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METHYLATION ANALYSIS OF THE CARBOHYDRATE PORTION OF FIBRONECTIN ISOLATED FROM HUMAN PLASMA

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Summary

Fibronectin was isolated from pooled human plasma by affinity chromatography on Sepharose-coupled gelatin and subjected to methylation analysis. The results indicate that the carbohydrate chains in fibronectin are very similar to those found in other serum glycoproteins, i.e. a common inner core structure consisting of two N-acetylglucosamine and three mannose residues attached to the polypeptide backbone via a N-acetylglucosamine-asparagine linkage. To this inner core are attached two side chains made up of N-acetylglucosamine, galactose and terminated by sialic acid.

Introduction

Fibronectin[a] in human plasma was known for a long time as cold insoluble globulin (1), because of its tendency to coprecipitate with proteins present in the cryoprecipitate (2). This cold insoluble globulin has been shown to be a high molecular weight glycoprotein composed of two polypeptide chains of approximately 250,000 daltons each (3). A surface protein similar to cold insoluble globulin in molecular weight and amino acid and carbohydrate composition (4) was isolated from spent culture medium of normal fibroblasts (3-8) and shown to be immunologically identical to cold insoluble globulin (2). The affinity of fibronectin for collagen has been demonstrated, and was used for a simple purification procedure (9). This paper describes the quantitation by gas chromatography-mass spectrometry after methylation and

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[a]Synonyms: cold insoluble globulin (CIg) for the plasma form, large external transformation sensitive (LETS) protein for the cellular form.

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subsequent conversion to alditol acetates of the carbohydrate structural units of fibronectin isolated from human plasma.

Materials and methods

Electrophoretically and immunologically pure fibronectin was isolated from pooled human plasma by affinity chromatography on Sepharose-coupled gelatin as described earlier (9). Monosaccharide composition was determined by gas chromatography after methanolysis and trifluoroacetylation of the glycoprotein samples by the procedure of Wrann and Todd (10). For methylation analysis, 0.5 mg of sample were methylated by the procedure of Hakomori (11), in which the methylsulfinyl anion was used to generate the polysaccharide alkoxide before the addition of the methyliodide. The methylsulfinyl anion was prepared as described by Sanford and Conrad (12). Acetolysis, reduction and acetylation of the permethylated polysaccharides were performed using the procedures described by Stellner et al (13). Identification of the partially methylated alditol acetates was carried out according to the method of Björndal et al (14) for the neutral sugars and according to the method of Stellner et al (13) for the amino sugar derivatives. Samples were analyzed in a Varian Model 2740 gas chromatograph with a flame ionization detector connected to a Dupont Model 21-492 B double focusing mass spectrometer. The alditol acetates derived from the methylated sugars were separated on a 6 ft column (2 mm inside diameter) of 3% ECNSS-M coated on Gas - Chrom Q (Applied Science Laboratories, Inc., State College Pa.), the oven temperature being programmed at 1^{o}/min from 140-190^{o}. The mass spectra of the carbohydrate derivatives were taken over a mass range of 35 to 600 atomic mass units using an ionizing potential of 70 ev. Scans were taken every 9 seconds and the spectra were processed using a Dupont Model 21-094 disc-based data system. Ovalbumin (Sigma Chem. Co.) and blood group substance H from pig stomach mucosa (15) were used as glycoprotein standards for retention times of some of the partially methylated alditol acetates.

Results

The results of the methylation analysis of fibronectin isolated from human plasma are summarized in Table 1 . The quantity of each carbohydrate derivative was determined as follows. The peak areas of the gas chromatogram of the individual methylated, acetylated derivatives of a particular sugar were totaled, and the fraction of the total for each was determined. The actual amount of each derivative was obtained by multiplying this fraction by the total amount of the respective sugar present in fibronectin, as determined by quantitative carbohydrate analysis as O- or N,O-trifluoroacetyl-methylglycoside (10). The data system used made it possible to obtain mass chromatograms for characteristic mass fragments observed in gas chromatography-mass spectrometry of partially methylated alditol acetates, which is

TABLE 1. STRUCTURAL UNITS OF THE CARBOHYDRATE MOIETY OF FIBRONECTIN.

Carbohydrate residue	Glycosidic linkage	nmoles carbohydrate / mg dry weight
Sialic acid	terminal	40
Galactose	4	52
	6	16
		68
Mannose	2	52
	4	5
	3,6	28
		85
N-Acetylglucosamine	4	93

especially advantageous in the analyses of glycoproteins with low carbohydrate content, where the background in the gas chromatograms is relatively high. In Figure 1 some of the mass chromatograms of a fibronectin sample are presented. The mass chromatogram for m/e 158 (a characteristic primary fragment for N-acetylamino sugar derivatives) shows only a single peak at scan number 319 (Figure 1,B). At the same scan number there is also a peak in the mass chromatogram of m/e 233 (Figure 1,D). The only N-acetylglucosamine derivative yielding m/e 233 is 3,6-di-O-methyl-1,4,5-tri-O-acetyl-2-deoxy-2-(N-methylacetamido)-glucitol (13), corresponding to a 4-linked N-acetylglucosamine residue in the untreated glycoprotein. In fact, an identical retention time and fragmentation pattern were observed for the corresponding standard. Therefore, all the N-acetylglucosamine residues in fibronectin are 4-linked in addition to their glycosidic linkage at C-1. The identification of the hexose derivatives was more complicated. A partially methylated mannitol acetate yields an almost identical fragmentation pattern as compared to the corresponding galactitol derivative. Hence they can be distinguished only by their different retention times. It was necessary to determine the exact retention times for some hexose derivatives using glycoprotein standards, which contain only one of the hexoses present in

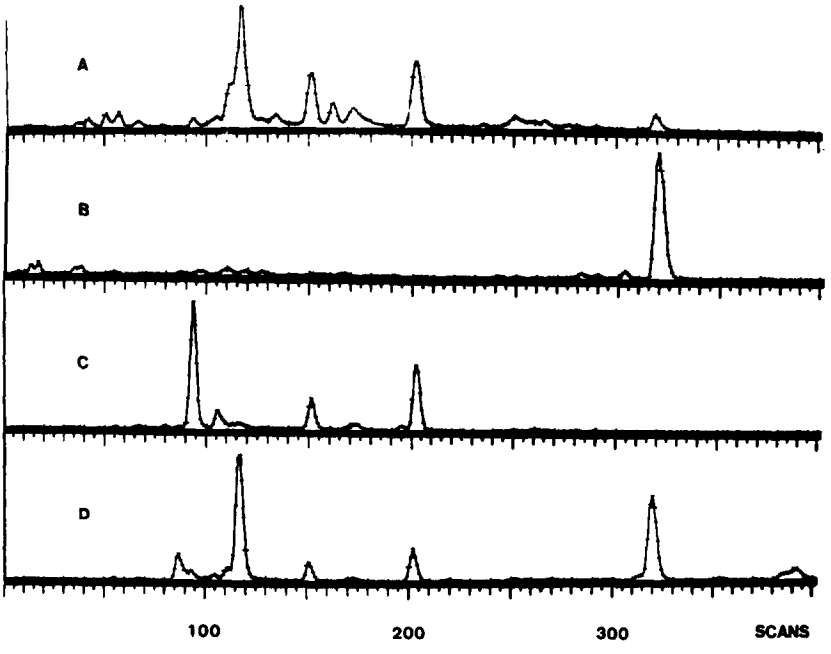

FIGURE 1 : mass chromatograms of m/e 117 (A), m/e 158 (B), m/e 189 (C) and m/e 233 (D) as obtained in gas chromatography-mass spectometry of fibronectin isolated from human plasma.

fibronectin, such as ovalbumin (mannose, no galactose) and blood group substance H (galactose, no mannose). Using these standards hexose residues in fibronectin could be confidently identified as 2-linked, 4-linked and 3,6-linked mannose at scan numbers 93, 110, 202 and 4-linked and 6-linked galactose at scan numbers 116 and 151, respectively (Figures 1,A,C,D). As expected for a glycoprotein with relatively low carbohydrate content (about 5%), the background in the gas chromatograms of the fibronectin samples was relatively high. This resulted in a few additional small peaks, which could not be assigned to any carbohydrate derivative.

Discussion

Structural studies on whole glycoproteins can give only limited information on the structure of their individual carbohydrate chains, because of the frequently observed heterogeneity within the same molecule (16).

Furthermore, underglycosylation during the biosynthesis may result in a large variety of incomplete chains of the same type. In the case of fibronectin isolated from human plasma, however, the small number of structural carbohydrate units found in this study indicate a rather small degree of heterogeneity in the carbohydrate portion of this molecule. After comparing the results of this study with structural studies on other serum glycoproteins with similar carbohydrate compositions, the following structure can be proposed for the majority of the carbohydrate chains of fibronectin.

$$\begin{matrix} \text{NeuNAc(2-4/6)Gal(1-4)GlcNAc(1-2)Man}_{(1-3)} \\ \text{NeuNAc(2-4/6)Gal(1-4)GlcNAc(1-2)Man}_{(1-3)} \end{matrix} \Big\}\text{Man(1-4)GlcNAc(1-4)GlcNAc---AsN}$$

As no N-acetylgalactosamine was found in the quantitative analysis of fibronectin, the carbohydrate linkage in fibronectin should be exclusively N-acetylglucosamine-asparagine. All of the N-acetylglucosamine residues are 4-linked. The only branching sugar found was 3,6-linked mannose. Characteristically, branched mannose residues in asparagine-linked carbohydrate chains of mammalian glycoproteins link only to other mannose residues or to N-acetylglucosamine in order to build the inner core of the chains (17).

Since no terminal galactose, mannose or N-acetylglucosamine could be found in this methylation analysis of human plasma fibronectin, all nonreducing termini of the carbohydrate chains are obviously occupied by sialic acid. This might be caused by the removal of glycoproteins devoid of sialic acid from the circulation by the liver (18). This hypothesis is compatible with our earlier findings that fibronectin isolated from spent culture medium of fibroblasts contains less sialic acid than that from human plasma (4).

Further delineation of the carbohydrate structure of fibronectin will require methylation analysis, enzymatic and partial acid hydrolysis and periodate oxidation of glycopeptides, preferentially of those carrying only a single carbohydrate chain.

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