2 and detached a 5K glycosylated polypeptide from E1, leaving an 18K nonglycosylated polypeptide embedded within the viral envelope (Sturman and Holmes, 1977; Sturman, 1981). It is not yet known whether the glycosylated portion of E1 which extends outside the viral membrane contains the amino terminal or carboxy terminal region of E1. However, this region appears to contain multiple oligosaccharide side chains, since on PAGE E1 migrates either as a broad, diffuse band of 20K to 23K or, under other conditions, as three or more discrete bands.

The role of glycosylation in the functions of E1 is not yet known. Glycosylation may affect the conformation of E1 or its orientation in the viral envelope. However, glycosylation of E1 may not be essential for virion formation since both nonglycosylated and glycosylated E1 are incorporated into A59 virions (data not shown) and the E1-like membrane proteins found in some other coronaviruses are apparently not glycosylated (Garwes, 1979). The novel O-linked post-translational glycosylation of E1 may be associated with its restricted intracellular migration. E1 is restricted to the perinuclear area of infected cells, in contrast to E2 which migrates rapidly to the plasma membrane (Doller and Holmes, 1980). Possibly the oligosaccharides of E1 act as signals for movement from the RER to other membranes. Oligosaccharides are important recognition signals for enzymes destined for lysosomes (Hasilik, 1980) and glycoproteins destined for endocytosis (Baenziger and Fiete, 1980; Stahl and Schlesinger, 1980).

The differential effects of tunicamycin on the synthesis and glycosylation of the two coronavirus envelope glycoproteins has permitted tentative assignment of functions to these glycoproteins. In MHV-infected cells treated with tunicamycin, virions which lack the E2 glycoprotein, and hence the peplomers, bud from intracytoplasmic membranes and are released from the cell (Figs. 2, 3, and 4). Thus the transmembrane glycoprotein E1 appears

to be the only envelope glycoprotein required for virus budding. Indeed, the location of E1 on intracytoplasmic membranes may determine the budding site of the coronavirus. Budding may occur where the viral nucleocapsid recognizes intracellular membranes altered by the addition of E1. The E2 glycoprotein appears to be essential for virus attachment to receptors on the plasma membrane. Virus lacking peplomers showed a markedly decreased infectivity. No virions were observed adsorbed to the plasma membrane of tunicamycin-treated cells although numerous virions lacking E2 were present in the medium (Figs. 2 and 3).

Many cellular glycoproteins such as fetuin, corneal proteoglycan, glycophorin, thyroglobulin, and immunoglobulins contain both asparagine-linked and serine- or threonine-linked oligosaccharides (Sharon and Lis, 1981). Further detailed study may reveal some O-linked oligosaccharides on viral glycoproteins now believed to contain only N-linked oligosaccharides. Indeed, the vaccinia virus hemagglutinin, a nonstructural glycoprotein, has both O- and N-linked oligosaccharide chains (Shida and Dales, 1981). To date, however, no structural viral glycoprotein except the E1 glycoprotein of MHV has been identified which might contain only O-linked carbohydrates.

It is unlikely that glycosylation of E1 is a virus-encoded process. Since MHV has a limited amount of genetic material and codes for only six species of mRNA (Siddell *et al.*, 1980; Rottier *et al.*, 1981), it is far more likely that glycosylation of E1 occurs via cellular processes. Production of the E1 glycoprotein may be a good model system for the study of tunicamycin-resistant glycosylation. In MHV-infected cells treated with actinomycin D to inhibit synthesis of cellular glycoproteins and with tunicamycin to inhibit synthesis of the N-linked viral glycoprotein E2, E1 is the major glycoprotein synthesized. This glycoprotein can be purified in large amounts from virions released from tunicamycin-treated cells. Thus, the coronavirus glycoprotein E1 may be a useful model for analysis of the synthesis, gly-

cosylation, and intracellular transport of O-linked glycoproteins.

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REFERENCES

- ANDREWES, C., PEREIRA, H. G., and WILDY, P. (1978). In "Viruses of Vertebrates," pp. 174-186 Bailliere Tindall, London.
- BAENZIGER, J. U., and FIETE, D. (1980). Galactose and N-acetylgalactosamine specific endocytosis of glycopeptides by isolated rat hepatocytes. *Cell* **22**, 611-620.
- BURKE, D., and KEEGSTRA, K. (1979). Carbohydrate structure of Sindbis virus glycoprotein E2 from virus grown in hamster and chicken cells. *J. Virol.* **29**, 546-554.
- CASH, P., HENDERSHOT, L., and BISHOP, D. H. L. (1980). The effect of glycosylation inhibitors on the maturation and intracellular polypeptide synthesis induced by snowshoe hare Bunyavirus. *Virology* **103**, 235-240.
- CHOPPIN, P. W., and SCHEID, A. (1980). The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. *Rev. Infect. Dis.* **2**, 40-61.
- COMPANS, R. W., HOLMES, K. V., DALES, S., and CHOPPIN, P. W. (1966). An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. *Virology* **30**, 411-422.
- DIGGELMAN, H. (1979). Biosynthesis of an unglycosylated envelope glycoprotein of Rous sarcoma virus in the presence of tunicamycin. *J. Virol.* **30**, 799-804.
- DOLLER, E. W., and HOLMES, K. V. (1980). "Different Intracellular Transportation of the Envelope Glycoproteins E1 and E2 of the Coronavirus MHV," p. 267. Amer. Soc. Microbiol., Washington, D. C. (Abstract).
- ELBEIN, A. D. (1979). The role of lipid-linked saccharides in the biosynthesis of complex carbohydrates. *Annu. Rev. Plant Physiol.* **30**, 239-272.
- GARWES, D. J. (1979). In "Viral Enteritis in Humans and Animals" (F. Bricout and R. Scherer, eds.). Inserm Colloque 90.
- GHOSH, H. P. (1980). Synthesis and maturation of glycoproteins of enveloped animal viruses. *Rev. Infect. Dis.* **2**, 26-39.
- GIBSON, R., SCHLESINGER, S., and KORNFELD, S. (1979). The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* **254**, 3600-3607.
- HASILIK, A. (1980). Biosynthesis of lysosomal enzymes. *Trends Biochem. Sci.* **5**(9), 237-240.
- KLENK, H.-D., and ROTT, R. (1980). Cotranslational and posttranslational processing of viral glycoproteins. *Curr. Top. Microbiol. Immunol.* **90**, 19.
- LAI, M. M. C., and STOHLMAN, S. A. (1978). RNA of mouse hepatitis virus. *J. Virol.* **26**, 236-248.
- LEAVITT, R., SCHLESINGER, S., and KORNFELD, S. (1977). Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. *J. Biol. Chem.* **252**, 9018-9023.
- LEHLE, L., and TANNER, W. (1976). The specific site of tunicamycin inhibition in the formation of dolichol-bound N-acetylglucosamine derivatives. *FEBS Lett.* **71**, 167-170.
- MACNAUGHTON, M. R. (1978). The genomes of three coronaviruses. *FEBS Lett.* **94**, 191-194.
- MORRISON, T. G., MCQUAIN, C. O., and SIMPSON, D. (1978). Assembly of viral membranes: Maturation of the vesicular stomatitis glycoprotein in the presence of tunicamycin. *J. Virol.* **24**, 368-374.
- NAKAMURA, K., and COMPANS, R. W. (1978a). Effects of glucosamine, 2-deoxy-D-glucose and tunicamycin on glycosylation, sulfation and assembly of influenza viral proteins. *Virology* **84**, 303-319.
- NAKAMURA, K., and COMPANS, R. W. (1978b). Glycopeptide components of influenza viral glycopeptides. *Virology* **86**, 432-442.
- NIEMANN, H., and KLENK, H.-D. (1981). In "The Biochemistry and Biology of Coronaviruses" (V. ter Meulen, S. Siddell, and H. Wege, eds.). Plenum, London.
- OLDEN, K., PRATT, R. M., and YAMADA, K. M. (1978). Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell* **13**, 461-473.
- PIZER, L. I., COHEN, G. H., and EISENBERG, R. J. (1980). Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J. Virol.* **34**, 142-153.
- PREHM, P., SCHEID, A., and CHOPPIN, P. W. (1979). The carbohydrate structure of the glycoproteins of the paramyxovirus SV5 grown in bovine kidney cells. *J. Biol. Chem.* **254**, 9669-9676.
- ROTHMAN, J. E., KATZ, F. N., and LODISH, H. F. (1978).

- Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. *Cell* 15, 1447-1454.
- ROTHMAN, J. E., and LODISH, H. F. (1977). Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (London)* 269, 775-780.
- ROTTIER, P. J. M., SPANN, W. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1981). Translation of three mouse hepatitis virus strain A59 subgenomic RNAs in *Xenopus laevis* oocytes. *J. Virol.* 38, 20-26.
- SCHWARZ, R. T., and DATEMA, R. (1980). Inhibitors of protein glycosylation. *Trends Biochem. Sci.* 5(3) 65-67.
- SCHWARZ, R. T., ROHRSCHEIDER, J. M., and SCHMIDT, M. F. G. (1976). Suppression of glycoprotein formation of Semliki Forest, influenza and avian sarcoma virus by tunicamycin. *J. Virol.* 12, 782-791.
- SCHWARZ, R. T., SCHMIDT, M. F. G., and DATEMA, R. (1979). Inhibition of glycosylation of viral glycoproteins. *Biochem. Soc. Trans.* 7, 322-326.
- SHARON, N., and LIS, H. (1981). Glycoproteins; research booming on long-ignored, ubiquitous compounds. *Chem. Eng. News* 59, 21-44.
- SHIDA, H., and DALES, S. (1981). Biogenesis of vaccinia: Carbohydrate of the hemagglutinin molecule. *Virology* 111, 56-72.
- SIDDELL, S. G., WEGE, H., BARTHEL, A., and TER MEULEN, V. (1980). Coronavirus JHM: Cell free synthesis of structural protein p60. *J. Virol.* 33, 10-17.
- SLOMIANY, A., and SLOMIANY, B. L. (1978). Structures of the acidic oligosaccharides isolated from rat sublingual glycoprotein. *J. Biol. Chem.* 253, 7301-7306.
- SPRIO, R. G., and BHOYROO, (1974). Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* 249, 5704-5717.
- STAHL, P. D., and SCHLESINGER, P. H. (1980). Receptor-mediated pinocytosis of mannose/N-acetylglucosamine-terminated glycoproteins and lysosomal enzymes by macrophages. *Trends Biochem. Sci.* 5(7), 194-196.
- STALLCUP, K. C., and FIELDS, B. N. (1981). The replication of measles virus in the presence of tunicamycin. *Virology* 108, 391-404.
- STURMAN, L. S. (1977). Characterization of a coronavirus. 1. Structural proteins: Effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* 77, 637-649.
- STURMAN, L. S. (1981). In "The Biochemistry and Biology of Coronaviruses" (V. ter Meulen, S. Siddell, and H. Wege, eds.). Plenum, London.
- STURMAN, L. S., and HOLMES, K. V. (1977). Characterization of a coronavirus. II. Glycoproteins of the viral envelope; tryptic peptide analysis. *Virology* 77, 650-666.
- STURMAN, L. S., HOLMES, K. V., and BEHNKE, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J. Virol.* 33, 449-462.
- STURMAN, L. S., and TAKEMOTO, K. K. (1972). Enhanced growth of a murine coronavirus in transformed mouse cells. *Infect. Immun.* 6, 501-507.
- TAKATSUKI, A., ARIMA, K., and TAMURA, G. (1971). Tunicamycin, a new antibiotic. *J. Antibiot.* 24, 215-223.
- THOMAS, D. B., and WINZLER, R. J. (1969). Structural studies on human erythrocyte glycoproteins. *J. Biol. Chem.* 244, 5943-5946.
- TOMITA, M., and MARCHESI, V. T. (1975). Amino-acid sequence and oligosaccharide attachment sites of human erythrocyte glycophorin. *Proc. Nat. Acad. Sci. USA* 72, 2964-2968.
- TYRRELL, D. A. J., ALEXANDER, D. J., ALMEIDA, J. F., CUNNINGHAM, C. H., EASTERDAY, B. C., GARWES, D. J., HIERHOLZER, J. C., KAPIKIAN, A., MACNAUGHTON, M. R., and MCINTOSH, K. (1978). Coronaviridae: Second Report. *Intervirology* 10, 321-328.
- WEGE, H., MULLER, A., and TER MEULEN, V. (1978). Genomic RNA of the murine coronavirus JHM. *J. Gen. Virol.* 41, 217-227.
- WEITZMAN, S., SCOTT, V., and KEEGSTRA, K. (1979). Analysis of glycopeptides as borate complexes by polyacrylamide gel electrophoresis. *Anal. Biochem.* 97, 438-449.