Carbohydrate Structure of Sindbis Virus Glycoprotein E2 from Virus Grown in Hamster and Chicken Cells

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Sindbis virus was used as a probe to examine glycosylation processes in two different species of cultured cells. Parallel studies were carried out analyzing the carbohydrate added to Sindbis glycoprotein E2 when the virus was grown in chicken embryo cells and BHK cells. The Pronase glycopeptides of Sindbis glycoprotein E2 were purified by a combination of ion-exchange and gel filtration chromatography. Four glycopeptides were resolved, ranging in molecular weight from 1,800 to 2,700. Structures are proposed for each of the four glycopeptides, based on data obtained by quantitative composition analyses, methylation analyses, and degradation of the glycopeptides using purified exo- and endoglycosidases. The largest three glycopeptides (S1, S2, and S3) have similar structures but differ in the extent of sialylation. All three contain *N*-acetylglucosamine, mannose, galactose, and fucose, in a structure similar to oligosaccharides found on other glycoproteins. Glycopeptide S1 has two residues of sialic acid, whereas glycopeptides S2 and S3 contain 1 and 0 residues of sialic acid, respectively. The smallest glycopeptide, S4, contains only *N*-acetylglucosamine and mannose, and is also similar to mannose-rich oligosaccharides found on other glycoproteins. Each of the complex glycopeptides (S1, S2, or S3) from virus grown in BHK cells is indistinguishable from the corresponding glycopeptides derived from virus grown in chicken cells. Glycopeptide S4 is also very similar in size, composition, and sugar linkages from virus derived from the two hosts. These results suggest that chicken cells and BHK cells have similar glycosylation mechanisms and glycosylate Sindbis glycoprotein E2 in nearly identical ways.

Sindbis is an alpha virus which can infect the cells of a wide variety of organisms (24). The Sindbis virion is comprised of a nucleocapsid surrounded by a lipid bilayer envelope which contains two virus-specific glycoproteins, E1 and E2 (8, 12, 30). During viral replication in an infected host cell, the viral glycoproteins are synthesized on membrane-bound polysomes, become membrane associated, and eventually are associated with the plasma membrane where viral maturation occurs (24).

Sindbis virus has a limited amount of genetic information with a genome sufficient to code for only six to eight proteins (24). This limited capacity is nearly completely utilized in coding for viral structural proteins and RNA polymerase functions (4, 24). This situation has led to the suggestion that the two viral glycoproteins are glycosylated principally, if not entirely, by host cell-specific enzymes (5). Evidence supporting this conclusion comes from the observation that

the oligosaccharide chains of the Sindbis glycoproteins closely resemble oligosaccharide chains found on plasma membrane glycoproteins in uninfected cells (15). Additional evidence was provided by Schlesinger et al. (31), who demonstrated that Sindbis glycoproteins have altered carbohydrate moieties when grown in a mutant cell line with altered glycosyl transferase activities. Thus, examining the structure of the carbohydrate found on the Sindbis glycoproteins from virus grown in different hosts is equivalent to examining the glycosylation of the same polypeptide sequence in these hosts. Comparisons of this type allow conclusions that are not possible when comparing glycoproteins where both the polypeptide sequence and the cell of origin are different.

Previously, it has been shown that the glycopeptides derived from the Sindbis glycoproteins are similar in size when Sindbis is grown *in vitro* in cells cultured from two quite divergent vertebrate species, namely, chickens and hamsters (7, 16). Host-specific differences in carbohydrate composition of the glycoproteins were suggested to be a result of differing degrees of completion

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of the oligosaccharide moieties, although other interpretations were possible (7, 16). In this report, the oligosaccharide structures of glycoprotein E2 are analyzed in greater detail. Structures are proposed for each of the E2 oligosaccharides based on compositions, sugar linkages, and sequential enzyme degradation. In all respects, the complex, galactose-containing oligosaccharide chains of E2 from virus grown in the two hosts are identical.

MATERIALS AND METHODS

Growth of cells and of Sindbis virus. BHK 21/13 cells and secondary chicken embryo cells were grown as described previously (33). Confluent monolayers of cells in glass roller bottles were infected with Sindbis virus at a multiplicity of 0.05 to 0.1 PFU/cell. After 24 h at 37°C, the culture medium was removed, and virus was purified as described below. To prepare radiolabeled virus, confluent monolayers of cells on plastic tissue culture plates were infected at a multiplicity of 10 to 50 PFU/cell in 0.5 ml of medium. After incubation at 37°C for 1 h, 5.0 ml of medium containing the desired radioactive precursor was added. All radioactive precursors were purchased from New England Nuclear Corp. The [6-³H]glucosamine (10 Ci/mmol) and [2-³H]mannose (2 Ci/mmol) were added at 10 μ Ci/ml, [1-¹⁴C]galactose (6 Ci/mmol) was added at 5.0 μ Ci/ml, and [1-¹⁴C]glucosamine hydrochloride (57 mCi/mmol) was added at 2.5 μ Ci/ml.

Purification of Sindbis virus and Sindbis proteins. All virus was purified by precipitation with polyethylene glycol (Carbowax 6000, Union Carbide Corp.) followed by sedimentation through a 15 to 30% sucrose gradient (9, 33). Sindbis virus was precipitated from sucrose gradient fractions by 10-fold dilution with water followed by centrifugation. Glycoprotein E1 was selectively solubilized by suspending this virus pellet in 0.5% Triton X-100 in 0.01 M potassium phosphate buffer at pH 6.0 (7). By using this procedure, we were able to rapidly purify glycoprotein E1 as well as a pellet containing only glycoprotein E2 plus the non-glycosylated core protein (7). This pellet was washed with 70% ethanol to remove glycolipids.

Pronase treatment of purified glycoproteins. Pronase (protease type VI from *Streptomyces griseus*) was purchased from Sigma Chemical Co. Preparative scale samples contain 25 to 30 mg of protein and 200,000 cpm of [³H]glucosamine-labeled proteins as a marker. Such samples were suspended in 0.1 M Tris-hydrochloride buffer (pH 8.0) and were digested with enzyme (40 U) for 24 h at 60°C. This digest was clarified by centrifugation at 10,000 $\times g$ for 15 min. The clear supernatant was removed and stored at -20°C. The pellet was suspended in 0.1 M Tris (pH 8.0) and digested with Pronase a second time as described above. This digest was again clarified by centrifugation, and the supernatant was removed, combined with the supernatant from the first digestion, and lyophilized. This material was suspended in 5.0 ml of deionized water and was applied to a Bio-Gel P-2 column (100 by 1.5 cm) to remove amino acids and small peptides. All glycopeptides from both of the Sindbis glycoproteins were found in the column void.

Fractions containing carbohydrate were pooled, lyophilized, and digested again with Pronase to ensure complete proteolysis.

For samples containing only small amounts of radiolabeled glycoproteins, a single Pronase digestion for 24 h at 60°C was sufficient to achieve complete proteolysis.

Sugar, sialic acid, and amino acid compositions. Neutral and amino sugars were quantitated by gas-liquid chromatography of the corresponding alditol acetates. Glycopeptide samples containing 100 to 200 μ g of carbohydrate and 10 to 25 μ g of myo-inositol as an internal standard were hydrolyzed with 2 N trifluoroacetic acid (Mallinkrodt) for 2 h under a nitrogen atmosphere at 121°C in screw-capped test tubes with Teflon liners. The hydrolysates were dried under a flow of dry nitrogen at 35°C. They were then reduced with sodium borohydride (Sigma) and acetylated with acetic anhydride (Fisher Scientific Co.) as described previously (1). After acetylation was complete, samples were concentrated and desalted as follows: 0.75 ml of toluene (Mallinkrodt, Scintillar grade) was added to each sample, and samples were dried under a stream of nitrogen at 20°C. The resulting powder was dissolved in 1.0 ml of distilled water, and this solution was quickly extracted with two 1.0-ml portions of chloroform (Spectranalyzed, Fisher). The combined chloroform extracts were then evaporated under a stream of nitrogen at 20°C to a final volume of 20 to 50 μ l, and 1.0- μ l portions were injected onto the gas chromatograph. Gas-liquid chromatography was performed on glass columns (120 cm by 2 mm) packed with 1% OV-275 (Applied Science Laboratories) on Gas-Chrom Q (Applied Science) as described previously (7). All the values presented (Table 1) represent averages of replicate injections from duplicate or triplicate analyses of each glycopeptide.

N-acetylneuraminic acid (NANA) compositions were determined by gas-liquid chromatography of the trimethylsilyl ether prepared as described by Reinhold (26). Derivatives were separated by chromatography on glass columns (120 cm by 2 mm) packed with 1% OV-17 (Applied Science) on Gas-Chrom Q (Applied Science) and were quantitated by comparison with a myo-inositol internal standard.

Amino acid compositions were determined by automated analysis of hydrolysates, using ninhydrin reagent. Glycopeptides were hydrolyzed under nitrogen in 6 N hydrochloric acid for 16 h at 100°C in glass tubes with Teflon-lined screw caps. Amino acids were separated and quantitated by using an automated Beckman amino acid analyzer.

Analysis of permethylated glycopeptides. Samples to be analyzed for sugar linkages were methylated by a modification of the procedure described by Sandford and Conrad (29). The permethylated glycopeptides were then run through a column (1.7 by 25 cm) of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) in chloroform:methanol (1:1, vol/vol) to remove dimethyl sulfoxide and excess reagents (39). The permethylated glycopeptides were then hydrolyzed in 2 N trifluoroacetic acid for 2 h at 121°C and were reduced and acetylated as described above. Partially methylated alditol acetates were separated by gas-liquid chromatography on 120-cm by 2-mm glass col-

TABLE 1. Carbohydrate compositions of the E2 glycopeptides of Sindbis virus grown in BHK or chicken (CEC) cells^a

Sugar	S1-BHK	S1-CEC	S2-BHK	S2-CEC	S3-BHK	S3-CEC	S4-BHK	S4-CEC
Fucose	0.38	0.64	0.41	0.46	0.32	0.64	<0.06	<0.06
Mannose	2.82	3.33	2.92	3.42	2.97	3.25	6.72	9.00
Galactose	2.16	2.73	2.31	2.46	2.06	2.37	<0.07	<0.10
Glucose	<0.15	0.80	<0.05	0.27	<0.05	0.67	<0.12	<0.15
Glucosamine	4.00	4.00	4.00	4.00	4.00	4.00	2.00	2.00
NANA	2.35	2.14	1.08	0.93	<0.19	<0.16	ND	ND

^a Values represent moles of sugar per 4.0 mol of *N*-acetylglucosamine (S1, S2, and S3) or moles of sugar per 2.0 mol of *N*-acetylglucosamine (S4). ND, Not determined.

umns packed with 3% ECNSS-M on Gas-Chrom Q (Applied Science) or 3% OV-225 (19, 39). Peaks were tentatively identified by retention times, and these identities were verified by combined gas chromatography-mass spectrometry (19, 39). Quantitation was achieved as described by Bjorndal et al. (3). The values presented (see Table 3) represent averages of replicate injections from a single methylation analysis.

Enzyme digestions. Neuraminidase from *Clostridium perfringens* was purchased from Sigma. β -Galactosidase, α -mannosidase, and β -*N*-acetylhexosaminidase were purified from jack bean meal by modifications of the procedures described by Li and Li (18; D. J. Burke, Ph.D. thesis, State University of New York at Stony Brook, 1976). All final enzyme preparations were free of α -galactosidase, α -fucosidase and β -mannosidase activities. All enzyme digests of glycopeptides were performed at 37°C in the presence of 0.02% sodium azide. Digestions were performed on samples containing less than 100 μ g of carbohydrate. Incubation conditions were as follows: neuraminidase (0.04 U) digestions were performed for 2 h in 50 mM phosphate buffer (pH 5.2); β -galactosidase (100 U) for 36 h in 50 mM citrate buffer (pH 4.0); α -mannosidase (100 U) for 48 h in 50 mM citrate buffer (pH 4.5); and β -*N*-acetylhexosaminidase (100 U) for 48 h in 50 mM citrate buffer (pH 4.5). In all cases except neuraminidase, enzyme was added in 3 equal portions during the course of digestion.

Endo-*N*-acetylglucosaminidase was purified through the G-200 step as described by Muramatsu et al. (22), yielding a preparation which contained both β -galactosidase and β -*N*-acetylhexosaminidase. Neuraminidase-treated Sindbis glycopeptides were incubated with this enzyme preparation for 16 h at 37°C in 0.05 M citrate-phosphate buffer (pH 6.0) and then analyzed by gel filtration.

Gel filtration. Bio-Gel P-2 and P-6 were purchased from Bio-Rad Laboratories. Bio-Gel P-2 chromatography of glycopeptides was performed on columns (120 by 2 cm) swollen and eluted with deionized water. Bio-Gel P-6 chromatography of glycopeptides was performed in columns (120 by 1 cm) in 50 mM NH_4HCO_3 .

RESULTS

Purification of the glycopeptides of glycoprotein E2. A pellet containing glycoprotein E2 plus the non-glycosylated core protein was prepared as described previously (7). In a typical preparation, this pellet contained 20 mg of E2

and 100,000 cpm of [³H]glucosamine-labeled marker. This pellet was digested twice with Pronase as described above, and then desalted on a Bio-Gel P-2 column. The desalted, carbohydrate-containing fractions were lyophilized, suspended in 2 mM Tris (pH 8.0), and applied to a diethylaminoethyl (DEAE)-Sephadex A-25 column. Typical elution profiles of E2 glycopeptides from Sindbis grown in BHK and chicken cells are shown in Fig. 1. There are three principal peaks in Fig. 1A. The fractions comprising peak A-I were pooled, lyophilized, suspended in 1.0 ml of 50 mM ammonium bicarbonate, and further purified by Bio-Gel P-6 column chromatography (Fig. 2A). The majority of the material present in glycopeptide peak A-I is present in two peaks (Fig. 2A). Additional experiments (data not shown) demonstrated that these peaks coelute with glycopeptides S3 and S4. Fractions were pooled as indicated by the bars in Fig. 2.

Likewise, glycopeptide peaks A-II and A-III (Fig. 1A) were purified further by chromatography on Bio-Gel P-6 (Fig. 2B and C). Peaks A-II and A-III consist principally of glycopeptides S2 and S1, respectively. Again, peak fractions were pooled as shown by the bars in Fig. 2B and C. Glycopeptide peak A-0 (Fig. 1A) was variable in amount and has not been further characterized.

Glycopeptides from glycoprotein E2 of virus grown in chicken cells were purified in an exactly analogous fashion. Three peaks were resolved on DEAE-Sephadex A-25 (Fig. 1B). The peaks were pooled and chromatographed on Bio-Gel P-6 (not shown). Glycopeptides S1 through S4 eluted in the same order from DEAE-Sephadex as did the glycopeptides of glycoprotein E2 from virus grown in BHK cells. As noted previously (7), glycopeptides S1 through S4 of E2 from virus grown in the two hosts exactly coelute on Bio-Gel P-6, although the relative amounts of each glycopeptide are host dependent.

Compositions of the purified glycopeptides. Table 1 gives the carbohydrate compositions of the four glycopeptide species purified from glycoprotein E2 of virus grown in BHK and chicken cells. Portions of the same preparations of glycopeptides S1 through S4 were

FIG. 1. DEAE-Sephadex chromatography of E2 glycopeptides. (A) Glycoprotein E2 of Sindbis grown in BHK cells was digested with Pronase as described in the text. The resulting glycopeptides were applied to a column (1.5 by 40 cm) of DEAE-Sephadex A-25 in 2 mM Tris (pH 8). The column was washed with this buffer and then eluted with a 0 to 0.2 M NaCl gradient. Portions of each fraction were assayed for radioactivity, and peak fractions were pooled as indicated by horizontal bars. (B) Glycopeptides were prepared from glycoprotein E2 of Sindbis grown in chicken embryo cells as described in the text. They were applied to a column of DEAE-Sephadex as described in (A), except that the salt gradient was to 0.15 M NaCl. Fractions (3 ml each) were collected, and 100- μ l portions of each fraction were assayed for radioactivity. Fractions were pooled as indicated by the horizontal bars.

analyzed for sugars, amino acids, and NANA, as described above. Values for glycopeptides S1, S2, and S3 are normalized to *N*-acetylglucosamine = 4.0 mol. Values for glycopeptide S4 are normalized to *N*-acetylglucosamine = 2.0 mol. The sialic acid contents of the S4 glycopeptides were not determined. However, they probably

FIG. 2. Bio-Gel P-6 chromatography of E2 glycopeptides from virus grown in BHK cells. (A) Fractions designated as A-I (Fig. 1A) from DEAE-Sephadex chromatography of E2 glycopeptides were pooled, lyophilized, and further purified by gel filtration on a column (1.0 by 100 cm) of Bio-Gel P-6 in 50 mM NH_4HCO_3 . Fractions (750 μ l each) were collected, and 50- μ l portions were assayed by scintillation counting. (B) The same experiment as in (A), except that the glycopeptides were from peak A-II of Fig. 1A. (C) The same experiment as in (A), using glycopeptide material from peak A-III of Fig. 1A.

do not contain sialic acid, since they do not change elution position after treatment with neuraminidase (33). None of the four glycopeptides contains measurable amounts of *N*-acetylgalactosamine. The ratio 4:3:2 for *N*-acetylglucosamine/mannose/galactose in S1, S2, and S3 was observed in several preparations of those glycopeptides. Glucose, however, was present in variable quantities in different preparations of glycopeptides. The data presented in Table 1 show the presence of small amounts of glucose in glycopeptides S1, S2, and S3 from chicken cells but its absence from the other glycopeptides. Other preparations of glycopeptides gave different results for glucose levels. This variability is taken as an indication that glucose is a

contaminant from some source other than the glycopeptides.

Amino acid compositions of the E2 glycopeptides for virus grown in BHK cells are given in Table 2. These values are normalized to aspartate = 1.0 mol. For preparations of glycopeptides S1, S2, and S3, there are 3.6 to 4.2 mol of *N*-acetylglucosamine (by gas chromatography) per mol of aspartate (by amino acid analysis). Glycopeptide S4 contains 1.3 mol of aspartate and 0.8 mol of phenylalanine per 2.0 mol of *N*-acetylglucosamine. Glycopeptides S1, S2, and S3 of virus grown in chicken cells contain the same amino acids as the corresponding glycopeptides of virus grown in BHK cells (not shown). The fact that S1, S2, and S3 contain the same amino acids supports the proposition that all three are attached to glycoprotein E2 at the same asparaginyl residue(s) of the polypeptide chain. Glycopeptide S4 has a much different carbohydrate composition and contains different amino acids, suggesting that it has a different carbohydrate attachment site than S1, S2, and S3.

Molecular weights of the E2 glycopeptides. As shown in Tables 1 and 2, glycopeptide S1 has an approximate composition of $\text{GlcNAc}_4\text{Man}_3\text{Gal}_2\text{NANA}_2\text{Fuc}_1\text{Asn}_1\text{Thr}_1\text{Ile}_1$. The molecular weight of this glycopeptide is 2,692. Similarly, glycopeptides S2 and S3 are 2,415 and 2,124 daltons, respectively, based on their compositions. An "average" glycopeptide

S4 contains seven mannose residues, two *N*-acetylglucosamine residues, one asparagine, and one phenylalanine per glycopeptide, yielding an average molecular weight of 1,819. These molecular weights are consistent with previously reported molecular weights for these glycopeptides (6) and with estimates made by gel filtration, using ovalbumin glycopeptides as markers (not shown).

Methylation analysis of E2 glycopeptides. Purified E2 glycopeptides S1, S2, S3, and S4 from virus grown in BHK cells or chicken cells were permethylated, hydrolyzed, reduced, and acetylated as described above. As shown in Table 3, E2 glycopeptides S1, S2, and S3 have very similar sugar linkages. Assuming a single 3,6-linked mannose residue, each glycopeptide then has about two residues of 2-linked mannose. Glycopeptide S3 contains only terminal galactose and S1 has only 3-linked galactose residues. The fact that the amount of 3-linked galactose corresponds to the number of sialic acid residues (Table 1) suggests that all 3-linked galactose residues are substituted with sialic acids at the nonreducing terminus. Further evidence for this contention is presented below. Glucosamine was found in all glycopeptides as the 4-linked derivative, although recovery of this derivative was low. Also, since some glucosamine residues are fucosylated (see below), some disubstituted glucosamine might have been expected. The low recovery of 4-linked and absence of disubstituted glucosamine residues is probably due to the resistance of methylated amino sugars to acid hydrolysis (36), the degradation of these derivatives during gas chromatography-mass spectrometry (36), and the low response of hexosamine derivatives in gas chromatography using flame ionization detectors. The 4-linked glucosamine residue was identified by its characteristic mass spectral fragments at *m/e* 158 and *m/e* 116 (3).

TABLE 2. Amino acid compositions of the Sindbis E2 glycopeptides^a

	S1	S2	S3	S4
Aspartate	1.0	1.0	1.0	1.0
Threonine	0.7	0.8	0.6	0.1
Isoleucine	0.8	0.7	0.8	<0.05
Phenylalanine	<0.05	<0.05	<0.05	0.6

^a Values represent moles of amino acid per 1.0 mol of aspartate.

TABLE 3. Methylation analysis of glycopeptides from Sindbis virus glycoprotein E2^a

Sugar	Position of methyl groups	Deduced linkage	BHK cells				Chicken cells ^b		
			S1	S2	S3	S4	S1	S3	S4
Fucose	2,3,4	Terminal	+	+	+	0	+	+	0
Mannose	2,3,4,6	Terminal	0	0	0	3.2	<0.1	<0.4	3.9
	3,4,6	2	1.5	2.3	1.5	2.3	2.1	1.6	2.2
	2,4	3,6 ^c	1.0	1.0	1.0	2.0	1.0	1.0	2.0
Galactose	2,3,4,6	Terminal	<0.2	1.5	1.5	0	0.4	2.0	0
	2,4,6	3	2.1	1.2	<0.2	0	2.3	0.4	0
Glucosamine	2,3,6	4	+	+	+	+	+	+	+

^a Terminal fucose and 4-linked *N*-acetylglucosamine are difficult to quantitate, and no attempt was made to do so. These derivatives were confirmed as being present in those samples indicated by +.

^b Glycopeptide S2 from chicken cells was lost during preparation.

^c All derivatives of glycopeptides S1, S2, and S3 were normalized to a value of 1.0 residue of 3,6-linked mannose. Derivatives from glycopeptide S4 were normalized to 2.0 residues of 3,6-linked mannose.

Glycopeptide S4 contains terminal mannose, 2-linked mannose, and 3,6-linked mannose residues in a molar ratio of approximately 3:2:2. Again, the 4-linked glucosamine derivative was difficult to estimate quantitatively, but was the only glucosamine derivative identified.

Exoglycosidase digestions of S1, S2, and S3. Neuraminidase converts glycopeptides S1 and S2 to glycopeptides which exactly coelute with S3 on Bio-Gel P-6 or Bio-Gel P-4 (16). The glycopeptide lacks sialic acid, and methylation analysis shows that galactose is present only as the nonreducing terminal sugar. All further glycosidase experiments were performed on the single glycopeptide peak eluting at the position of S3 after neuraminidase treatment of glycopeptides S1, S2, and S3.

An "S3" peak was present in this manner from Sindbis labeled with [^3H]glucosamine and [^{14}C]galactose (Fig. 3). This glycopeptide was then treated with β -galactosidase, and the products were again analyzed by Bio-Gel P-6 chromatography (Fig. 3). All ^{14}C label was found at an elution volume characteristic on monosaccharides. A single peak of ^3H label eluted at a position slightly smaller than S3. This peak contained no detectable ^{14}C label. Digestion of this agalactoglycopeptide with β -hexosaminidase resulted in the formation of two labeled products. One of these eluted from Bio-Gel P-6 at the same volume as *N*-acetylglucosamine, whereas the second eluted at a volume somewhat smaller than the agalactoglycopeptide. These two peaks contained approximately equal amounts of ^3H label. Digestion of intact glycopeptide S1, S2, or S3 with β -hexosaminidase or α -mannosidase resulted in no change of elution position and no release of monosaccharides.

These results indicate that glycopeptide S1 contains nonreducing termini with the sequence $\text{NANA2} \rightarrow 3\text{Gal1} \rightarrow 4\text{GlcNAc}$. The glycosidic linkage of galactose to glucosamine must be to the C4 of glucosamine, since 4-linked glucosamine is the only glucosamine derivative observed in methylation analysis. Based on the molecular weight values estimated by gel filtration and composition, there must be two such sequences per molecule of S1. Glycopeptide S2 contains one chain of this same trisaccharide and one chain of $\text{Gal1} \rightarrow 4\text{GlcNAc}$, whereas S3 contains two chains of the galactose-glucosamine disaccharide at its nonreducing termini. Identical results are obtained in these experiments when S1, S2, and S3 are purified from virus grown in BHK or chicken cells.

Digestion of glycopeptides S1, S2, and S3 with partially purified endo-*N*-acetylglucosaminidase D. Endo-*N*-acetylglucosaminidase D was purified from *Streptococcus pneu-*

FIG. 3. Digestion of the glycopeptides of glycoprotein E2 with β -galactosidase. Glycoprotein E2 preparations labeled with [^3H]glucosamine and [^{14}C]galactose were pooled, digested with Pronase, and then analyzed by gel filtration on Bio-Gel P-6. Peaks S1, S2, and S3 were pooled, adjusted to pH 5.2, treated with neuraminidase, concentrated by lyophilization, and chromatographed on Bio-Gel P-6 (upper panel). The radioactivity eluting with glycopeptide S3 was pooled, adjusted to pH 4.0, digested with β -galactosidase, concentrated by lyophilization, and then chromatographed on Bio-Gel P-6 (bottom panel).

moniae through the G-200 step as described by Muramatsu et al. (22). This preparation contained both β -galactosidase and hexosaminidase activities. If glycopeptides S1, S2, and S3, labeled with [^3H]glucosamine, are digested with neuraminidase plus this partially purified enzyme mixture, four new peaks of glucosamine label are seen on Bio-Gel P-6 (Fig. 4). The first one to elute (Fig. 4, peak I) from the column labels only with glucosamine and fucose (Fig. 4), and not with mannose or galactose (not shown). Peak II coelutes with NANA. Peak IV elutes at the same position as *N*-acetylglucosamine.

Peak III labels with glucosamine (Fig. 4) and also is the major product when mannose-labeled glycopeptides are digested with endoglucosaminidase D (not shown). Sufficient glycopeptide material was subjected to endoglucosaminidase D digestion so that chemical analysis could be

FIG. 4. Bio-Gel P-6 chromatography of endo-*N*-acetylglucosaminidase D digestion products. Sindbis virus was grown in BHK cells in the presence of [^3H]fucose or [^{14}C]glucosamine. Intact virus with both labels was then digested with Pronase, and the resulting glycopeptides were desalted on Bio-Gel P-2. The desalted glycopeptides were digested with neuraminidase plus endoglycosidase D as described in the text. The digestion products were then analyzed on a column (1.0 by 110 cm) of Bio-Gel P-6 in 50 mM NH_4HCO_3 .

carried out on the products. This chemical analysis showed that peak III contains only glucosamine and mannose in a 1:3 molar ratio. Methylation analysis showed that peak III contained terminal and 3,6-linked mannose residues in a 2:1 ratio. *N*-acetylglucosamine was present as the 4-linked derivative. Based on these data and on the specificities of the enzymes used to generate this fragment (37), it is proposed that this fragment has the structure:

Glycopeptides S1, S2, and S3 contain no terminal mannose residues (Table 3), but instead contain two 2-linked mannose residues. The fragment described above could be obtained by the sequential degradation of glycopeptide S1, S2, or S3 by neuraminidase, α -galactosidase, and hexosaminidase, followed by further cleavage of the remaining core by endo-*N*-acetylglucosaminidase D, as shown schematically in Fig. 5.

FIG. 5. Structure proposed for the carbohydrate portion of glycopeptide S1 of glycoprotein E2. Horizontal dashed lines show the positions of hydrolysis by enzymes.

Peak I has been tentatively identified as the peptide-containing fragment resulting from endo-*N*-acetylglucosaminidase D digestion (Fig. 5). A glycopeptide consisting of a single residue *N*-acetylglucosamine attached to a single residue of asparagine was generated by endo-*N*-acetylglucosaminidase D action upon a purified ovalbumin glycopeptide and was shown to elute from Bio-Gel P-6 at a position similar to peak I. Both peak I and peak II (NANA) elute earlier than predicted by their molecular weights. This is most likely due to the charge exclusion phenomenon observed using Bio-Gel columns (10). All fucose label elutes with peak I, showing that all fucose is linked to the glucosamine which is attached to asparagine.

Enzymatic degradation of the S4 glycopeptides. The mannose-rich glycopeptide S4 of glycoprotein E2 from Sindbis grown in either BHK or chicken cells is a glycopeptide that is heterogeneous with respect to its size on Bio-Gel P-6 or P-4 columns. However, no attempt was made to further purify the subcomponents of this glycopeptide, due to insufficient quantities of materials. The S4 glycopeptides from virus grown in either host contained only glucosamine and mannose (Table 1). By using the exo- α -mannosidase from jack bean meal, it is possible to remove 60% of [^3H]mannose label from these glycopeptides as the monosaccharide. Similar results are obtained using S4 glycopeptides of virus grown in either host. When the S4 glycopeptides are digested with endo-*N*-acetylglucosaminidase D, as shown schematically in Fig. 5.

aminidase D, variable results are obtained, depending on the host cell in which the virus was grown. When BHK cells are the host, some preparations of virus give S4 glycopeptides that are completely digested, whereas other preparations give S4 glycopeptides that are partially or completely resistant to digestion by endo-*N*-acetylglucosaminidase D. However, when chicken cells are the host, the S4 glycopeptides are resistant to digestion. The exact molecular basis for this difference is not known.

DISCUSSION

A proposed structure for glycopeptide S1 of Sindbis glycoprotein E2 from virus grown in either BHK or chicken cells is shown in Fig. 5. This structure is the one most consistent with all the data presented above. Glycopeptides S2 and S3 are variants of glycopeptide S1, differing only in sialic acid content.

Studies on the S4 glycopeptides are less complete, due to the fact that they are an unresolved mixture. The heterogeneity of S4 is reminiscent of that found in other mannose-rich glycopeptides (2). The data presented here suggest that the S4 glycopeptides may have structures similar to other "high-mannose" oligosaccharides (17, 35), but other structures cannot be ruled out. Glycopeptide S4 of glycoprotein E2 is probably attached at a site or sites different from glycopeptides S1, S2, and S3. The best evidence for this is that glycopeptide S4 contains phenylalanine but no isoleucine or threonine. Amino acid analysis of glycopeptide S4 of E2 from virus grown in chicken embryo cells has not been done. However, E2 glycopeptide S4 from virus grown in chicken cells is similar in carbohydrate composition (Table 1), size (7), and sugar linkages (Table 3) to the analogous glycopeptide of virus grown in BHK cells.

The structures proposed for glycopeptides S1, S2, and S3 are hardly unique in nature. Similar structures are present on the envelope glycoproteins of vesicular stomatitis virus (10, 25) and Semliki Forest virus (21). Similar structures have been found on a variety of different soluble glycoproteins (17). Some of these oligosaccharides have the same core structure but differ in the fine structure of the sugar sequence on the nonreducing terminus (13, 17, 25, 35). A difference between the Sindbis glycopeptides and many of the similar structures found elsewhere is the linkage of *N*-acetylneuraminic acid to galactose. In most such glycopeptides, sialic acid is attached either to the C₆ of galactose or to a mixture of C₆ and C₃ (17, 34). In the E2 glycopeptides, only 3-linked galactose is found.

The fact that glycoprotein E2 of Sindbis gly-

cosylated so similarly in cells from such divergent species implies that these species have conserved the enzymes or enzyme complexes involved in glycosylation. Robbins et al. (27) have shown that cells from a variety of species produce very similar oligosaccharide-lipid intermediates. It has been suggested that the carbohydrates of such intermediates are transferred to Sindbis glycoproteins in virus-infected cells (27, 32). In cells infected with vesicular stomatitis virus, the vesicular stomatitis virus glycoprotein is apparently glycosylated by direct transfer of a large, mannose-rich oligosaccharide from a lipid intermediate (14). This oligosaccharide is subsequently trimmed before the addition of GlcNAc-Gal-NANA side chains (14, 28). If such mechanisms apply to the synthesis of the Sindbis glycopeptides as well, the results reported here support the concept that the enzymes involved are highly conserved.

The results presented here also demonstrate the importance of the polypeptide sequence in determining the nature of the carbohydrate which is to be added to it. Thus, Sindbis glycoprotein E2 acquires a complex acidic oligosaccharide and a high-mannose oligosaccharide when grown in BHK and chicken cells, whereas the vesicular stomatitis virus glycoprotein acquires two complex acidic oligosaccharides when grown in a variety of cells including BHK cells (10, 25). Another example is seen in the linkage of sialic acid to galactose. Although chicken cells, and probably BHK cells, have the capability to attach sialic acid to the hydroxyl group at either the carbon 3 or carbon 6 of galactose (15), sialic acid is attached only to the 3 position of the Sindbis glycoproteins.

However, certain aspects of the oligosaccharide structure are host specific. For example, the extent of sialylation of the complex oligosaccharide of the Sindbis glycoproteins varies with the host. Glycopeptide S1 (two sialic acid residues) is the predominant species when Sindbis is grown in BHK cells. Thus, the determinants of carbohydrate addition to polypeptides are complex, including both the polypeptide sequence (and possibly configuration) as well as cell-specific factors. A similar conclusion was reached earlier by Etchison and Holland (11) based on sugar composition studies of vesicular stomatitis virus grown in different hosts. Further studies will be necessary to define the controlling determinants in more detail.

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