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IDENTIFICATION OF GLYCOSPHINGOLIPID-GLYCOPROTEIN HYBRIDS IN GASTRIC EPITHELIUM

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SUMMARY - A glycolipid fraction, obtained from dog gastric epithelium by detergent solubilization, was found to contain the glycoconjugates with features common to both glycosphingolipids and glycoproteins. Three such compounds have been purified to homogeneity and their composition and structural characteristics determined. The purified glycosphingolipid-glycoprotein hybrids had a molecular weight in the range of 14,000 and contained 88.2 - 91.1% carbohydrate, 3.1 - 4.5% protein, 2.0 - 2.4% sphingosine, and 1.1 - 1.9% fatty acids. The oxidation of the olefinic bond of sphingosine followed by β-elimination reaction led to the release from each compound of glucose and mannose containing oligosaccharides. Deglycosylation with trifluoromethanesulfonic acid exposed the core regions of the hybrid molecules which were found to consist of sphingosine, glucose, N-acetylglucosamine and amino acids. The data suggest that glycosphingolipid-glycoprotein hybrid molecules are held together by amide linkages between the sphingosine and aspartic or glutamic acid.

INTRODUCTION - In the cell membranes the glycosphingolipids may be classified into those surrounding functional membrane proteins (1-3), those which serve as receptors and cell surface markers (4-8), and those constituting the components of the membrane matrix (9,10). The most actively investigated are the glycosphingolipids which serve as membrane receptors and cell surface markers. Implication of these glycolipids in number of functions that occur at the cell surface such as contact inhibition, intercellular adhesion, immunological specificity and hormone receptors, overshadowed the importance of glycosphingolipids as constituents of membrane matrix. The studies on membrane matrix constituents are further hindered by technical difficulties mainly due to lack of methodology allowing to disassemble rigidly associated molecules while preserving their intergrity.

Previously, we have shown that the membrane matrix glycosphingolipids can be released from their native inaccessible environment in the form of complexes

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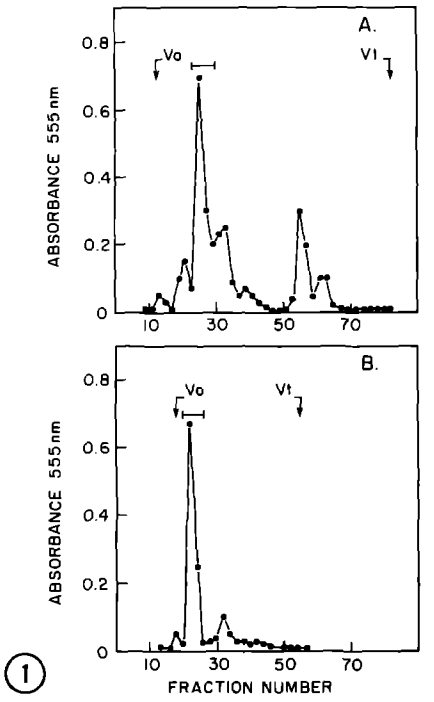
with glycoproteins (11,12). Here, we report some of the chemical characteristics of these glycosphingolipid-glycoprotein hybrid molecules and the type of linkage between them.

MATERIALS AND METHODS - Dog stomachs, dissected 5h prior to experiment and shipped in crushed ice, were obtained from Rockland Co. (Gilbertsville, PA). Ribonuclease A (RN-ase, grade I), deoxyribonuclease I (DN-ase I, grade I) and protease (pronase from Streptomyces griseus) were purchased from Sigma Chem. Co. (St. Louis, MO). The protein assay kit, Bio-Gel P-60, P-30, P-10 and reagents for gel electrophoresis were supplied by Bio-Rad (Richmond, CA). Silica gel HPK plates were from Whatman (Clifton, NJ). All other reagents and solvents were of analytical grade.

The glycosphingolipid-glycoprotein complexes were prepared from dog gastric mucosa as described previously (12). Briefly, the preparation of gastric epithelium was subjected consecutively to RNase, DNase and pronase digestion, and then to β-elimination reaction. The resulting glycosphingolipid enriched fraction was extracted with chloroform/methanol (2:1, 1:1, v/v), and partitioned with water. The aqueous portion of the system was adjusted to 1% with Zwittergent-14, and after 24h solubilization was subjected to Bio-Gel P-60 column chromatography. The eluates were monitored for protein, carbohydrate and sphingosine (13-15). Fractions containing the glycosphingolipids were pooled, dialyzed and lyophilized. The lyophilized material, following solubilization was then chromatographed on Bio-Gel P-30 and P-10 columns. The resulting glycoconjugate fraction was peracetylated and subjected to chromatography on thin-layer plates (12).

Gel electrophoresis of the purified glycoconjugate fraction was performed in 1% SDS with 7% polyacrylamide gel (16). Deglycosylation of the isolated glycosphingolipid-glycoprotein complexes was performed with trifluoromethanesulfonic acid at 0°C for 2h (17). The deglycosylated material was recovered by dialysis and purified by thin-layer chromatography in chloroform/methanol/water (65:35:8, v/v/v). Following recovery, the deglycosylated samples were analyzed for the content and composition of sphingosine, fatty acids, carbohydrate, and amino acids (14,15,18,19). The separation of the oligosaccharides, obtained after oxidation of the olefinic bond of sphingosine and β-elimination reaction (20), was performed on thin-layer plates developed in 1-propanol/water/acetic acid (3:3:1 and 3:2:2, v/v/v).

RESULTS - Application of the detergent solubilized glycoconjugate fraction to a Bio-Gel P-60 column produced several fractions, one of which contained the glycosphingolipid-glycoprotein complexes (12). The chromatography of this material on Bio-Gel P-30 and P-10 columns is shown in Figure 1. The bulk of glycoconjugate material, eluted from P-30 column in the included volume (Fig. 1A), gave on P-10 column a well defined peak which emerged just after the void volume (Fig. 1B). On SDS-polyacrylamide gel electrophoresis, such obtained material gave a single band with an apparent m.w. of 14,000 which stained with both periodate-Schiff and Coomassie blue reagents (Fig. 2). Following peracetylation, however, this material resolved into three distinct



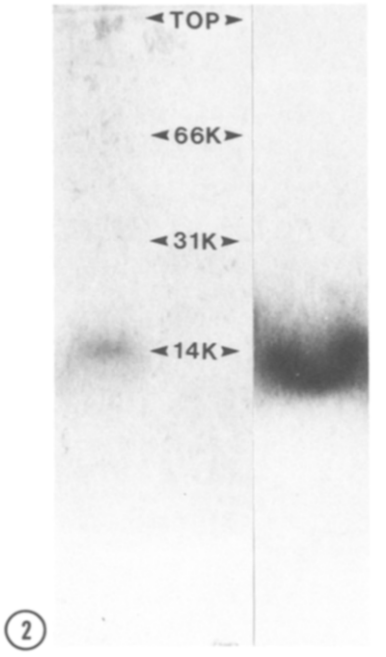


Fig. 1. Gel filtration chromatography of glycospingolipid-glycoprotein hybrids from dog gastric mucosa on (A) Bio-Gel P-30 (1.2 x 154cm) and (B) Bio-Gel P-10 (0.9 x 159cm) columns. The glycosphingolipid-glycoprotein complex fraction from P-60 column was suspended in 0.5M NaCl - 0.1% Zwittergent--14 and chromatgraphed on Bio-Gel P-30 in the above solvent. The material pooled from the fractions as indicated was then chromatographed on Bio-Gel P-10 equilibrated and eluted with 0.5M NaCl - 0.1% Zwittergent-14. The elution of glycosphingolipid-glycoprotein complexes was monitored for carbohydrate with periodic acid/Schiff reaction (24). The material eluted from P-10 column was pooled as indicated (panel B) and used in this study.

Fig. 2. SDS-polyacrylamide gel electrophoresis of the glycosphingolipid-glycoprotein hybrid fraction recovered from Bio-Gel P-10 column (Fig. 1B). The samples containing 4µg protein were incubated with 1% SDS and ß-mercaptoethanol (16), and subjected to electrophoresis in 7% polyacrylamide gel. The gel on the left was stained for protein with Coomassie blue and the one on the right was stained for carbohydrate with periodic acid/Schiff reagent. The arrowheads indicate position of the molecular weight markers.

components on thin-layer chromatography. The thin-layer chromatogram of the isolated glycosphingolipid-glycoprotein hybrids is shown in Figure 3.

The purified fractions exhibited similar chemical composition and contained 88.2 - 91.1% carbohydrate, 3.1 - 4.5% protein, 2.0 - 2.4% sphingosine, and 1.1 - 1.9% fatty acids. The carbohydrate analysis revealed for each fraction a high content N-acetylglucosamine, mannose and galactose. The glucose was present in equimolar proportions with sphingosine. The molar ratios of carbohydrates found in the hydrolyzates of separated glycoconjugate hybrids

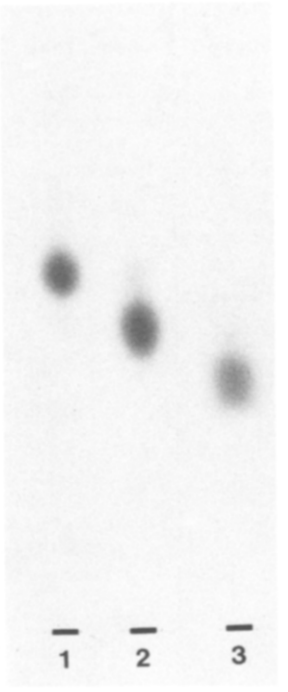


Fig. 3. Thin layer chromatography of the peracetylated glycosphingolipid-glycoprotein hybrids purified from the fraction recovered from P-10 column. The plate was developed in 1-propanol/water (7:3, v/v) and the glycosphingolipid--glycoprotein hybrids were visualized with orcinol spray.

are given in Table I. Oxidation of the olefinic bond of sphingosine followed by β-elimination reaction resulted, from each fraction, in the liberation of two major types of oligosaccharides one enriched in mannose and N-acetylglucosamine (Table II, oligosaccharides Ib, IIb, IIIb), and the other containing glucose and N-acetylgalactosamine (Table II, oligosaccharides Ia, IIa, IIIa).

Table I. The composition and molar ratios of carbohydrates in the glycosphingolipid-glycoprotein hybrids purified from dog gastric epithelium

Compound	Molar ratios						
	Fuc	Man	Gal	Glc	GlcNAc	GalNAc	Sia
I	4.91	13.90	22.20	1.00	14.40	4.08	0.89
II	4.75	6.74	23.65	1.00	11.80	2.88	1.31
III	7.30	12.11	17.58	1.00	8.71	4.81	1.17

The molar ratios are expressed relative to glucose.

Table II. The composition and molar ratios of carbohydrates in the oligosaccharides released from glycosphingolipid-glycoprotein hybrids by the oxidation of the sphingosine olefinic bond followed by β-elimination reaction

Oligosaccharide	Molar ratios						
	Fuc	Man	Gal	Glc	GlcNAc	GalNAc	Sia
Ia	3.11	-	7.09	1.00	0.40	3.87	-
Ib	1.00	6.12	6.82	-	6.29	-	0.87
IIa	2.71	-	8.73	1.00	-	3.05	-
IIb	1.00	2.91	6.95	-	5.22	-	1.09
IIIa	6.10	-	10.04	1.00	-	4.53	-
IIIb	1.00	8.80	5.16	-	6.94	-	1.17

The oligosaccharides Ia, Ib, IIa, IIb, IIIa and IIIb were obtained from I, II and III glycosphingolipid-glycoprotein hybrids, respectively.

Examination of the products of the glycoconjugate hybrids treated with trifluoromethanesulfonic acid revealed the glycolipid-like properties of the deglycosylated material. The deglycosylated material was readily soluble in organic solvents, and stained for carbohydrate and amino acids on thin-layer chromatography. Chemical analyses indicated that the deglycosylated core of each studied compound contained sphingosine, glucose, mannose, N-acetylglucosamine and amino acids. The amino acids, separated on thin-layer plates in the form of dabsyl derivatives, were identified as aspartic acid, glutamic acid, serine, threonine and lysine. These data indicated that the investigated compounds contained constituents which are characteristic to both glycosphingolipids and glycoproteins.

DISCUSSION - The studies on the interaction between glycosphingolipids and membrane bound glycoproteins are difficult to perform in situ, and therefore most of the research is conducted on simple models (21). Yet, from the functional point of view it is important to determine the exact structure of membrane matrix and to characterize the interacting molecules. The physicochemical investigations confirm the hydrogen bonding between glycosphingolipids and proteins (9,22), and the existence of such glycosphingolipid-protein complexes have been described (23). Complexes of this type, however, are easily

separable by column chromatography (23). From the results presented in this communication, it appears that some of the glycosphingolipids are also involved in the interaction with other molecules stronger than that imposed by hydrogen bonding. The glycosphingolipid-glycoprotein hybrids described here were inseparable by column chromatography, SDS-gel electrophoresis or by thin layer chromatography. These compounds were resistant to β-elimination reaction, unless the olefinic bond of sphingosine was subjected to oxidation. Upon this treatment, two major types of oligosaccharides were generated. One type of oligosaccharides contained glucose, an indicator of glycosphingolipid derivation and the other type consisting of mannose and N-acetylglucosamine which are characteristic components of N-glycosidic type glycoproteins. This indicated that glycosphingolipid and glycoprotein components of the hybrid molecules were held together by covalent bonds, but not by ester or O-glycosidic type. The data on the composition of the core regions of these compounds strongly suggest that the linkage between the glycoprotein and glycosphingolipid components was of amide type, and most likely involved an amino group of sphingosine and carboxyl group of acidic amino acid. In the light of the above findings, it appears that the core region of each of the isolated hybrids consists of Glc-Sphingosine-NH-OC-Peptide-GlcNAc.

Since amino acid analysis indicated the presence of two acidic amino acids (glutamic and aspartic acid), it may be that either of the two is involved in an amide linkage with sphingosine, or perhaps that aspartic acid is involved in N-glycosidic linkage with N-acetylglucosamine only and glutamic acid is linking the glycopeptide with glycosphingolipid. This, however, remains to be established. Nevertheless, the results of this study provide a clear evidence that in membranes the interaction between glycosphingolipids and glycoproteins is by far more complex than simple nonionic or hydrogen type. Knowledge of the type of interactions occurring in the membranes may ultimately aid in our understanding how the proteins, glycoproteins and lipids are anchored within the membrane network.

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