GLYCOPEPTIDES FROM PLASMA MEMBRANES AND MICROSOMES OF RAT LIVER*

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Received 17 August 1970
Revised version received 14 September 1970

1. Introduction

Previous work in this laboratory [1] has demonstrated that the structural protein fraction prepared from rat liver microsomes consists of glycoproteins with 1.5% of the carbohydrate moieties made up of mannose, galactose, glucose, hexosamine and sialic acid. For chemical characterization of the carbohydrate moieties we attempted to isolate glycopeptides by proteolytic digestion. Two types of glycopeptides, i.e. one with acidic and another with neutral oligosaccharides, could be isolated. They apparently resembled those isolated by Li et al. [2] from an unfractionated membranous fraction of microsomes but differed significantly in carbohydrate composition from the latter.

The microsomal structural protein fraction is derived largely from proteins of endoplasmic reticulum and partly from those of plasma membranes, Although they could not be separated from each other due to close similarity in gross chemical and physical properties [1], it seemed interesting to see if each type of oligosaccharide mentioned above might correspond to each membrane structure. There are a few methods available for isolation of plasma membranes with a high degree of purity directly from rat liver homogenate, but these methods can not be used on a large scale. In our experiments, only about 1 mg (as protein) of plasma membranes was usually obtained starting from 10 g wet weight of rat liver, whilst 150–200 mg (as protein) of microsomes could readily be prepared, of which about 20 mg was accounted for by plasma membranes as judged by assaying 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), a marker enzyme for plasma membranes. We, therefore, prepared a certain amount of hepatic plasma membranes with labelled carbohydrate moieties from rats which had received ^{14}C-glucosamine, and digested their structural protein (or glycoprotein) together with the microsomal structural protein fraction. By following radioactivity during the isolation of glycopeptides, we could identify glycopeptides from plasma membranes. Actually, we found that the radioactivity is detected only in the acidic oligosaccharides, indicating that plasma membranes possess acidic oligosaccharides as constituents, whereas neutral oligosaccharides reside only in endoplasmic reticulum (or the closely related subcellular organelles included in the microsomal fraction).

2. Materials and methods

The structural protein fraction of microsomes was prepared from livers of female Wistar rats according to Miyajima et al. [1]. To prepare labelled plasma membranes, 4 μCi of 1-^{14}C-glucosamine (11.5 mCi/mmole) dissolved in 0.3 ml of saline was injected to each rat, and plasma membranes were isolated accord-

* Supported by grants from the Ministry of Education, Japan, and Toyo Rayon Science Foundation.
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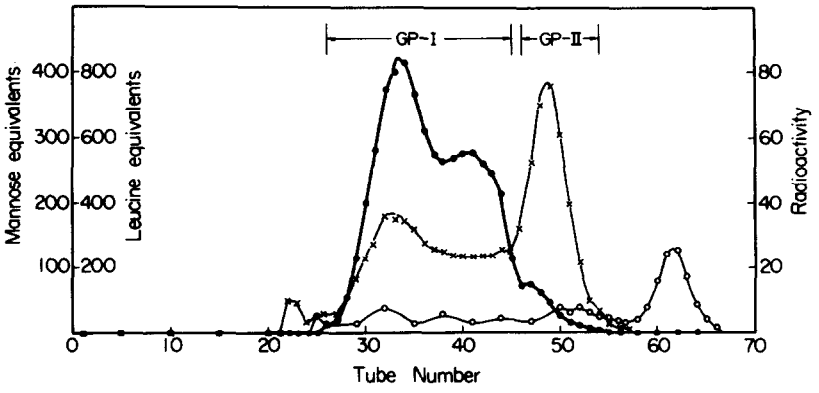

Fig. 1. Gel filtration of the final pronase digest of microsomal *structural protein* mixed with plasma membranes labelled with ^{14}C-glucosamine. The digest (12 mg) was dissolved in 4.8 ml of 0.05 M pyridine–acetic acid buffer of pH 5.0, and applied to a column of Sephadex G-50 (2.0 X 100 cm) equilibrated with the same buffer. Elution was carried out with the same buffer at a flow rate of 15 ml/hr, and 5 ml fractions were collected. Aliquots were used for the orcinol reaction, the ninhydrin reaction and the radioactivity determination. x—x: Mannose equivalents (nmoles/ml). o—o: Leucine equivalents (nmoles/ml). •—•: Radioactivity (dpm/0.3 ml).

ing to Neville [3] 3 hr after the isotope injection when the maximum incorporation had been reached. Specific activity of 5010 dpm per mg protein was obtained with activity located entirely in hexosamine and sialic acid. Contamination with endoplasmic reticulum in the isolated plasma membranes was estimated to be 9–15%, by assaying glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9), a marker enzyme for endoplasmic reticulum.

Neutral sugars were determined by gas-liquid chromatography of the trimethylsilyl ether derivatives of methylglycosides according to Miyajima et al. [1] after hydrolysis in 1 N HCl at 100° for 8 hr. Hexosamines and amino acids were determined in a Hitachi amino acid analyzer after hydrolysis in 6 N HCl at 100° for 16 hr. However, to determine specific radioactivities of glucosamine, the samples were hydrolyzed in 2 N HCl at 100° for 16 hr, and the hydrolysates were treated with resins as described previously [1]. The neutralized eluates were used for the Elson-Morgan reaction [4] and for the determination of radioactivity. Sialic acid was determined according to Warren [5] after hydrolysis in 0.1 N H_2SO_4 at 80° for 1 hr. Specific radioactivities of sialic acid were determined on the eluates of Dowex-1 chromatography of the hydrolysates [6].

Chromatographic fractions were monitored by the ninhydrin reaction according to Yemm and Cocking [7] and by the orcinol reaction according to Hewitt [8].

Paper electrophoresis was carried out in pyridine–acetic acid–water (1:10:89, v/v), pH 3.6, at a potential gradient of 50 V per cm for 1.5 hr. Spots were stained by the chlorination method [9].

3. Results and discussion

The structural proteins were digested with pronase in 0.1 M borate buffer, pH 8.0, containing 0.01 M calcium acetate at 37° by the procedure used to prepare glycopeptides from a plasma glycoprotein [10]. The recovery of carbohydrate in the glycopeptide fractions after three cycles of the digestion was about 80%, but galactosamine and most of glucose contained in the structural protein fraction remained in the undigested materials which were insoluble at the end of the first digestion.

The labelled plasma membranes were treated with phenol in the presence of sodium dodecylsulphate to remove lipids in a manner similar to that applied to whole microsomes [1] prior to being mixed with the

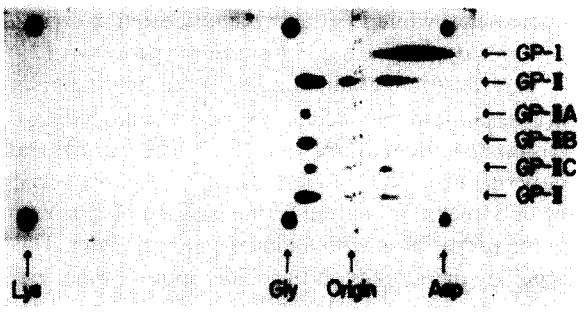

Fig. 2. Paper electrophoresis of the isolated glycopeptides. The conditions are described in the text. Reference amino acids are aspartic acid (Asp), glycine (Gly) and Lysine (Lys). Two different amounts of the sample were used for GP-II.

microsomal structural protein fraction for the pronase digestion.

Fig. 1 shows a gel filtration pattern on a Sephadex G-50 column of the final pronase digest obtained from 8.5 mg (5.6 mg protein) of the labelled plasma membranes mixed with 555 mg of the structural protein fraction. It is clear that GP-I contained radioactivity parallel to the orcinol values whereas GP-II had very little radioactivity. It should be noted that the recovery of radioactivity in the glycopeptides from the plasma membranes was nearly quantitative, only a few per cent being found in the undigested fraction.

Each glycopeptide fraction was passed through a column of Dowex-50 X8 (H^+, 20–50 mesh) to remove carbohydrate-free peptides.

GP-I was acidic, and showed an elongated spot on paper electrophoresis as shown in fig. 2. On DEAE-Sephadex A50 equilibrated with 0.001 M phosphate buffer of pH 7.1, GP-I showed at least 9 peaks on elution with increasing concentration of phosphate buffer up to 0.05 M, the first peak eluting with 0.005 M buffer. Thus, GP-I was apparently composed of a large number of glycopeptides. However, it should be noted that all the peaks had radioactivities nearly in proportion to the orcinol values. For analysis, unfractionated GP-I was used.

GP-II could be fractionated into three peaks (GPs-IIA, IIB and IIC) on DEAE-Sephadex A50 as shown in fig. 3, of which GP-IIC contained substantial radioactivity while other peaks eluting with 0.001 M buffer had very little activity. As shown in fig. 2, GPs-IIA and IIB appeared homogeneous and identical to each other on paper electrophoresis migrating as fast as neutral amino acids. GP-IIC was still heterogeneous revealing three spots, of which one acidic spot migrating as fast as the slower moving part of the elongated spot of GP-I was radioactive.

Amino acid and carbohydrate compositions and specific radioactivities of the isolated glycopeptides are shown in table 1.

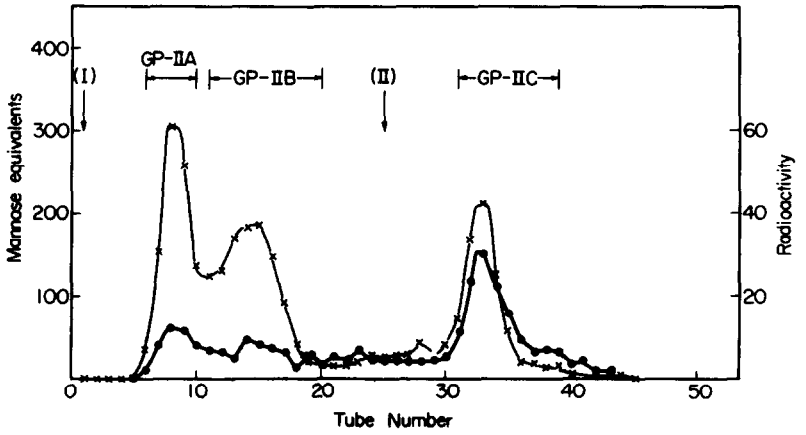

Fig. 3. Chromatography of GP-II on DEAE-Sephadex A50. 2.6 mg of GP-II dissolved in 1.4 ml of 0.001 M phosphate buffer, pH 7.1, was applied to a column of DEAE-Sephadex A50 (0.9 X 16 cm) equilibrated with the same buffer. Elution was carried out initially with the same buffer (I), and then with 0.05 M buffer (II). Flow rate was 4 ml/hr, and 1.4 ml fractions were collected. Aliquots were used for the orcinol reaction and the radioactivity determination. x—x: Mannose equivalents (nmoles/ml). •—•: Radioactivity (dpm/0.5 ml).

Table 1
Amino acid and carbohydrate compositions and radioactivities of the isolated glycopeptides.

	GP-I	GP-IIA	GP-IIB
*Composition**			
Aspartic acid	1.0	1.0	1.0
Threonine	0.38	0.11	0.39
Serine	0.35	0.22	0.56
Glycine	0.25	0.37	0.18
Glutamic acid	0.21		0.17
Proline	0.20		0.15
Alanine	0.12	0.14	0.14
Valine	0.07	0.11	0.10
Glucosamine	3.85	1.85	1.82
Mannose	1.93	5.00	5.37
Galactose	2.60		
Fucose	0.35		
Glucose	0.17	0.50	0.16
Sialic acid	3.12		
Specific radioactivity (dpm per μmole)			
Glucosamine	1660	205	192
Sialic acid	1360		

* Values are expressed as moles per mole of aspartic acid.

GP-I is acidic containing sialic acid, but differs in carbohydrate composition from one of the two glycopeptides (*Glycopeptide I*) isolated by Li et al. [2] from the unfractionated membranous fraction of microsomes from which the structural protein used in our experiments is prepared. In GP-I the molar ratio of mannose/galactose is 0.74 and that of sialic acid/glucosamine 0.81, whereas in *Glycopeptide I* the ratios are 7.2 and 0.23, respectively. GPs-IIA and IIB differ only in amino acid composition and are neutral with no sialic acid, thus resembling another glycopeptide (*Glycopeptide II*) of Li et al. [2], but differing from the latter in that GP-II contains 2 moles of glucosamine and 5 moles of mannose per mole of aspartic acid in contrast to 4 moles and 9 moles, respectively, in *Glycopeptide II*.

The differences between our results and those of Li et al. [2] are probably due to differences in the conditions of the experiments and appear to reflect the difference in the object of study, since we attempted to isolate glycopeptides from structural elements of microsomes while Li et al. were interested in glycopeptides participating in biosynthetic processes [2].

Our most remarkable finding is that plasma membranes contain only acidic oligosaccharides. Glucosamine specific radioactivity of GPs-IIA and IIB was only one eight that of GP-I. These small values could be due to contamination of the isolated plasma membrane preparation with endoplasmic reticulum. The specific radioactivity in the acidic spot on the paper electrophoretogram obtained from GP-IIC was 1210 dpm per μmole glucosamine. Whether this small amount of acidic glycopeptide with somewhat lower specific activity compared to whole GP-I originated from plasma membranes is at present unknown. It would be interesting to see if endoplasmic reticulum contains acidic oligosaccharides in addition to the neutral oligosaccharides.

Acknowledgements

The authors are grateful to Dr. J.E.Scott, Rheumatism Research Unit, Canadian Red Cross Memorial Hospital, England, for his kind help in the preparation of the manuscript.

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