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Coronavirus Glycoprotein E1, a New Type of Viral Glycoprotein

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The carbohydrate contents of coronavirus glycoproteins E1 and E2 have been analyzed. E2 has complex and mannose-rich-type oligosaccharide side-chains, which are attached by *N*-glycosidic linkages to the polypeptide. Glycosylation of E2 is initiated at the co-translational level, and it is inhibited by tunicamycin, 2-deoxy-glucose, and 2-deoxy-2-fluoro-glucose. Thus, E2 belongs to a glycoprotein type found in many other enveloped viruses. E1, in contrast, represents a different class of glycoprotein. The following observations indicate that its carbohydrate side-chains have *O*-glycosidic linkage. (1) The constituent sugars of E1 are *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, and neuraminic acid; mannose and fucose are absent. (2) The side-chains can be removed by β-elimination. (3) Glycosylation of E1 is not sensitive to the compounds interfering with *N*-glycosylation. E1 is the first viral glycoprotein analyzed that contains only *O*-glycosidic linkages. Coronaviruses are therefore a suitable model system to study biosynthesis and processing of this type of glycoprotein.

1. Introduction

Coronaviruses are a group of positive stranded, RNA-containing viruses, which cause respiratory, gastroenteric and central nervous disorders in humans and animals (McIntosh, 1974; Tyrrell *et al.*, 1978; Robb & Bond, 1979; Nagashima *et al.*, 1978,1979). Gerdes *et al.* (1981) have reported serological cross-reactivity between viruses isolated from multiple sclerosis patients and mouse hepatitis virus A59, a prototype coronavirus that has been characterized in some detail at the molecular level. It contains three structural proteins: a nucleocapsid protein N (M_r 50,000), which is phosphorylated (Stohlman & Lai, 1979), and the two envelope glycoproteins E1 and E2. Glycoprotein E1 is probably a transmembranal matrix protein with the carbohydrate moieties residing on the outside of the virus particle (Sturman, 1977). E2 appears in the mature virion in two forms (M_r 180,000 and M_r 90,000), both of which constitute the club-shaped peplomers that protrude from the viral membrane and surround the particle like a corona. By treatment of purified virus with trypsin *in vitro* the M_r 180,000 species of E2 can be converted into the M_r 90,000 cleavage products (Sturman & Holmes, 1977).

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The glycosylation of viral glycoproteins has been studied in detail for several enveloped viruses, including alpha-, rhabdo- and myxoviruses (for a review, see Klenk & Rott, 1980). All these viruses share a common pathway of glycosylation: in a co-translational event, the nascent polypeptide chain extending into the lumen of the rough endoplasmic reticulum is glycosylated by transfer en bloc of an activated oligosaccharide from a dolichol-linked intermediate Dol-P-P-$(GlcNAc)_2Man_9Glc_3$ (Struck & Lennarz, 1980). This initial step requires a tripeptide sequence (H_2N-Asn-X-Ser(Thr)-COOH) in the peptide chain, and the resulting carbohydrate protein linkage is of the *N*-glycosidic type between *N*-acetylglucosamine and asparagine. On the way to the plasma membrane, the core oligosaccharides are trimmed and new sugars, including galactose, *N*-acetylglucosamine, fucose and neuraminic acid, are added in the Golgi apparatus. From this organelle, viral glycoproteins are transported to the plasma membrane, where they are sequestered into budding virus particles.

We report here that glycoprotein E1 of coronaviruses does not follow this generally accepted glycosylation pathway. We show that glycosylation of E1 is a post-translational event, and cannot be inhibited by tunicamycin and other glycosylation inhibitors that interfere with the formation of *N*-glycosidic linkages. The constituent analysis of the carbohydrate side-chains of E1, as well as their sensitivity to mild alkaline treatment, suggest an *O*-glycosidic type of linkage between oligo-saccharides and polypeptide that has not been described before for viral structural proteins.

2. Materials and Methods

(a) *Viruses and cells*

Mouse hepatitis virus (MHV) A59 and its host cell line, the 17 clone 1 line of spontaneously transformed BalbC3T3 cells, were obtained from Dr L. S. Sturman, Albany, N.Y. The bovine coronavirus L9 was grown in primary bovine fetal thyroid cells as described by Storz *et al.* (1981*a*). Virus was purified according to Sturman *et al.* (1980). Virus titers were determined by plaque assays with 0·8% (w/v) agar in the overlay medium.

(b) *Labeling of viral polypeptides*

To obtain radioactive virus particles, [6-^{3}H]glucosamine (25 Ci/mmol; Amersham-Buchler, Braunschweig, F.R.G.), [1-^{3}H]galactose (40 Ci/mmol), [1-^{3}H]fucose (4·6 Ci/mmol) or [2-^{3}H]mannose (16 Ci/mmol) was added to the growth medium at a final concentration of 2 μCi/ml after the 1 h adsorption period of the virus. ^{14}C-labeled protein hydrolysate (55 mCi/matom) was used at 0·5 μCi/ml. (9, 10(*n*))-[^{3}H]palmitic acid was used at 50 μCi/ml (Schmidt & Schlesinger, 1980).

To analyze intracellular synthesis of viral polypeptides, monolayers of 17Cl1 cells on 5 cm Petri dishes were infected at a multiplicity of infection of 50 plaque-forming units/cell. At 16 h post infection, the medium was replaced by medium without leucine. After 2 h leucine starvation, the cells were pulse-labeled for 15 min by the addition of 300 μCi [^{3}H]leucine/ml, and were either lysed directly for immunoprecipitation or were chased in medium containing unlabeled leucine for the times indicated. In experiments where tunicamycin was used, the antibiotic (1 μg/ml; Eli Lilly, Indianapolis) was added at the beginning of the starvation period and was present throughout the rest of the experiment.

(c) *Preparation of antiserum*

Purified virus (5×10^9 p.f.u.†) emulsified in complete Freund's adjuvant was injected intradermally into a New Zealand white rabbit. Five weeks later, the rabbit was boosted by subcutaneous injection of a virus suspension (10^9 p.f.u.) in saline. One week later, the rabbit was bled and the virus neutralization titer of the serum determined by plaque inhibition tests. The serum titer (50% reduction of plaques) was 1 : 7000. For immunoprecipitation, the serum was purified from host cell-specific antibodies by passage over a Sepharose 4B-affinity column, to which cell extract from non-infected 17CL1 cells was covalently coupled.

(d) *Immunoprecipitation*

Labeled cells were lysed at 0°C in RIPA buffer (50 mM-Tris·HCl (pH 7·2), 10 mM-EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0·1% (w/v) SDS, and 5% (v/v) Trasylol, Bayer AG, Germany) and clarified by centrifugation at 10,000 *g* for 4 min: 100 μl of the supernatant were incubated for 60 min on ice with 10 μl of rabbit antiserum. The immune complexes were adsorbed to a suspension of protein A-bearing *Staphyloccus aureus* (Kessler, 1975) in RIPA buffer containing 10% (v/v) fetal calf serum, and the mixture was washed 4 times with wash buffer (10 mM-sodium phosphate (pH 7·2), 10 mM-EDTA. 1 M-NaCl, 1% (v/v) Triton X-100, 5% (v/v) Trasylol). The final pellet was resuspended in sample buffer and subjected to electrophoresis as described below.

(e) *Polyacrylamide gel electrophoresis and isolation of viral glycopeptides*

Analytical 5% to 15% (w/v) polyacrylamide gradient slab gels containing 0·1% (w/v) SDS and 4 M-urea were used (Laemmli, 1970). Samples were heated to 100°C for 2 min in 80 mM-Tris·HCl (pH 6·8), 0·1% (v/v) glycerol, 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. The resolved radiolabeled protein bands were localized by fluorography (Laskey & Mills, 1975), using Kodak XAR-5 film. For preparative isolation of viral glycoproteins, cylindrical 10% polyacrylamide gels (7 mm × 100 mm) were used. They were sliced and the radioactivity of sister gels was determined by liquid scintillation counting (Schwarz & Klenk, 1974). Viral glycoproteins were recovered by freezing and thawing, elution with 0·1% (w/v) SDS in water, filtration through 0·45 μm Millipore filter units and repeated precipitation in 90% (v/v) aqueous acetone at 0°C. Preparations were then dialyzed against distilled water and lyophilized. Radiolabeled glycopeptides were obtained by digestion with 1% (w/v) Pronase at 50°C in 1 M-Tris·HCl (pH 8·0), 10^{-4} M-$CaCl_2$ for 48 h with a second addition of Pronase after 24 h. Insoluble material was removed by centrifugation and the supernatant was extracted with chloroform/methanol (2 : 1, v/v). The upper phase containing essentially all the radiolabel was lyophilized, and desalted on a Biogel P2 column (1 cm × 35 cm). Further fractionation of E1 glycopeptides was obtained by affinity chromatography on a column (1 cm × 10 cm) of wheat germ agglutinin-Sepharose 4B (Pharmacia). Desalted glycopeptides were applied in 5 mM-sodium acetate buffer (pH 5·2) to the column equilibrated with the same buffer. The column was washed with this buffer, and adsorbed material was eluted with buffer containing 0·1 M-*N*-acetylglucosamine. Both the unbound and the bound fraction were desalted and freed from *N*-acetylglucosamine by chromatography on Biogel P2 as described above, using distilled water as an eluent. Binding studies of E2 glycopeptides to concanavalin A-Sepharose 4B (Pharmacia) were performed as described by Krusius & Finne (1977).

(f) *Digestion with glycosidases*

Glycopeptides were treated with 5 units of neuraminidase from *Vibrio cholerae*

† Abbreviations used: p.f.u., plaque-forming unit; SDS, sodium dodecyl sulfate; MHV, mouse hepatitis virus.

(Behringwerke) in 0·2 ml of 10 mM-Tris-acetate (pH 6·8), 2 mM-$CaCl_2$ at 37°C for 24 h. The incubation mixture was applied to a Biogel P6 column (1 cm × 150 cm) and eluted with 0·1 M-pyridine acetate (pH 5·2). Endo-β-galactosidase from *Escherichia freundii* was a kind gift from Dr M. N. Fukuda (Seattle). E1 glycopeptides were incubated at 37°C with 25 milliunits of enzyme in 0·2 ml of 0·1 M-sodium acetate (pH 5·8) for 18 h. Samples were analyzed on a column of Biogel P4 (minus 400 mesh; 1 cm × 150 cm).

Endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*) was purchased from Seikagaku Kogyo, Japan. E2 glycopeptides were incubated with 25 milliunits of the enzyme in 0·05 ml of 0·05 M-citrate/phosphate buffer (pH 5·5). Incubations were for 18 h at 37°C under a toluene atmosphere. The enzyme-treated glycopeptides were then analyzed on a Biogel P4 (minus 400 mesh) column as described above.

(g) *Alkali borohydride treatment*

For the analysis of amino acids, radiolabeled glycoproteins E1 (M_r 23,000) and E2 (M_r 90,000) were subjected to β-elimination conditions according to Pigman & Downs (1977), using porcine submaxillary gland protein as a control. Amino acid analyses were performed on a Biotronic LC6000 amino acid analyzer as described by Spackman *et al.* (1958). For the preparation of reduced oligosaccharides, the method of Carlson (1974) was applied with the following modifications: samples were kept at 45°C for 10 h in 0·1 M-NaOH, containing 1 M-$NaBH_4$ and 0·1% (w/v) SDS. After cooling to 0°C, the pH of the mixture was adjusted to 5·0 by the addition of 4 M-aqueous acetic acid, and samples were pssed over a Dowex 50WX2 (200 to 400 mesh; H^+ form; SERVA) column (1 cm × 8 cm) and washed with 1% (v/v) acetic acid. The eluted material was lyophilized and boric acid was removed by repeated evaporation with methanol at 30°C.

High-voltage paper electrophoresis of released oligosaccharides was conducted on Whatman no. 3MM paper in pyridine/acetic acid/water (10 : 4 : 86, by vol.), pH 5·3, at 25 V/cm for 6 h. The paper was then cut into 0·5 cm strips and the radioactivity was determined by liquid scintillation counting. Molecular weights of the released oligosaccharides were determined on a calibrated Biogel P4 (minus 400 mesh, batch no. 163034) column (1 cm × 150 cm) in 0·1 M-pyridine acetate (pH 5·3). Fractions of 0·43 ml were taken and assayed for radioactivity by liquid scintillation counting.

(h) *Carbohydrate constituent analysis*

All reagents were of ultrapure grade or freshly distilled. Desalted glycopeptides containing 5 to 10 μg of total sugar were hydrolyzed in 0·25 M-H_2SO_4 in 90% (v/v) aqueous acetic acid at 80°C for 8 h, reduced and peracetylated as described by Stellner & Hakomori (1974). Peracetylated alditol acetates were analyzed on a Finnigan model 4021 combined gas chromatograph/mass spectrometer, using a 25 m fused silica capillary column with Dexsil 410 as the wall coating phase. Sugar ratios were calculated on the basis of the total reconstructed ion chromatograms, applying response factors that were determined with corresponding commercial standards.

Paper chromatography of re-*N*-acetylated hydrolysates was carried out on Whatman 3MM paper sheets in the descending manner using ethyl acetate/pyridine/water (4 : 1 : 1, by vol.) as the solvent.

3. Results

(a) *Analysis of radiolabeled mouse hepatitis virus proteins*

MHV A59 was grown in the presence of ^3H-labeled sugars or ^{14}C-labeled protein hydrolysate, and purified virus preparations were analyzed by SDS/polyacrylamide gel electrophoresis (Fig. 1). ^{14}C-labeled amino acids were mainly incorporated into the nucleocapsid protein N and into the low molecular weight glycoprotein E1

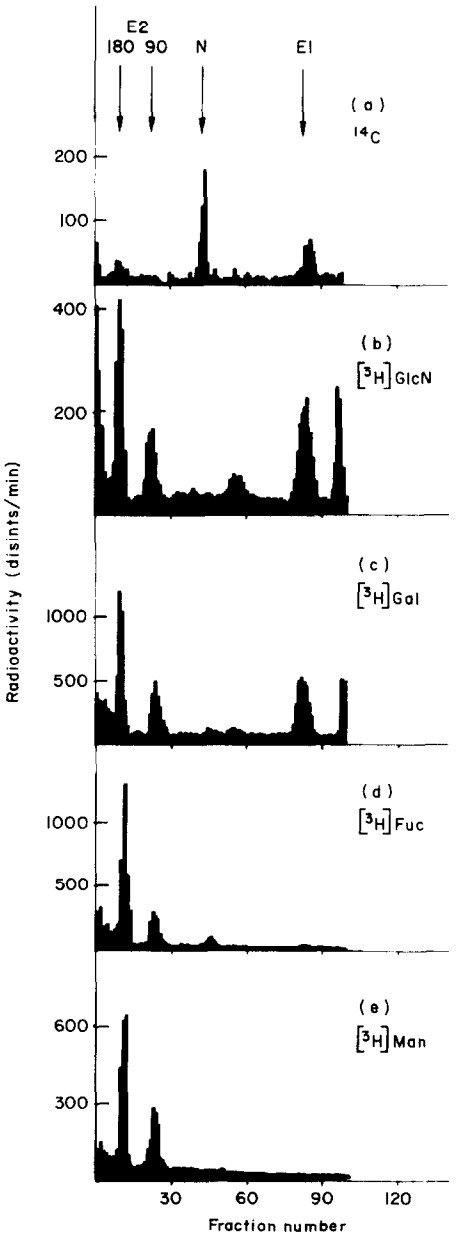

FIG. 1. Incorporation of radioactive amino acids (^{14}C) and sugars (^{3}H) into coronavirus A59 polypeptides. Coronavirus A59 was grown in the 17 clone 1 line of spontaneously transformed BalbC3T3 cells in the presence of radioactive markers as indicated. Virus was harvested 30 h post infection, purified and analyzed on cylindrical SDS-containing 10% (w/v) polyacrylamide gels. Gels were sliced and radioactivity of the resolved polypeptides (indicated in (a)) determined by liquid scintillation counting.

(Fig. 1(a)). The peripheral spike glycoprotein E2 (M_r 180,000) and its proteolytic M_r 90,000 cleavage products were poorly labeled. This is probably due to partial loss of spike peplomers during virus purification, which is a common observation with coronaviruses. The analysis of MHV labeled with the radioactive sugars [6-^{3}H]glucosamine, [1-^{3}H]galactose, [1-^{3}H]fucose and [2-^{3}H]mannose showed that each of these sugars is present in E2. This was not unexpected, since this carbohydrate pattern is found in many other viral glycoproteins. E1, however, was not labeled with either fucose or mannose (Fig. 1(d) and (e)). The absence of mannose is significant, since mannose is an essential constituent of the carbohydrate core structures of *N*-glycosidically linked side-chains. The radioactive band in Figure 1(b) and (c) migrating faster than glycoprotein E1 is probably glycolipid. This is suggested by the observations that this material is not labeled by amino acids, mannose or fucose, and that it is removed from the gel after fixation with organic solvents, such as isobutanol (Fig. 2).

In order to investigate whether the presence of F1 with its abnormal labeling characteristics is unique for the murine coronavirus A59 or a general coronavirus-specific phenomenon, we have also analyzed the glycosylation pattern of the enteropathogenic bovine coronavirus L9 grown in primary bovine fetal thyroid cells (Storz *et al.*, 1981*a*,*b*; Fig. 2). A low molecular weight glycoprotein co-migrating with the murine E1 in SDS/polyacrylamide gel electrophoresis was present in the bovine virus preparations. This polypeptide readily aggregated to material of high molecular weight when boiled in the presence of reducing agents (data not shown), as does glycoprotein E1 of the murine virus (Sturman, 1977). Metabolic labeling indicated that E1 of the bovine virus could again be labeled only with galactose and glucosamine but not with fucose or mannose.

(b) *Labeling with [^{3}H]palmitic acid*

In order to investigate whether glycoprotein E1 purified by preparative polyacrylamide gel electrophoresis was possibly contaminated with tightly bound glycolipids, we analyzed A59 virus grown in the presence of [^{3}H]palmitic acid. Figure 3 shows that E1 was not labeled under these conditions. Thus, glycolipid contamination could be excluded. However, significant amounts of label were incorporated into glycoprotein E2 and its proteolytic cleavage products. Therefore, it appears that E2 contains covalently bound fatty acids.

(c) *Sugar composition*

The different labeling characteristics of A59 glycoproteins E1 and E2 were confirmed by sugar constituent analyses of E1 and E2 glycopeptides, which were obtained from the purified polypeptides by digestion with Pronase and gel filtration on Biogel P6. E1 glycopeptides were further fractionated by chromatography on wheat germ agglutinin bound to Sepharose 4B into an unbound fraction (63% of the applied material) and a bound fraction (37%), which was eluted with 0·1 M-*N*-acetylglucosamine. The major sugar constituents of the bound and the unbound fraction were galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and neuraminic acid (Fig. 4 and Table 1). The latter sugar was identified by high-voltage paper electrophoresis of neuraminidase-treated E1 glycopeptides (Fig. 5).

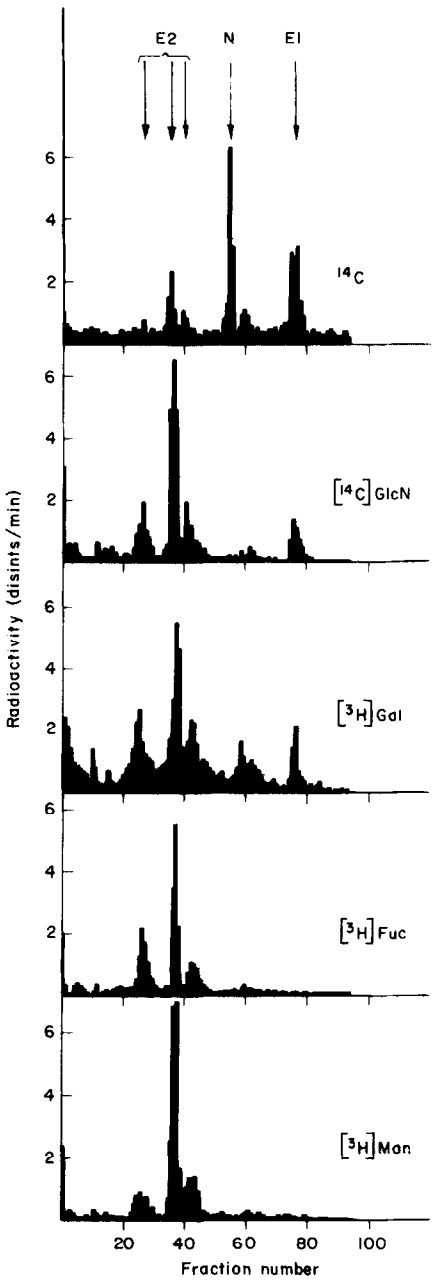

FIG. 2. Analysis of bovine coronavirus L9 polypeptides. L9 was grown in primary bovine fetal thyroid cells in the presence of radioactive amino acids (^{14}C) and sugars (^{3}H). Virus was harvested 30 h post infection and analyzed on SDS-containing 5% to 15% polyacrylamide gradient gels. Polypeptides migrating with an apparent molecular weight of 180,000, 90,000 and 65,000 were designated as E2 species on the basis of their related carbohydrate pattern.

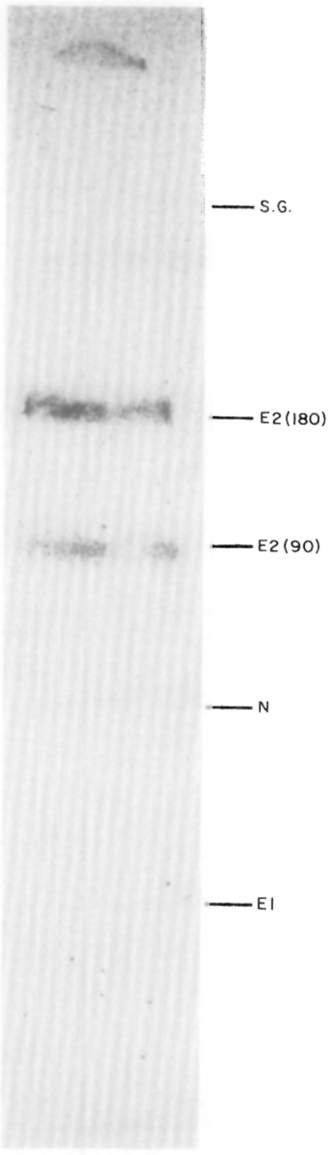

FIG. 3. Labeling of coronavirus A59 with [^{3}H]palmitic acid. A59 was grown in the presence of [9, 10-^{3}H]palmitic acid and harvested 18 h post infection. Purified virus was analyzed on an SDS-containing 5% to 15% polyacrylamide gradient gel. No label was incorporated into the nucleocapsid protein N and glycoprotein E1, indicating that metabolic conversion of label into amino acids is negligible and aggregation of E1 with glycolipids can be excluded. S.G. indicates the beginning of the separation gel.

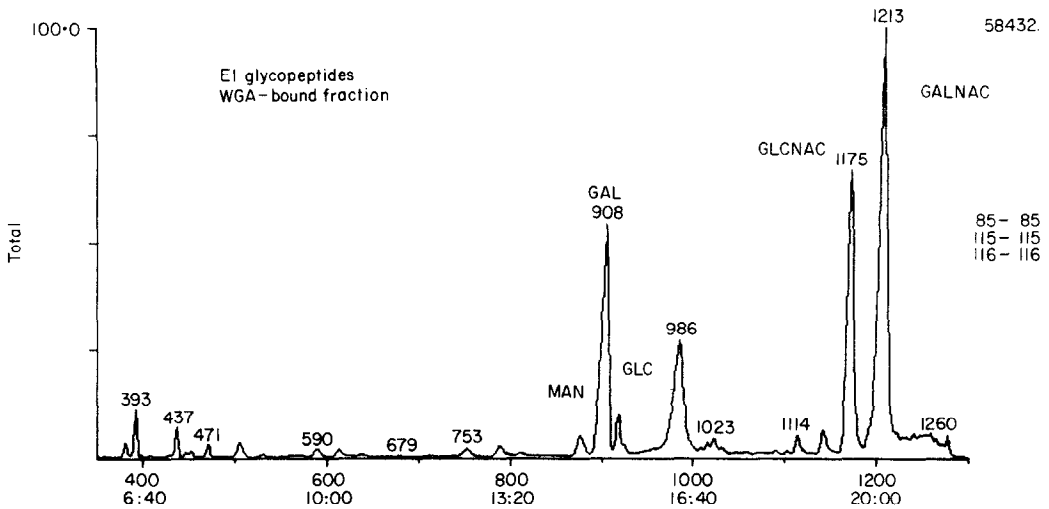

FIG. 4. Reconstructed total mass fragmentogram of alditol acetates obtained from the wheat germ agglutinin-bound fraction of E1 glycopeptides from coronavirus A59. E1 glycopeptides binding to wheat germ agglutinin-Sepharose 4B (about 5 μg of total sugar) were hydrolyzed, reduced, peracetylated and analyzed on a 25 m fused silica capillary column by combined gas chromatography/mass spectrometry. A temperature program of 2 deg.C/min from 150 to 230°C was applied after a 5 min delay of the starting temperature. Peaks were identified according to their retention time (abscissa) and their fragmentation pattern. The peak with scan number 986 did not show sugar characteristic fragmentation.

TABLE 1

Sugar composition of glycopeptides from E1 and E2 of coronavirus A59

	E1				E2	
	WGA†-bound		WGA-unbound			
Sugar constituent	%	ratio	%	ratio	%	ratio
Fucose	0	0	0	0	3·5	0·47
Mannose	2·54	0·09	3·05	0·18	22·2	3·00‡
Galactose	28·87	1·00‡	17·02	1·00‡	21·3	2·87
Glucose	2·26	0·12	8·76	0·51	6·8	0·92
GlcNAc	28·73	1·00	15·73	0·92	46·3	6·27
GalNAc	59·50	2·16	68·87	4·04	0	0
Neuraminic acid§	+		+		+	

† WGA, wheat germ agglutinin.
‡ Ratios based on this derivative.
§ Determined by high-voltage paper electrophoresis.

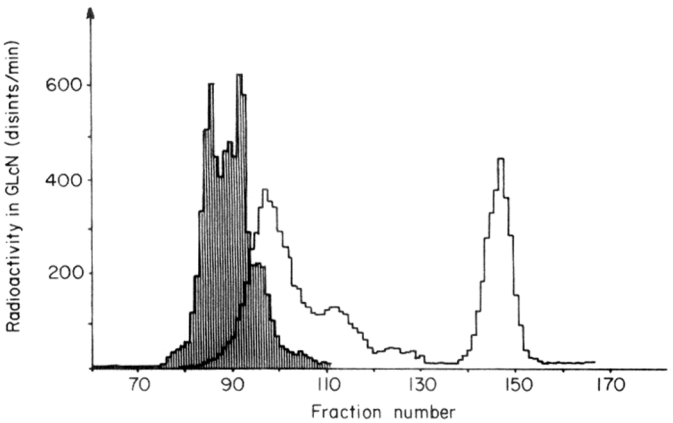

FIG. 5. Biogel P6 chromatography of E1 glycopeptides from A59. [^{3}H]glucosamine-labeled E1 glycopeptides were applied to a Biogel P6 column (1 cm × 150 cm) and eluted with water containing 0·02% (w/v) NaN_3 (shaded profile). Fractions of 1 ml were taken and radioactivity of portions was determined by liquid scintillation counting. The glycopeptides eluted at molecular weights between 2200 and 1800. After enzymatic removal of neuraminic acid residues, a reduction in molecular weight to about 1500 was observed for the main fraction (open profile). About 30% of the label eluted in the position of free *N*-acetylneuraminic acid (fraction 142 to 152). This material was lyophilized and co-migrated with *N*-acetylneuraminic acid standard in high-voltage paper electrophoresis.

Both fractions contained negligible amounts of mannose and no fucose. The presence of minute amounts of glucose is considered a contamination, since no radioactive glucose was detected in hydrolysates of [^{3}H]galactose-labeled glycopeptides by paper chromatography. Due to metabolic conversion, radioactive *N*-acetylgalactosamine was detected with this method in hydrolysates of E1 glycopeptides that had been labeled with either [1-^{3}H]galactose or [6-^{3}H]glucosamine (data not shown). Both the wheat germ agglutinin-bound and unbound fraction were resistant to treatment with *endo*-β-galactosidase from *Escherichia freundii*. This enzyme hydrolyzes Gal-β-1 → 4-GlcNAc linkages in polylactosaminyl structures, but does not allow branching of the galactose residue (Fukuda, 1981).

The sugar composition of E2 glycopeptides showed the typical composition of *N*-glycosidically linked carbohydrate side-chains commonly found in viral glycoproteins. Treatment of [^{3}H]glucosamine-labeled glycopeptides with *endo*-β-*N*-acetylglucosaminidase H from *Streptomyces griseus* followed by analysis on a Biogel P4 (minus 400 mesh) column revealed that 15% of the material was sensitive to this enzyme, indicating the presence of mannose-rich side-chains in E2. This was further evidenced by binding studies of E2 glycopeptides to a column of concanavalin A covalently linked to Sepharose 4B: 54% of glucosamine-labeled glycopeptides did not bind and were eluted with buffer; 32% could be eluted with 20 mM-α-methylmannoside; and 14% showed a high affinity to the lectin and were eluted with 200 mM-α-methylmannoside (data not shown).

(d) *β-Elimination*

A standard procedure to distinguish *N*-glycosidic carbohydrate–protein linkages

from *O*-glycosidic linkages is the susceptibility of the latter to mild alkaline degradation (Zinn *et al.*, 1977). We have applied this method for sugar-labeled glycoproteins E1 and E2 and analyzed released oligosaccharides after passage over Dowex 50 WX 2 (H^+ form) by high-voltage paper electrophoresis and gel filtration. About 95% of the carbohydrate label of E1 was recovered in the runthrough of the ion-exchange column, whereas essentially all the label of E2 was retained. In high-voltage paper electrophoresis at pH 5·3, 80% of the E1-derived oligosaccharides were charged and migrated towards the anode. When E1 labeled with ^{14}C-labeled amino acids was used in a similar β-elimination reaction and subjected to electrophoresis without prior ion-exchange treatment, all the label remained at the origin. This indicates that, under the alkaline conditions of the elimination reaction, no significant breakdown of the polypeptide backbone of E1 occurs. The oligosaccharides released from [^3H]galactose-labeled E1 were resolved by gel filtration on a calibrated Biogel P4 column into four different peaks, eluting at fractions 168, 181, 208 and 215 (Fig. 6). Using oligosaccharides of the mannose-rich type as standards, their molecular weights were determined as 1550, 1100, 570 and 480, respectively. The structural analysis of these fractions is under investigation.

For mucine-type glycoproteins, which contain exclusively *O*-glycosidic carbohydrate side-chains linked to serine and threonine residues, it has been shown that these amino acids are converted into alanine and α-aminobutyric acid, respectively, when the β-elimination was followed by palladium-catalyzed hydrogenation (Pigman & Downs, 1977). Using porcine submaxillary mucine, we were able to confirm such a conversion. However, when we carried out amino acid analyses of E1 that had been subjected to β-elimination under reducing conditions, we were unable to detect a decrease in the serine and threonine content of the

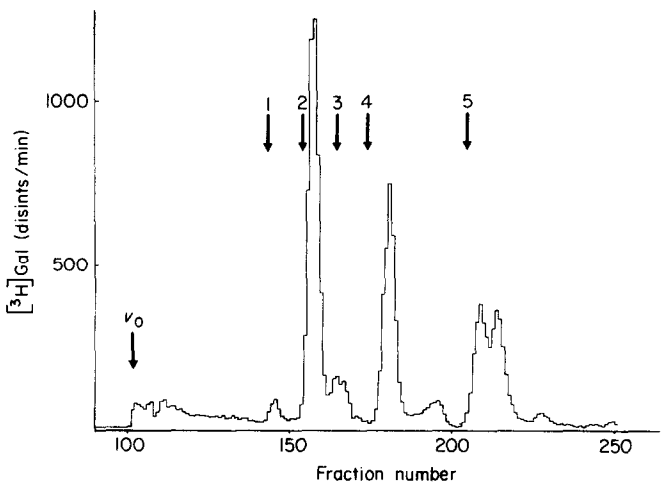

FIG. 6. Molecular weights of the oligosaccharides obtained by β-elimination from E1 of coronavirus A59. Glycoprotein E1 was subjected to β-elimination and released oligosaccharides were analyzed on a Biogel P4 (minus 400 mesh) column (1 cm × 150 cm). Fractions of 0·43 ml were taken and assayed for radioactivity. The following compounds were used for the calibration of the column as indicated by arrows: V_0, (exclusion volume) bovine serum albumin; (1) $Man_9GlcNAc$ (M_r 1752); (2) $Man_8GlcNAc$ (M_r 1580); (3) $Man_7GlcNAc$ (M_r 1408); (4) $Man_6GlcNAc$ (M_r 1236); and (5) sialyl-lactose (M_r 634).

glycoprotein, or a concomitant increase in the alanine and α-aminobutyric acid content. The failure to identify serine and threonine as attachment sites for the oligosaccharides could be explained by the fact that only a small proportion of these residues are glycosylated. It is also conceivable that the reduced solubility of the carbohydrate-free E1 (K. Holmes, personal communication) does not allow proper hydrogenation of the unsaturated amino acids formed during β-elimination.

(e) *Intracellular forms of the viral glycoproteins*

The intracellular synthesis of viral polypeptides is shown in Figure 7. Infected 17Cl1 cells were pulse-chase labeled 18 hours post infection. Cytoplasmic lysates were immunoprecipitated with a serum raised against purified virus. Three major virus-specific proteins were precipitated, forming single bands at M_r 180,000 and M_r 50,000, and a double band at M_r 20,000 and M_r 23,000. They co-migrated (data not shown) with the virion proteins E2 (M_r 180,000), N (M_r 50,000), and E1 (M_r 23,000). In addition, four minor species were precipitated (at M_r values of 120,000, 90,000, 65,000 and 38,000). The M_r 90,000 species, which appeared with leucine labeling only after 80 minutes of chase, co-migrated with the virion E2

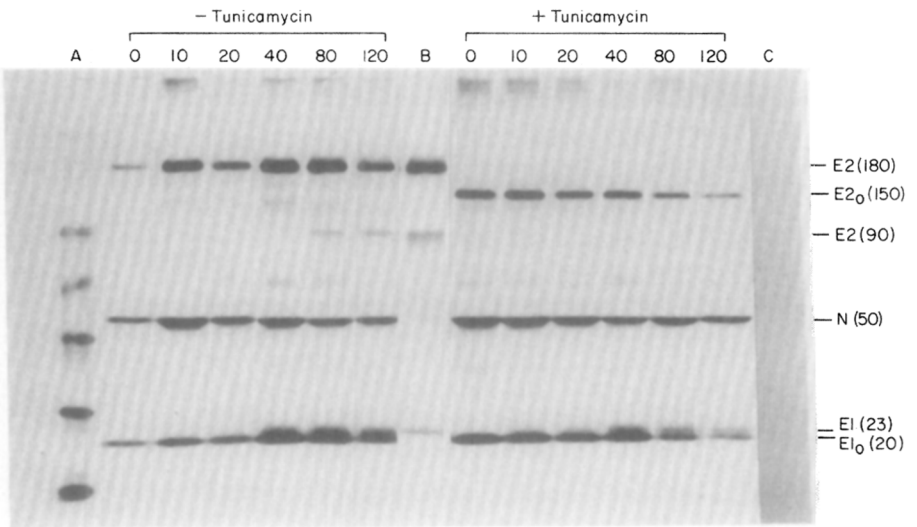

FIG. 7. Intracellular forms of A59 polypeptides and effects of tunicamycin. Left side: A59-infected 17Cl1 cells (multiplicity of infection 50 p.f.u./cell) were pulse-labeled 18 h post infection for 15 min with [^{3}H]leucine and chased for the times indicated. Virus-specific polypeptides were immunoprecipitated from cytoplasmic lysates and analyzed on an SDS-containing 5% to 15% (w/v) polyacrylamide slab gels. Right side: Sister cultures of the previous experiment were treated similarly, but tunicamycin (1 μg/ml) was added to the medium 2 h before the pulse and was present throughout the experiment.

Track A shows ^{14}C-labeled molecular weight markers (Amersham): myosin M_r 200,000, phosphorylase b M_r 92,000, bovine serum albumin M_r 69,000, ovalbumin M_r 46,000, carbonic anhydrase M_r 30,000 and lysozyme M_r 14,300. Track B shows the immunoprecipitate of infected cells that were pulse-labeled with [1-^{3}H]galactose (500 μCi/ml) and chased for 30 min in medium containing additional unlabeled glucose. Track C shows the immunoprecipitate obtained from infected, leucine-labeled cells with control rabbit serum.

(M_r 90,000), indicating that the proteolytic cleavage of the M_r 180,000 species is a post-translational event.

Two intracellular forms of the E1 protein have also been reported with the murine coronavirus JHM, and it has been suggested (Siddell *et al.*, 1981) that the larger species (M_r 25,000) is derived by glycosylation from the smaller (M_r 23,000). The concept, that the same type of precursor–product relationship exists between the M_r 23,000 and the M_r 20,000 species of E1 of coronavirus A59, is suggested by our observation that the smaller form is already labeled after a short pulse, whereas the larger appears only after a longer chase. As shown before, the carbohydrate moiety of glycoprotein E1 could be labeled only with radioactive galactose and glucosamine. Both of these sugars were incorporated into the M_r 23,000 species of the E1 polypeptide and not the M_r 20,000 species, in pulse-chase labeling experiments (Fig. 7, track B). This suggests that glycosylation of E1, like proteolytic cleavage of E2, is a post-translational event.

(f) *Effects of glycosylation inhibitors on the synthesis of E1 and E2*

Tunicamycin inhibits the formation of dolichol-linked *N*-acetylglucosaminyl pyrophosphate, which serves as an essential intermediate in the *N*-glycosylation of glycoproteins (for a review, see Schwarz & Datema, 1980). The effects of this inhibitor on the synthesis of viral glycoproteins in infected cells are also shown in Figure 7. At a tunicamycin concentration of 1 μg/ml, protein synthesis was only slightly reduced. Furthermore, glycosylation of E1 followed essentially the same kinetics as in the absence of the inhibitor. E2, however, showed a drastic decrease in its apparent molecular weight, yielding a M_r 150,000 species, designated $E2_0$. In a similar experiment, this species could not be labeled with galactose (data not shown). This indicates that the M_r 150,000 species is the non-glycosylated form of E2. An inhibitory effect of tunicamycin on the synthesis of E2 has also been reported by Holmes *et al.* (1981). However, these authors were unable to demonstrate the synthesis of the non-glycosylated E2 analog.

The presence of tunicamycin and of other inhibitors, such as 2-deoxy-arabinohexose (2-deoxy-glucose) and 2-deoxy-2-fluoro-glucose, known to interfere with the formation of *N*-glycosidic linkages, did not inhibit virus particle formation. This is demonstrated in Figure 8, which shows the glycoproteins of purified virus particles formed in the presence of these inhibitors. The compounds interfered with the glycosylation of E2, but had no significant effect on E1. Particles formed in the presence of tunicamycin and 2-deoxy-glucose lacked detectable amounts of E2. 2-Deoxy-2-fluoro-glucose caused heterogeneous E2. This is probably due to glycosylation *via* an alternative pathway, in which shorter oligosaccharides are transferred from the lipid-linked intermediate to the nascent polypeptide, which are not processed in the way outlined in the Introduction (Datema *et al.*, 1980).

The specific infectivity of virus particles synthesized in the presence of these inhibitors was determined by plaque assays. Tunicamycin, as well as 2-deoxy-glucose, reduced the infectivity by a factor of more than 10^4. This indicates that glycosylation of E2 is an essential requirement for the infectivity of coronavirus particles.

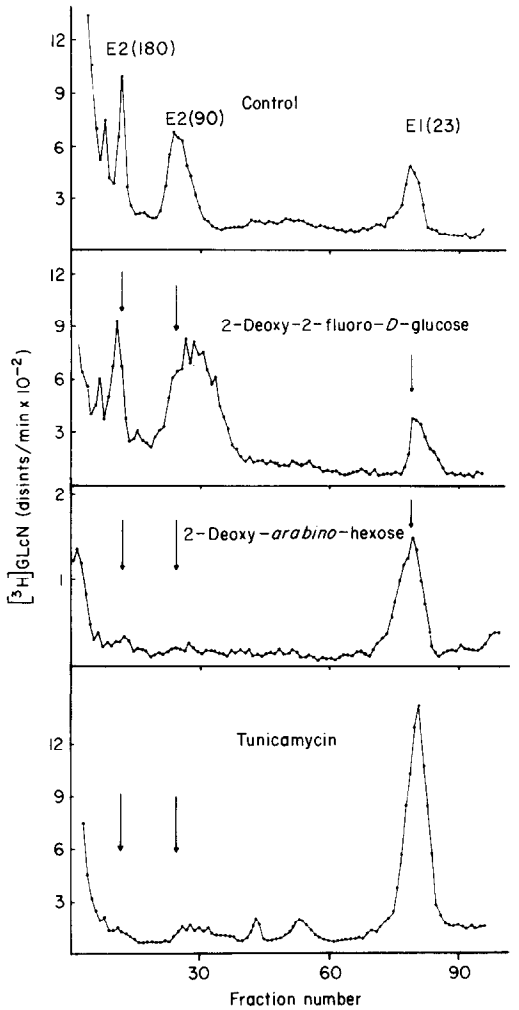

FIG. 8. Effects of glycosylation inhibitors on the glycoproteins and the infectivity of A59 particles. 17Cl1 cells were infected with coronavirus A59 (multiplicity of infection, 8 p.f.u./cell) and incubated for 20 h in medium containing [6-^{3}H]glucosamine (2 μCi/ml). 2-Deoxy-2-fluoro-D-glucose (Calbiochem), 2-deoxy-arabinohexose (Serva), or tunicamycin (Eli Lilly) were present at concentrations of 8 mg/ml, 20 mg/ml and 1 μg/ml, respectively. The infectivity of A59 particles released into the culture fluid was determined by plaque assays. The titers were 6×10^6 p.f.u./ml (for the control virus), $1{\cdot}8 \times 10^6$ p.f.u./ml (fluoro-glucose), 3×10^2 p.f.u./ml (deoxyglucose), and 2×10^2 p.f.u./ml (tunicamycin). Purified virus preparations were analyzed on cylindrical SDS/10% (w/v) polyacrylamide gels, which were sliced and counted as described in Materials and Methods.

4. Discussion

Our data demonstrate that the two glycoprotein species of the coronavirus envelope differ in several important structural and biosynthetic properties. These include the structure of the carbohydrate side-chains, the covalent binding of fatty acids, the processing steps at the co-translational and post-translational level, and

the sensitivity to glycosylation inhibitors. Because of these differences, it is now clear that E1 and E2 belong to two distinct glycoprotein classes.

Glycoprotein E2 has carbohydrate side-chains with *N*-glycosidic linkages, as is the case with glycoproteins of rhabdoviruses, ortho- and paramyxoviruses, alphaviruses, and oncornaviruses. This conclusion is based on the following observations. (1) We show by metabolic labeling and by chemical analysis that E2 contains glucosamine, mannose, galactose, fucose and neuraminic acid. Basically, the same carbohydrate composition has been found with the glycoproteins of all the other viruses. Most of the E2 side-chains are of the complex type. About 15% of the glycopeptides derived from E2 are sensitive to treatment with endoglycosidase H and show strong binding to concanavalin A, indicating that these side-chains belong to the mannose-rich type. (2) The side-chains of E2 cannot be removed by β-elimination. (3) Glycosylation of E2 is inhibited by tunicamycin and 2-deoxyglucose, and it is altered by 2-deoxy-2-fluoro-glucose. These compounds interfere specifically with the biosynthesis of oligosaccharides linked by *N*-glycosidic linkages to the polypeptide. In the presence of tunicamycin, E2 is synthesized in its non-glycosylated form. This polypeptide appears to be quite stable in the infected cell, but the available evidence indicates that it is not incorporated into virus particles which, according to the data presented here and for similar studies by Sturman (1981), are still being formed under these conditions. (4) The observation that the unglycosylated E2 polypeptide can be detected only in the presence of inhibitors of glycosylation indicates that glycosylation of this glycoprotein is initiated at the co-translational level. This is, again, typical for *N*-glycosidic carbohydrate protein linkages.

There are yet other aspects in which E2 resembles viral glycoproteins with well-established *N*-glycosidic linkages. Some of these, such as the G protein of vesicular stomatitis virus, glycoprotein E1 and E2 of alphaviruses, the hemagglutinin of influenza virus and the F protein of paramyxoviruses are acylated (Schmidt, 1982), and we have found now that coronavirus protein E2 contains also fatty acid in covalent linkage. Post-translational proteolytic cleavage is another modification that is observed both with E2 of coronaviruses and with a whole series of other viral glycoproteins; e.g. with the influenza virus hemagglutinin, the F and HN glycoproteins of paramyxoviruses, the precursor of the E2 and E3 glycoproteins of alphaviruses, and the envelope glycoproteins of type C retroviruses (for a review, see Klenk & Rott, 1980).

The first piece of evidence indicating that E1 belongs to a different class of glycoproteins was its carbohydrate composition. E1 contains glucosamine, galactosamine, galactose and neuraminic acid. The absence of mannose and the presence of galactosamine are highly suggestive for *O*-glycosidically linked oligosaccharides that always contain galactose, galactosamine and neuraminic acid. Glucosamine has been observed occasionally in such oligosaccharides; e.g. in glycoproteins with blood group I activity (Feizi *et al.*, 1971) and in rat sublingual glycoprotein (Slomiany & Slomiany, 1978). The *O*-glycosidic nature of the carbohydrate–protein linkages in E1 was further substantiated by the finding that the oligosaccharide side-chains can be removed

by β-elimination, a standard procedure to distinguish *O*-glycosidic from *N*-glycosidic side-chains. The exact structure of the side-chains is not known, but our data show that there are at least four differently sized oligosaccharides that can be distinguished as two groups by their affinity to wheat germ agglutinin.

Whereas glycosylation of glycoproteins with *N*-glycosidic linkages is initiated at the co-translational level, glycosylation of E1 appears to be exclusively a post-translational event. This is suggested by the observation that a presumably carbohydrate-free precursor of E1 (M_r 20,000) is present in infected cells which, as indicated by pulse-chase experiments, is subsequently converted into the glycosylated E1 (M_r 23,000). As pointed out above, Siddell *et al.* (1981) have described a similar post-translational modification of a low molecular weight glycoprotein in coronavirus JHM, and these authors also proposed a glycosylation event to explain the change in the apparent molecular weight.

In the presence of tunicamycin, where glycosylation of E2 is completely inhibited, the non-glycosylated E1 (M_r 20,000) is processed into the glycosylated species (M_r 23,000) with the same kinetics as in the absence of the antibiotic. 2-Deoxyglucose and 2-deoxy-2-fluoro-glucose, which also interfere with *N*-glycosylation have no effect on the glycosylation of E1. In the light of investigations reported by Gahmberg *et al.* (1980), these observations further support the concept of *O*-glycosidic linkages in E1. When these authors studied the effects of tunicamycin on the glycosylation of glycophorin, which contains both *N* and *O*-glycosidic side-chains, they found that the drug interfered only with the attachment of the *N*-glycosidic side-chain and had no effect on the *O*-glycosidic linkages.

It will be interesting to see if the structural differences between E1 and E2 can be correlated to differences in the function of the two glycoproteins. There is evidence that E2 is necessary for the cell-fusing capacity of the virus (Holmes *et al.*, 1981) and that proteolytic cleavage is essential for this activity (Storz *et al.*, 1981). It is therefore, tempting to speculate that E2 initiates infection as do the glycoproteins of ortho- and paramyxoviruses. Glycoprotein E1, on the other hand, has been suggested to play an important role in envelope formation and in the interaction between the envelope and the nucleocapsid (Sturman *et al.*, 1980). In this respect, it is interesting to note that, in contrast to most other enveloped viruses, coronaviruses mature by budding from the endoplasmic reticulum (McIntosh, 1974). Whether the unusual carbohydrate pattern of E1 can be correlated to the unusual budding site remains to be seen.

Despite the fact that a large variety of secretory and membrane proteins of great biological interest are glycoproteins with *O*-glycosidic linkages (for a review, see Montreuil, 1980), intracellular transport and processing of these glycoproteins is still only poorly understood. We know of only two other reports where evidence for the existence of *O*-glycosidic linkages in viral glycoproteins has been obtained. These glycoproteins are the hemagglutinin of vaccinia virus, which is found in the infected cell but not in virus particles (Shida & Dales, 1981), and herpes simplex virus glycoproteins (Olofsson *et al.*, 1981). These glycoproteins contain both *O*-glycosidic and *N*-glycosidic linkages. E1 is the first example of a viral glycoprotein that contains exclusively *O*-glycosidic linkages. Providing all the advantages of a

virus system for studying structure and biosynthesis of macromolecules. coronaviruses may serve, therefore, as an ideal tool to investigate the biogenesis of glycoproteins with *O*-glycosidic linkages. The fact that the virus provides with E2 an internal control for a glycoprotein with *N*-glycosidic linkages further stresses the model character of the system.

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