GLYCOPROTEIN E1 OF CORONAVIRUS A59: A NEW TYPE OF VIRAL GLYCOPROTEIN

Heiner Niemann and Hans-Dieter Klenk

Institut für Virologie FB Humanmedizin
Frankfurter Str. 107
6300 Giessen

INTRODUCTION

Glycosylation of viral glycoproteins as a co- and posttranslational event has been studied in a large number of viral systems (for review see Klenk and Rott, 1980). From these data a general picture can be drawn:

In a first step the nascent polypeptide chain extending into the lumen of the rough endoplasmic reticulum (RER) is glycosylated by an en bloc transfer of the oligosaccharide from a dolichol-linked intermediate Dol-P-P-$(GlcNAc)_2$ Man_9 Glc_3. This glycosylation step requires a tripeptide-sequence (H_2N·Asn-X-Ser(Thr)·COOH) and the resulting carbohydrate-protein linkage is of the N-glycosidic type between N-acetylglucosamine and asparagine. During transport of the glycoprotein to smooth membranes the carbohydrate side chains become trimmed by specific glycosidases. After sequential removal of the glucoses, a varying number of mannose residues may be cleaved off to yield the generally heterogeneous "mannose rich" side chains, also found in glycoproteins of mature virions.

If trimming of the side chains proceeds to a $Man_5(GlcNAc)_2$-Asn-species, re-addition of N-acetylglucosamine, galactose, fucose, and neuraminic acid will occur to form various "complex type" side chains. This presumably takes place in the Golgi. From here the viral glycoproteins are transported to the plasma membrane where they are sequestered into budding virus particles.

In this paper we describe a new type of viral glycoprotein having a carbohydrate composition and characteristics hitherto unknown for viral glycoproteins.

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MATERIALS AND METHODS

Virus and Cells

The A59 strain of murine coronavirus was grown in the 17 clone 1 line of spontaneously transformed Balb C 3T3 cells. Both the virus and the cell line were kindly provided by Dr. L. S. Sturman. Virus was radiolabeled by the addition of radioisotopes to the growth medium, reinforced Eagle's medium containing 10 % fetal calf serum, after the 1 hr adsorption period. Virus was harvested 30 hrs after infection, collected by centrifugation (2 hrs, 53 700 xg) and purified by isopycnic centrifugation on a 30 to 50 % (w/w) sucrose gradient.

SDS-polyacrylamide gel electrophoresis

Initially cylindrical gels containing 10 % polyacrylamide were employed essentially as described by Laemmli (1970).

For preparative isolation of radiolabeled glycoprotein E1 samples were prepared according to Sturman et al. (1980). Glycoprotein was recovered from crushed gels after freezing and thawing by elution with 0.1 % SDS, filtration through o.45 µm Millipore filter units and precipitation in 90 % aqueous acetone at 0^{o} C. Preparations were then exhaustively dialysed against distilled water and lyophilized.

Isolation of E1 glycopeptides

Radiolabeled glycoprotein was digested with predigested pronase at 50^{o} C in 1.0 M-Tris-chloride containing 10^{-4} M $CaCl_2$ at pH 8.0 for 48 hrs with a second addition of protease after 24 hrs. Insoluble degradation products were removed by centrifugation and the supernatant was extracted with chloroform/methanol (2/1). The upper phase containing essentially all the radioactive label was desalted by gel filtration on a Biogel P2 column (1x35 cm).

E1 glycopeptides were further fractionated by affinity chromatography on WGA-Sepharose 6B as described by Krusius and Finne (1978).

Release of carbohydrate by alkali-borohydride-treatment

Glucosamine labeled E1 was subjected to β-elimination conditions according to Carlson (1968). In short, lyophilized E1 was incubated in a solution containing 0.05 M NaOH and 1.0 M $NaBH_4$ at 45^{o} C for 10 hrs. After cooling to room temperature excessive borohydrate was destroyed by the addition of glacial acetic acid to pH 5.0 and boric acid was chased by repeated evaporation with methanol. Aliquots were spotted onto Whatman 3 MM paper sheets and subjected to high voltage paper electrophoresis in pyridine-acetic acid-water (4/10/86) at pH

4.5. Electrophoresis was carried out at 35 V/cm for 6 hrs. The paper was then cut into 0.5 cm stripes and counted for radioactivity.

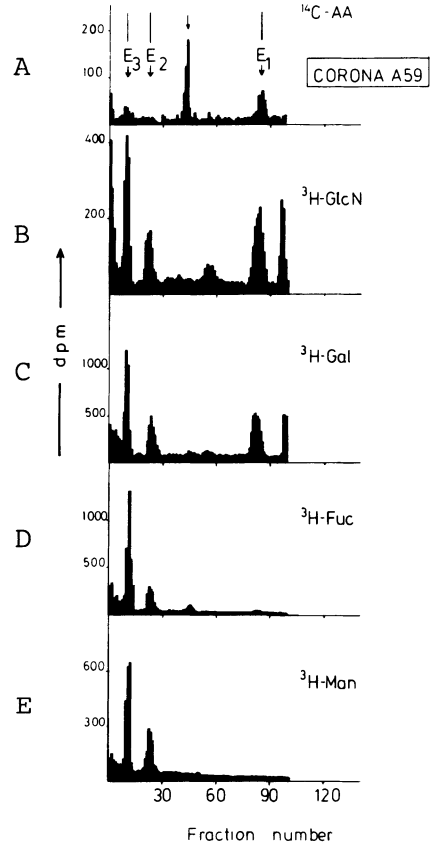

Fig. 1 Incorporation of radioactive markers into coronavirus A59 polypeptides

Coronavirus A59 was grown in spontaneously transformed Balb C 3T3 cells in the presence of ^{14}C amino acids (panel A) or radiolabeled sugars as indicated (panels B to E). Virus was harvested 30 hrs post-infection and analyzed on cylindrical 10 % polyacrylamide gels. Whereas E2 and E3 could be metabolically labeled with glucosamine, galactose, fucose, and mannose, E1 was only labeled with galactose and glucosamine.

Note that the incorporation of labeled amino acids into E2 and E3 is comparatively small indicating a numerical abundance of E1.

Sugar constituent analyses

All reagents were of ultrapure grade or freshly distilled. Desalted glycopeptides containing 5 - 10 µg of total sugar were hydrolyzed in 0.5 N H_2SO_4 in 90 % aqueous acetic acid at 80° C for 8 hrs, reduced and peracetylated as described by Stellner and 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 a stationary phase. Sugar ratios were calculated on the basis of the total reconstructed ion chromatograms.

RESULTS

Incorporation of radioactive sugars into viral glycoproteins

The polypeptide pattern of coronavirus A59 grown in the 17 clone 1 line of spontaneously transformed Balb C 3T3 cells was previously characterized in detail (Sturman, 1977; Sturman and Holmes, 1977; Sturman et al., 1980). Four size classes of polypeptide species were observed in SDS polyacrylamide gel electrophoresis: E1 (23 K), N (50 K), E2 (90 K), and E3 (180 K).

When coronavirus A59 was grown in the presence of ^{14}C-amino acid hydrolysate (Fig. 1A), label was mainly incorporated into the nucleocapsid protein N and into the low molecular weight glycoprotein E1 whereas E2 (and E3) were only poorly labeled. Panels B to E show the glycosylation pattern of A59 obtained by labeling with [6-^{3}H]glucosamine, [1-^{3}H]galactose, [2-^{3}H]mannose, and [1-^{3}H]fucose. All four sugar markers were incorporated into E2 (and E3), whereas E1 was labeled only with galactose and glucosamine. These labeling data cannot be explained on the basis of any structure or carbohydrate composition reported so far for viral glycoproteins. It should be noted that glucosamine label may be metabolically converted into galactosamine and neuraminic acid. A metabolical conversion of sugar label into amino acids on the other hand can be excluded, since under the described conditions no label was incorporated into the nonglycosylated N-protein.

In order to investigate, whether the presence of E1 with its abnormal labeling characteristics is unique for the murine coronavirus A59 or a rather general corona-specific phenomenon, we have also studied the glycosylation pattern of bovine coronavirus L9 grown in primary bovine fetal thyroid cells (J. Storz et al., this meeting). A corresponding low molecular weight glycoprotein comigrating with the murine E1 in SDS-polyacrylamide gel electrophoresis was found in the bovine virus. In analogy this glycoprotein

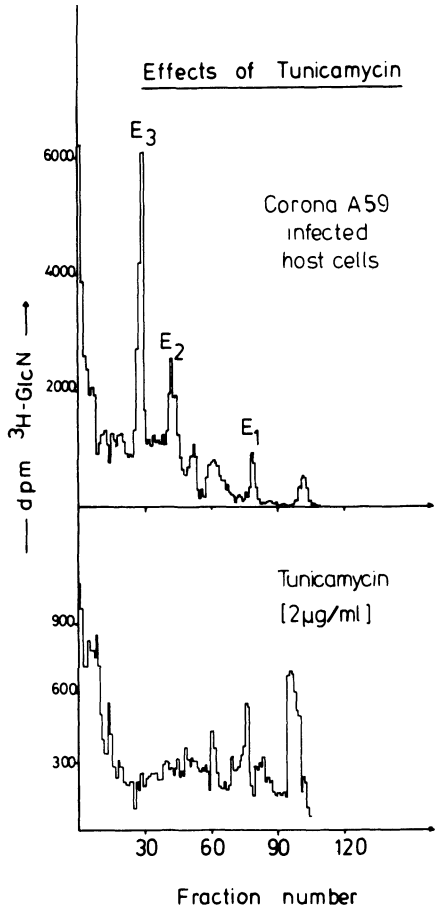

Fig. 2 Effects of tunicamycin

Upper panel: A59-infected 17 Cl 1 were labeled with ^{3}H-glucosamine. 20 hrs after infection cells were extracted with a buffer containing 6 M urea, 4 % SDS in 0.0625 M Tris-HCl at pH 6.7. The extract was analyzed on a 5 to 15 % polyacrylamide gradient gel.

Lower panel: same as above, but tunicamycin (2 µg/ml) was added after infection.

The glycosylation of E2 and E3 is inhibited under these conditions, whereas E1 is made in normal amounts. The virus titer dropped from 5 x 10^{7} PFU/ml in the control to 1 x 10^{2} PFU/ml in the supernatant ot tunicamycin-treated cells.

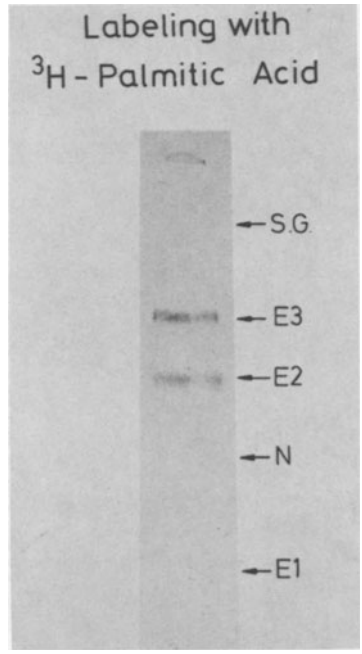

Fig. 3 Labeling with ^{3}H-palmitic acid

Coronavirus was grown in the presence of ^{3}H-palmitic acid (25 µCi/ml). Virus was harvested 18 hrs after infection. E2 and E3 are the only polypeptides showing incorporation of fatty acids. (S. G. indicates the begin of the separation gel).

could be labeled only with galactose and glucosamine but not with fucose and mannose. Furthermore this polypeptide readily aggregated to a 38K species when boiled in the presence of reducing agents as described for the murine glycoprotein E1 (Sturman, 1977).

Effects of tunicamycin

Tunicamycin is a drug known to inhibit the formation of dolichol-linked N-acetyl-glucosamine (Dol-P-P-(GlcNAc)), which serves as an acceptor in the formation of the dolichol-bound oligosaccharide Dol-P-P-$(GlcNAc)_2$ Man_9 Glc_3 (for review see Schwarz and Datema, 1980). In the first glycosylation event this oligosaccharide is then N-glycosidically linked to specific asparagine residues on nascent polypeptides in the RER. It was therefore of interest to study the effects of this drug on the glycosylation of E1. Figure 2 shows the viral glycoproteins in infected cells that were labeled

with radioactive glucosamine for 18 hrs with or without tunicamycin. Production of infective virus particles was reduced by more than five log steps in the presence of tunicamycin. In this case glycosylation of E2 was completely inhibited, whereas E1 incorporated glucosamine label in normal amounts.

This again indicates that E2 is glycosylated in a different biochemical pathway from all the viral glycoprotein hitherto described (similar results were obtained by K. Holmes, personal communication).

Labeling with ^{3}H-palmitic acid

It was recently established that attachment of fatty acids to glycoproteins synthesized at membrane-bound ribosomes is another kind of posttranslational modification (Schmidt and Schlesinger, 1980). When A59 was grown in the presence of [9,10-^{3}H]palmitic acid label was only incorporated into E2 and E3 but not into E1 (Fig. 3). Although we do not know the absolute number of fatty acid residues per E2 molecule, the numerical abundance of E1 in the virus particle, which is indicated by the ratio of ^{14}C amino acid label incorporated into E1 and E2 (Fig. 1A) argues against any undetected presence of fatty acids in E1.

This finding also excludes a contamination of E1 with glycolipids.

Alkali-borohydride treatment of E1

Carbohydrate side chains linked O-glycosidically to serine or threonine can be released from the polypeptide under alkaline and reducing conditions in a β-elimination reaction. Since this reaction is hindered when these amino acids are located at the termini of proteolytic peptides, β-elimination was carried out with undigested glucosamine labeled E1. The released products were analyzed by high voltage paper electrophoresis (Fig. 4). About 90 - 95 % of the carbohydrate label could be released under conditions established for the elimination of carbohydrate side chains from hog submaxillary mucin (Carlson, 1968). A considerable degradation of the elimination products is indicated by the fact that about 20 % of the label comigrated in electrophoresis with free N-acetylneuraminic acid. Control material which was only shortly exposed to alkaline and reductive conditions but then neutralized remained at the origin. N-glycosidic linkes are only slightly affected by this treatment as was shown for influenca virus glycoproteins (Keil et al., 1979).

Molecular weight and carbohydrate constituent analyses of E1 glycopeptides.

Glucosamine labeled glycoprotein E1 was digested with pronase

and the degradation products were chromatographed on a calibrated Biogel P6 column (Fig. 5). A remarkable degree of heterogeneity of the eluted material was observed, partly due to a varying substitution of the side chains with neuraminic acid. After neuraminidase treatment (vibrio cholerae) or partial acid hydrolysis (1 % acetic acid, 80 min at 100° C) the molecular weight of the main glycopeptide fraction was reduced from about 2000 dalton to 1500 dalton.

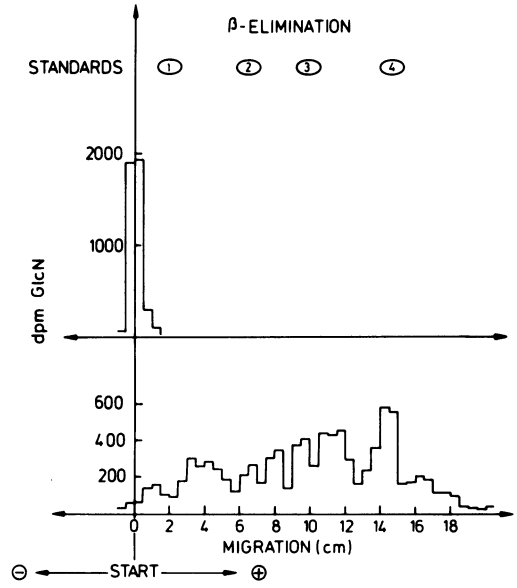

Fig. 4 β-Elimination reaction

^{3}H-glucosamine labeled E1 was treated with 1 M $NaBH_4$ in 0.05 N NaOH at 45° C for various length of time. The reaction products were analyzed by high voltage paper electrophoresis.

Upper panel: all sugar label remains at the origin, when E1 is shortly exposed to alkaline conditions but immediately neutralized.

Lower panel: E1 elimination products obtained after 10 hrs of incubation at 45° C. When ^{14}C-serine labeled E1 was treated similarly all radioactivity remained at the origin.

Standards applied: 1,2 porcine submaxillary mucin oligosaccharides, 3 neruaminyl-lactose, 4 N-acetyl-neuraminic acid.

Fig. 5 Elution pattern of E1 glycopeptides from a Biogel P6 column

^{3}H-glucosamine labeled E1 glycopeptides were applied to a Biogel P6 column (1 x 150 cm) and eluted with 0.02 % NaN_3 in water. The dark elution profile shows glycopeptides prior to neuraminidase treatment which elute at molecular weights of about 2200 to 1800 dalton. After enzymatic removal of neuraminic acid residues a reduction in molecular weight to about 1500 dalton for the main fraction is observed (white profile). About 30 % of the label elutes in the position of free N-acetyl-neuraminic acid.

For sugar constituent analyses further purification steps of the glycopeptides were necessary and involved extraction of pronase digest with chloroform/methanol (2/1) and affinity chromatography on a wheat germ agglutinin-Sepharose 6B column. 37 % of the radioactivity applied bound tightly to the column and could be specifically eluted with 0.1 M N-acetylglucosamine. Both the unbound and the bound fraction were desalted on Biogel P2 and subjected to sugar constituent analyses. Sugar derivatives were identified as peracetylated alditol acetates by combined gas chromatography-mass spectrometry.

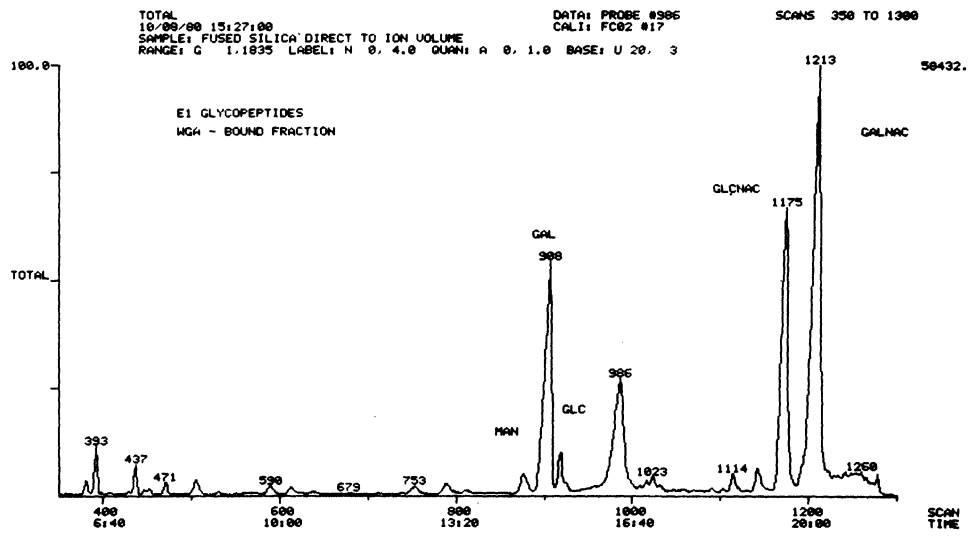

Fig. 6 Total reconstructed ion chromatogram of the alditol acetates obtained from E1 glycopeptides

The sugar constituents of the WGA-bound E1 glycopeptides containing about 5 µg of total sugar were hydrolyzed and converted into alditol acetates. Combined gas chromatographic-mass spectrometric analyses were carried out on a Finnigan model 4021 mass spectrometer equipped with a 25 m fused silica capillary column. A temperature program of 2^{o} C/min from 150^{o} to 230^{o} C was applied after a 5 min delay of the starting temperature. Peaks were identified according to their retention time and their fragmentation.

Table 1 Sugar composition of E1 and E2 glycopeptides

Sugar Constituent	E1 WGA-bound %	E1 WGA-bound ratio	E1 WGA-unbound %	E1 WGA-unbound ratio	E2 %	E2 ratio
Fucose	0	0	0	0	3.5	0.47
Mannose	2.54	0.17	3.05	0.18	22.2	3.00
Galactose	28.87	2.00	17.02	1.00	21.3	2.87
Glucose	2.26	0.25	8.76	0.51	6.8	0.92
GlcNAc	28.73	2.01	15.73	0.92	46.3	6.27
GalNAc	59.50	4.21	68.87	4.04	0	0
Neuraminic Acid	+		+		+	

As summarized in table 1 both the unbound and the bound fraction of E1-glycopeptides contain negligible amounts of mannose. The absence of fucose is also consistant with the labeling data. Glucose is considered to be a contamination since no radioactive glucose was detected in hydrolysates of ^{3}H-galactose labeled glycopeptides by paper chromatography.

In comparison to the WGA-bound fraction the unbound fraction contains about the double amount of N-acetylgalactosamine. The carbohydrate composition of E2 glycopeptides (Table 1, right column) shows the presence of fucose and mannose. E2 carbohydrate side chains may thus very well represent the commonly found complex type side chains containing an internal mannose-trisaccharide core structure which is N-glycosidically linked to asparagine via a chitobiose-unit.

DISCUSSION

Coronavirus A59 contains three glycoprotein species: E1, E2, and E3. Whereas E2 (90 K) and E3 (180 K) can be metabolically labeled with [1-^{3}H]fucose, [2-^{3}H]mannose, [6-^{3}H]glucosamine, and [1-^{3}H]galactose, E1 (23 K) was shown to incorporate label only with the two latter markers. Using the same experimental approach a similar species corresponding to E1 was also found in a bovine transmissible gastroenteritis virus grown in bovine fetal thyroid cells. These coronavirus specific labeling characteristics of glycoprotein E1 are different from all other viral glycoproteins hitherto described.

Glycosylation of E1 is not inhibited by tunicamycin, indicating that different from other viral glyoproteins including coronavirus E2, dolichol-linked N-acetylglucosamine is not involved in the biosynthesis of E1 carbohydrates.

Furthermore, essentially all the carbohydrate could be released under alkaline conditions, supporting the concept of O-glycosidic linkages in E1. Absolute proof must await the identification of the amino acid counter part(s) in the carbohydrate protein linkage, experiments which are currently underway. Amino acid analyses of E1 glycopeptides have shown serine and threonine residues as prominent constituents. Sugar constituent analyses of E1 glycopeptides revealed galactose, N-acetylglucosamine, N-acetylgalactosamine, and neuraminic acid. This sugar profile is quite typical for mucin glycoproteins, which are known to contain O-glycosidic linkages between N-acetylgalactosamine and serine or threonine.

These findings raise the question as to whether glycoprotein E1 is synthesized by a different pathway involving different cellular compartments, as described in the introduction. In this context it is of interest that K. Holmes reported that by means of fluores-

camine labeled antibodies E1 could only be detected in perinuclear regions of infected host cell, whereas E2 was found throughout the cytoplasm (Doller and Holmes, 1980). A novel biosynthetic pathway for the glycoprotein E1 is further substantiated by our finding that E1 in contrast to E2 could not be labeled with ^{3}H-palmitic acid. This posttranslational attachment of fatty acids has been reported for several membrane bound viral glycoproteins and has been localized to the Golgi.

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