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Comparison of the Carbohydrate of Sindbis Virus Glycoproteins With the Carbohydrate of Host Glycoproteins

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The carbohydrate portions of the Sindbis virus glycoproteins were compared with the carbohydrate portions of cell surface glycoproteins from uninfected host cells. Comparisons of the size of glycopeptides were made using gel filtrations. Comparisons of sugar linkages were made by methylation analysis. The conclusion was that the Sindbis carbohydrate is similar to a portion of the host carbohydrate. Thus, the Sindbis carbohydrate structures appear to be structures normally made in the uninfected host cell, but which are added to the Sindbis glycoproteins in virus-infected cells.

Key words: **Sindbis, glycoproteins, cell surface**

Considerable progress has been made in the past few years toward elucidating the structure of the carbohydrate portion of glycoproteins, principally soluble glycoproteins (1, 2). Of the membrane glycoproteins studied to date, most have carbohydrate structures similar to carbohydrate structures found on soluble glycoproteins (1, 2). The most common type of carbohydrate found on glycoproteins are oligosaccharides which are attached to the polypeptide chain by an N-glycosidic linkage to an asparagine residue (1). This type of oligosaccharide can be subdivided into 2 distinct groups (1). The first contains only glucosamine and mannose and is usually rich in mannose. The second is more complex, containing galactose, fucose, and sialic acid as well as glucosamine and mannose. Within each group most oligosaccharides have many structural features in common, with some differences observed between the oligosaccharides of various glycoproteins. One difficulty

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in assessing the importance of the gross similarities or subtle differences in the oligosaccharides is that the glycoproteins which have been examined are not only from diverse sources, but also have different amino acid sequences. Thus, the determinants of the observed similarities and differences may be the alteration in the polypeptide sequences or in the enzymes which glycosylate them. This problem, when combined with the natural heterogeneity of the carbohydrate portion of glycoproteins (1), has made it difficult to draw conclusions about the importance of differences or similarities which have been observed.

To circumvent this problem, we have attempted 2 different approaches. The first is to examine the structure of the carbohydrate found on a particular polypeptide sequence when it is glycosylated in 2 different cells. The second, which is the focus of this paper, is to examine the structure of the carbohydrate found on different polypeptide sequences glycosylated within the same cell.

The experimental system we have chosen for the first approach is to grow Sindbis virus in cultured cells from 2 different organisms and then to examine the carbohydrate portion of the Sindbis virus glycoproteins. Sindbis virus is a small lipid-enveloped virus which contains only 3 polypeptides: 1 nonglycosylated capsid protein (designated C) and 2 glycosylated envelope proteins (designated E1 and E2). The genome of Sindbis is a single-stranded RNA of 4×10^6 daltons, with coding capacity for a maximum of 400,000 daltons of protein. At least 75–80% of the coding capacity is utilized in coding for the viral structural proteins and the polymerase enzymes necessary to make new viral RNA. This leaves little or no viral information to code for enzymes involved in adding carbohydrate to the viral glycoproteins. Based on this argument, it has been postulated that the envelope glycoproteins of Sindbis virus are glycosylated by host-specific enzymes (3–5).

Strauss et al. (4) and Burge and Strauss (5) have examined the glycoproteins of Sindbis virus grown in chicken embryo cells and baby hamster kidney (BHK) fibroblasts. They observed that Sindbis virus grown in BHK cells contains more sialic acid than virus grown in chick cells (4), but in other respects the carbohydrate from virus grown in the 2 hosts was basically similar (5). We have extended their original observations and have examined the carbohydrate of each of the glycoproteins separately (6). Both glycoproteins contain more galactose and sialic acid when virus is grown in BHK cells (6). In addition, glycoprotein E1 from virus grown in BHK cells is deficient in a mannose-rich oligosaccharide that is present when virus is grown in chick cells (6). In other respects, the carbohydrate added by the 2 hosts appears identical.

More detailed structural studies indicate that glycoprotein E2 contains 2 distinct oligosaccharides (7). One glycopeptide, a mannose-rich structure, is indistinguishable from virus grown in either host (7). A second glycopeptide, similar to the complex glycopeptide of IgG (1), is also identical for virus grown in both hosts (7). However, this latter glycopeptide demonstrates microheterogeneity in the extent of sialylation, having forms which contain 0, 1, or 2 sialic acid residues (6). When virus is grown in BHK cells a larger proportion of this glycopeptide consists of the structure containing 2 sialic acid residues (6). Other than this difference in the relative amounts of the structures differing in the amount of sialic acid, the Sindbis glycoprotein E2 has exactly the same carbohydrate structure regardless of the host in which the virus was grown (7).

This finding can be interpreted in at least 2 ways. The first interpretation is that glycosylation mechanisms have been sufficiently conserved during evolution so that these 2 widely divergent cells glycosylate the same polypeptide sequence in the same way. Alternatively, it is possible that Sindbis virus in some way alters the host glycosylation

mechanisms so that Sindbis-specific oligosaccharides are added to the viral polypeptide. In an attempt to distinguish between these 2 possibilities we have compared Sindbis glycopeptides with glycopeptides from uninfected host cells. If the first assumption is correct, and Sindbis is simply picking up oligosaccharide structures normally made in the host, then uninfected cells should contain some structures identical to the Sindbis glycopeptides. The results of the comparisons are presented here.

MATERIALS AND METHODS

Growth of Cells and Virus

Primary chick cells were prepared from 10-day-old chicken embryos (8). The cells were transfered 1 time before using them for the growth of Sindbis. Chick cells and BHK 21/13 cells were maintained as described previously (6). Sindbis virus was grown and purified as described previously (6, 8).

Preparation of Trypsinate From Uninfected Chick Cells

A confluent monolayer of secondary chick cells was washed 5 times with phosphate-buffered saline. The cells were then treated with a trypsin solution (crystalline trypsin, obtained from Worthington Biochemicals Corporation, 0.1 mg/ml in phosphate-buffered saline) for 15 min at 37°C followed by the addition of an excess of soybean trypsin inhibitor. The cells, which were removed from the plate, were collected by centrifugation at 1,000 × g for 5 min. The supernatant solution containing the solubilized cell surface molecules was then subjected to a second centrifugation at 40,000 × g for 30 min to remove additional particulate material. The supernatant solution from this second centrifugation was designated as trypsinate.

Preparation of Glycopeptides and Analysis on BioGel P-6

Proteolytic digestion of glycoproteins was carried out using the protease mixture from Streptomyces griseus as described previously (8). The glycopeptides were fractionated using gel filtration on BioGel P-6 and fractions analyzed by liquid scintillation counting as described previously (3, 8).

Methylation Analysis of Glycopeptides

Complete methylation of glycopeptides was achieved using the procedure described by Hakomari (9). The methylation reactions were carried out and the methylated products were purified on an LH-20 column as described previously (10). The methylated glycopeptides were hydrolyzed under nitrogen atmosphere using 2 N trifluoroacetic acid (TFA) at 121°C for 2 h. The alditol acetate derivatives were prepared as described previously (6). The resulting partially methylated alditol acetates were separated on a Hewlett-Packard 5710A gas chromatograph using a glass column packed with either 1% OV-225 on Gas Chrom Q or 3% ECNSS-M on Gas Chrom Q (both from Applied Science Laboratories, State College, Pennsylvania). Quantitation was achieved by integration of peak areas with a Hewlett-Packard 3370-B integrator. The derivatives were identified using combined gas chromatography-mass spectrometry as described by Bjorndal et al. (11). The combined gas chromatography-mass spectrometry was carried out on a Hewlett-Packard system; a 5710A gas chromatograph was interfaced to a 5982 mass spectrometer. Spectra were recorded every 10 sec and stored in a 5933 A data system for later retrieval.

The sugar linkages were deduced from the observed derivatives assuming the pyranose ring form for all sugars. Thus the derivative 2,3,4,6-tetramethyl-1,5-diacetylmannitol is assumed to arise from terminal mannose.

Molar response factors were assumed to be equal for all of the neutral sugar derivatives (11). This was clearly not correct for the amino sugar derivative; therefore, no attempt was made to quantitate the derivatives of glucosaminitol (12).

RESULTS

Previous results had shown that the Sindbis glycopeptides could be separated into 4 distinct size classes using gel filtration on BioGel P-6 (2). In order to determine whether uninfected host cells had glycopeptides of the same size as Sindbis, gel filtration was used to compare the cell glycopeptides with the Sindbis glycopeptides. Subconfluent monolayers of secondary chick cells were labeled for 24 h with [^{14}C]glucosamine. Trypsin solubilized surface components were then prepared as described in Materials and Methods. An aliquot of the [^{14}C]glucosamine-labeled trypsinate was mixed with an aliquot of [^{3}H]glucosamine-labeled Sindbis virus grown in secondary chick cells. This mixture was digested exhaustively with pronase and one half of the digested mixture was analyzed by gel filtration as shown in Fig. 1. The remaining half of the mixture was heated in a boiling water bath for 5 min to inactivate the pronase, adjusted to pH 5.2 with citric acid, and then treated with neuraminidase for 2 h at 37°C as described previously (3). The neuraminidase-treated glycopeptides were then analyzed by gel filgration on BioGel P-6 as shown in Fig. 2. Experiments in which the isotopic labels were reversed gave results identical to those shown in Fig. 1 and 2.

The Sindbis glycopeptides give rise to 4 peaks designated S1 to S4 (8). The uninfected host trypsinate gives rise to a heterogeneous mixture of glycopeptides, including a large peak at the column void volume and a peak which elutes at the position of authentic glucosamine. It is important to note however, that included in this mixture of glycopeptides are species of the size of the Sindbis glycopeptides. When the mixture of glycopeptides is treated with neuraminidase, the pattern of the Sindbis glycopeptides simplifies to only 2 peaks, S3 and S4, as demonstrated previously (3). At the same time, the heterogeneity of trypsinate glycopeptides is greatly reduced. There is still a peak comigrating with glucosamine and a new peak of radioactivity which moves at the position of authentic sialic acid. About two thirds of the remaining radioactivity elutes as 3 poorly resolved peaks, one eluting considerably before S3, one approximately the same as S3, and one approximately with S4. Here the conclusion seems clear that at least a portion of the cell surface glycopeptides from unifected host cells are similar in size to the glycopeptides of Sindbis virus.

Further support for the contention that the Sindbis glycopeptides are structures found in unifected host cells was sought from chemical analysis of the glycopeptides from the 2 sources. Previous studies had shown that the Sindbis glycoproteins contained the sugars normally found in glycoproteins, i.e., glucosamine, mannose, galactose, fucose, and sialic acid (1, 2, 8). Since these sugars are so widely distributed in glycoproteins this information would be of little assistance in supporting the hypothesis that Sindbis was not altering host glycosylation. Therefore it was decided to examine the sugar linkages found in Sindbis and host cell glycopeptides. This procedure was used to ascertain if any new linkages were present in the Sindbis glycopeptides or whether all the linkages found in Sindbis were also present in glycopeptides of uninfected cells.

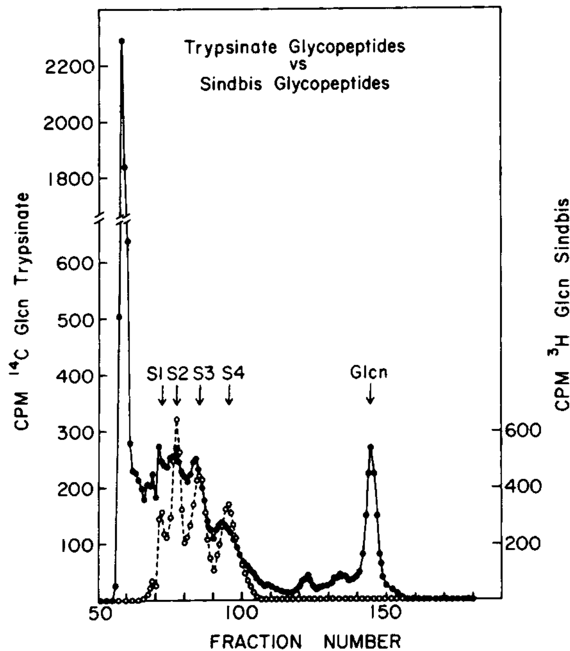

Fig. 1. Comparison of the glycopeptides from Sindbis virus with those from uninfected chick cell typsinate. [^{3}H]Glucosamine-labeled Sindbis virus (o --- o) was mixed with [^{14}C]glucosamine-labeled trypsinate (•—•) from uninfected chick cells. This mixture was digested exhaustively with protease and the resultant glycopeptides applied to a BioGel P-6 column (1.0 × 110 cm). The column was eluted with 0.1 Tris, pH 8, collecting 0.5-ml fractions. The amount of radioactivity in each fraction was determined by liquid scintillation counting.

To obtain adequate quantities of viral glycopeptides, 25 mg of purified Sindbis virus was mixed with purified [^{3}H]glucosamine-labeled Sindbis and subjected to exhaustive proteolytic digestion. The radiolabel was used to locate the glycopeptide containing fractions in the subsequent steps described below. The glycopeptides were separated from degraded peptides and amino acids by gel filtration on BioGel P-2. The glycopeptides, which eluted in the column void volume, were pooled, concentrated, and resubjected to exhaustive proteolytic digestion. The glycopeptide fraction was again purified and desalted by gel filtration on BioGel P-2 using the column eluent. The resulting mixture of Sindbis glycopeptides was subjected to methylation analysis as described in Materials and Methods. Glycopeptides were prepared and analyzed from Sindbis grown either in chick cells or BHK cells. The results of these analyses are shown in Table I.

Glycopeptides were prepared from trypsinate of uninfected secondary chick cells in an analogous manner. Specifically, trypsinate was prepared from 16 roller bottles of secondary chick cells using the procedure described in Materials and Methods. This trypsinate was mixed with [^{3}H]glucosamine-labeled trypsinate prepared separately and a glycopeptide fraction was prepared exactly as described for the Sindbis glycoprotein. The results of a methylation analysis of the trypsinate glycopeptides are also shown in Table I.

Terminal fucose is present in all 3 preparations of glycopeptide, but becasue it is present in small quantities it was difficult to accurately quantitate the levels of this derivative. Mannose is present in only 3 different linkages in the Sindbis glycopeptides. In the

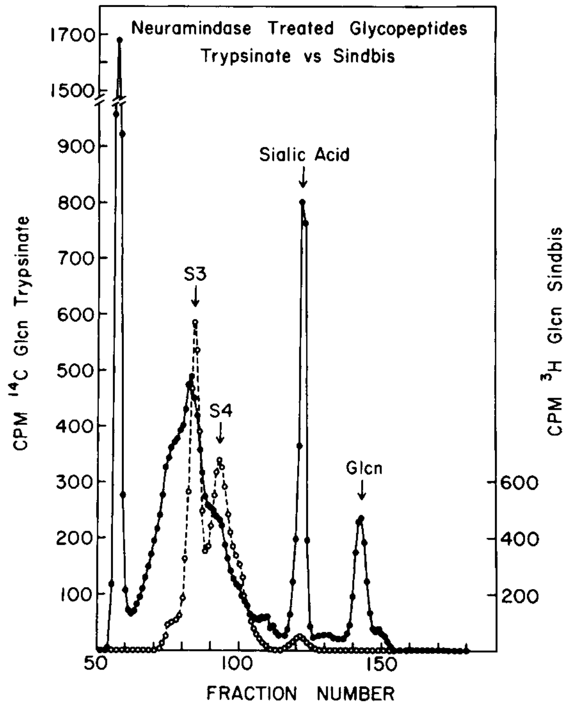

Fig. 2. Comparison of neuraminidase treated glycopeptides from Sindbis virus with those from uninfected chick cell trypsinate. [^{3}H]Glucosamine-labeled Sindbis virus (o - - - o) was mixed with [^{14}C]-glucosamine-labeled trypsinate (•—•) from uninfected chick cells. This mixture was digested exhaustively with protease, followed by boiling, then treatment with neuraminidase. The desialylated glycopeptides were analyzed on a BioGel P-6 column as described in Fig. 1.

cell surface glycopeptides, these same 3 linkages account for most of the mannose; however, there is, in addition, a small amount of 2,4-linked mannose. Other mannose derivatives, if present, represent considerably less than 10% of the total mannose in the glycopeptides.

Galactose is found in only 2 different linkages in the Sindbis glycopeptides. The cell surface glycopeptides contain these same 2 linkages in addition to 6-linked galactose. No branched galactose residues were found. Glucosamine was found only in the single linkage shown in Table I. No attempt was made to quantitate this derivative due to the lack of a molar response factor as discussed in Materials and Methods. The derivative corresponding to 4-linked glucosamine was a major peak in the chromatogram. Peaks one tenth as large could have been detected. Thus one can conclude that any other derivatives of glucosamine must have constituted a minor component of the total glucosamine present in the glycopeptides.

DISCUSSION

The data presented here suggest that the oligosaccharides added to the Sindbis glycoproteins by both chick cells and BHK are not unique, Sindbis-specific structures but rather are similar or identical to some of the structures found in uninfected host cells. The uninfected host cell has additional oligosaccharide structures which are not added to the Sindbis glycoprotein. Thus, the Sindbis glycoproteins act not only as an accurate probe of

TABLE I. Molar Ratios of Sugar Linkages

Deduced linkage	Sindbis-BHK	Sindbis-CEC	Uninfected chick trypsinate
Terminal Fucose	+	+	+
Terminal mannose	2.6	3.6	2.0
2-Linked mannose	6.7	7.0	7.6
3,6-Linked mannose	4.0	4.0	4.0
2,4-Linked mannose	0	0	+
Terminal galactose	2.1	1.4	1.8
3-Linked galactose	3.7	3.1	4.8
6-Linked galactose	0	0	1.7
4-Linked glucosamine	+	+	+

host glycosylation and reflect structures normally made in the host, but they also are selective probes and reflect only a portion of the structures made in the host.

These conclusions are supported by both types of evidence presented here. The comparison of glycopeptides by gel filtration shows that uninfected cells contain glycopeptides of the same size as the Sindbis glycopeptides. The host cell surface glycopeptides consists of a heterogeneous mixture of glycopeptides ranging from those as small as Sindbis glycopeptide S4 to structures considerably larger than glycopeptide S1. Experiments using galactose and fucose to label cell surface glycopeptides show that the peak at the void volume of the column labels poorly with these sugars. This suggests that the peak at the void volume includes some glycopeptides but consists mainly of glucosamine-rich materials such as glycosaminoglycans.

Sindbis glycopeptide S4 has previously been shown to be rich in mannose (6, 8), with a structure typical of the high mannose N-glycosidically-linked glycopeptides (1, 7). The uninfected host glycopeptides include structures of the same size as S4 (Fig. 1). Sindbis glycopeptides S1, S2, and S3 have been shown to have a more complex carbohydrate composition (6, 8), typical of the complex N-glycosidically-linked glycopeptides (1). Glycopeptides S1, S2, and S3 differ principally in the degree of sialylation, containing 2, 1, and 0 residues of sialic acid, respectively (6, 7). This is consistent with the observation that neuraminadase treatment converts glycopeptides S1 and S2 to species which coelute with glycopeptide S3 (Ref. 3; Fig. 2). The heterogeneity of the host surface glycopeptides is also greatly reduced by treatment with neuraminadase. The resulting broad peak of desialyted glycopeptides probably consists of a mixture of a number of different structures. Included in this mixture are structures the same size as Sindbis glycopeptides S3 and S4.

While it is necessary to show that the uninfected host cells have glycopeptides the same size as the Sindbis glycopeptides, further evidence is necessary to support the hypothesis that the structures are the same. The data in Table I demonstrate that all of the sugar linkages present in the Sindbis glycopeptides are also present in glycopeptides of uninfected cells. The similarity of the linkages found is quite striking. Uninfected chick cell glycopeptides contain all of the linkages found in the Sindbis glycopeptides. In addition, the chick cell glycopeptides contain at least 2 linkages not found in the Sindbis glycopeptides. Again these data support the hypothesis that Sindbis is acting as an accurate but selective probe of host glycosylation mechanisms. Final proof of this hypothesis will

require the demonstration that a host glycoprotein contains glycopeptides identical to the Sindbis glycopeptides. We are currently examining this possibility.

The data presented here support, but do not prove, the original assumption that Sindbis is glycosylated by host specified enzymes. This conclusion, combined with the observations that Sindbis protein E2 is glycosylated nearly identically in 2 different hosts (7), suggests that the glycosylation mechanisms are highly conserved between these 2 hosts. While this conclusion is currently limited to only 2 cells, chick cells and BHK cells, preliminary results using other cells suggest that it may be more general. This apparent conservation of glycosylation mechanisms has broad implications for understanding the structure, biosynthesis, and function of the N-glycosidically-linked carbohydrates found on glycoproteins.

The observation described earlier that the host cell makes structures which are not utilized to glycosylate the Sindbis proteins raises the interesting question of how many different N-glycosidically-linked glycopeptide structures are made in a cell. One approach to answering this question is to examine different polypeptide sequences glycosylated in the same cell. This can be accomplished by growing different viruses in the same host cell and analyzing the carbohydrate added to the different viral glycoproteins by the host cell. This approach has been taken by Burge and Huang (13) and Sefton (14). The data presented here also give some indication of the diversity encountered within one cell. There seems to be a complex mixture of glycopeptides in chick cells as analyzed by gel filtration (Fig. 1). This pattern is greatly simplified if the glycopeptides are treated with neuraminidase. This suggests that a considerable portion of the diversity is caused by heterogeneity in the amount of sialic acid, as in the case with the Sindbis glycopeptides (Fig. 2). The function of this heterogeneity, if any, remains unclear; however, in some systems the absence of sialic acid has been shown to have important consequences (15, 16).

The diversity of sugar linkages found in the cell glycopeptides is surprisingly small, with most of the sugars existing in only 1 or 2 different linkages. This observation, combined with the recent observations of Muramatsu et al. (17, 18), provides evidence that there are a relatively limited number of N-glycosidically-linked glycopeptide structures. Muramatsu et al. (17, 18) demonstrated that most of the mannose-labeled (N-glycosidically-linked) glycopeptides from human fibroblasts were hydrolyzed by either endoglucosaminidase D or endoglucosamidase H. They interpret this to mean that most of the glycopeptides are similar in structure to thyroglobulin Unit A (Sindbis S4) or thryglobulin Unit B (Sindbis S1, S2, and S3) glycopeptides. Further experiments will be necessary to define exactly how many different types of N-glycosidically-linked oligosaccharides are made within a particular cell.

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