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Carbohydrate Structure of the Major Glycopeptide From Human Cold-Insoluble Globulin

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Cold-insoluble globulin (CIg) is a member of a group of circulating and cell-associated, high-molecular-weight glycoproteins termed fibronectins. CIg was isolated from human plasma by affinity chromatography on gelatin-Sepharose. SDS-polyacrylamide gel electrophoresis of the purified glycoprotein gave a double band that migrated near myosin. The CIg glycopeptides were released by pronase digestion and isolated by chromatography on Sephadex G-50. Affinity chromatography of the major G-50 peak on Con A-Sepharose resulted in two fractions: one-third of the glycopeptides were unbound and two-thirds were weakly bound (WB). Sugar composition analysis of the unbound glycopeptides by GLC of the trimethylsilyl methyl glycosides gave the following molar ratios: sialic acid, 2.5; galactose, 3.0; N-acetylglucosamine, 4.9; and mannose, 3.0. Sugar composition analysis of the WB glycopeptides gave the following molar ratios: sialic acid, 1.7; galactose, 2.0; N-acetylglucosamine, 4.1; and mannose, 3.0. The WB CIg glycopeptides cochromatographed on Sephadex G-50 with WB transferrin glycopeptides giving an estimated molecular weight of 2,800. After degradation with neuraminidase alone or sequentially with β-galactosidase the CIg and transferrin glycopeptides again cochromatographed. Methylation linkage analysis of the intact and the partially degraded glycopeptides indicated that the carbohydrate structure of the major human CIg glycopeptide resembles that of the major glycopeptide from transferrin.

Key words: **cold-insoluble globulin, carbohydrate structure, human plasma**

Cold-insoluble globulin (CIg) is a component of plasma and a member of a group of high-molecular-weight glycoproteins which are known collectively as fibronectins [1]. The

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Abbreviations used: CIg, cold-insoluble globulin; LETS, large, external, transformation-sensitive; WB, weakly bound; SDS, sodium dodecyl sulfate; Con A, concanavalin A; PAS, periodic acid-Schiff; SFA, surface fibroblast antigen; CSP, cell surface protein.

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cellular forms have been variously referred to as LETS [2], galactoprotein a [3, 4], SFA [5], CSP [6], and Zeta [7], while the circulating form, CIg, has been shown to be very similar to another serum protein, opsonic, α_2 SB glycoprotein [8].

The cell-associated and circulating fibronectins confer, to varying degrees, several adhesive properties to cultured cells. Comparison of the biologic activities of the two forms from chicken and from man have shown that they have identical activities with regard to mediating cell attachment to collagen and cell spreading on culture substrata. However, the cellular glycoproteins are 50 times more reactive in restoring normal morphology to transformed cells and 150 times more reactive in hemagglutination [9].

It appears that the two forms, while sharing many structural similarities, may also exhibit some differences. Attempts to produce a monospecific antiserum which does not cross-react with both forms have thus far not been reported successful [10, 11]. In addition, fibronectins from both sources have been reported to have similar isoelectric points and to produce similar CnBr cleavage products [12]. However, in some gel electrophoresis systems, the plasma and cellular forms have been shown to behave differently. The circulating fibronectins migrate as a doublet, while the cellular glycoproteins form a more slowly migrating, single, diffuse band [9, 13, 14].

It is unknown whether these differences will be accounted for by variations in the amino acid and/or the carbohydrate composition of the cellular and the plasma glycoproteins. In the first part of a study designed to compare the carbohydrate structure of glycopeptides isolated from human plasma and cell-associated fibronectins, we have determined the chemical structure of the major glycopeptide from CIg.

MATERIALS AND METHODS

CIg was isolated from fresh human serum by affinity chromatography according to the method of Engvall and Ruoslahti [15]. Whole blood (600 ml) was obtained and allowed to clot for 1 h at room temperature (25°C). Subsequent steps in the CIg isolation were carried out at 4°C. The blood was centrifuged for 15 min at 1,000g and the resulting serum was fractionated on a gelatin-Sepharose column containing 10 mg gelatin (Knox) per milliliter Sepharose. The column was washed with ten volumes of phosphate-buffered saline, pH 7.2, containing 0.01 M sodium citrate and the bound material was eluted with 200 ml of 1 M sodium iodide, dialyzed overnight against distilled water, and lyophilized. The product was assayed for protein according to the method of Lowry et al [16] and for homogeneity by SDS-polyacrylamide gel electrophoresis on 7.5% gels [17] in the presence of 2-mercaptoethanol with CIg complexed to fibrinogen (A. B. Kabi Company, Stockholm) as a standard. The gels were stained for protein with Coomassie blue [18] or for carbohydrate with PAS [19].

CIg, transferrin (human, Calbiochem), and fetuin (type III, Sigma Chemical Company) were digested with pronase and the solubilized glycopeptides were isolated by chromatography on a 30 × 1.5-cm Sephadex G-50 column. Columns were eluted with 0.1 M pyridine acetate buffer, pH 5.0. Fractions (1 ml) were collected and assayed for hexose by the method of Dubois et al [20]. An aliquot of the CIg, transferrin, and fetuin glycopeptides was N-acetylated with [^{3}H]-acetic anhydride [21], mixed with the corresponding unlabeled glycopeptides and subjected to further fractionation on Con A-Sepharose [22]. The sugar composition of the unbound and WB CIg glycopeptides was determined after methanolysis by gas-liquid chromatography of the trimethylsilyl methyl glycosides [23, 24] on a Perkin-Elmer gas chromatograph, model 3920, equipped with a flame ionization detector and a 1.9-meter × 2-mm column of 3% OV-101 on 100–120 Supelcoport (Supelco., Inc.,

Bellefonte, Pennsylvania). Carrier gas was nitrogen at 45 ml/min. Peaks were integrated with a Spectra-physics "Minigrator." Amino acid analysis of the CIg glycopeptides was performed on a modified Technicon Amino Acid Analyzer by Dr. S. K. Chan, University of Kentucky.

The CIg and transferrin glycopeptides which were weakly bound to Con A-Sepharose were partially degraded with neuraminidase (type V, Sigma Chemical Company) alone or sequentially with a β-galactosidase and β-N-acetylhexosaminidase isolated from jack bean meal [25, 26] (gifts of Dr. Y.-T. Li, Tulane University, New Orleans). The resulting glycopeptides were chromatographed on a 30 × 1.5-cm Sephadex G-50 column. Fractions (1 ml) were collected and assayed for radioactivity. Fractions containing either the intact or partially degraded glycopeptides were pooled and linkage positions were determined by methylation analysis [Björndal et al, 27, 28] as modified by Järnefelt et al [29]. Instrumental conditions for the mass spectrometer were as follows: Finnigan model 3300-6110 with chemical ionization using methane at 1 torr and source temperature of 60°C; transfer lines were kept at 250°C. Other source parameters were as follows: collector, 34.2 V; electron multiplier, 1,950 V; emission current, 60 mA; ionizing electron energy, 150 eV; ion energy,

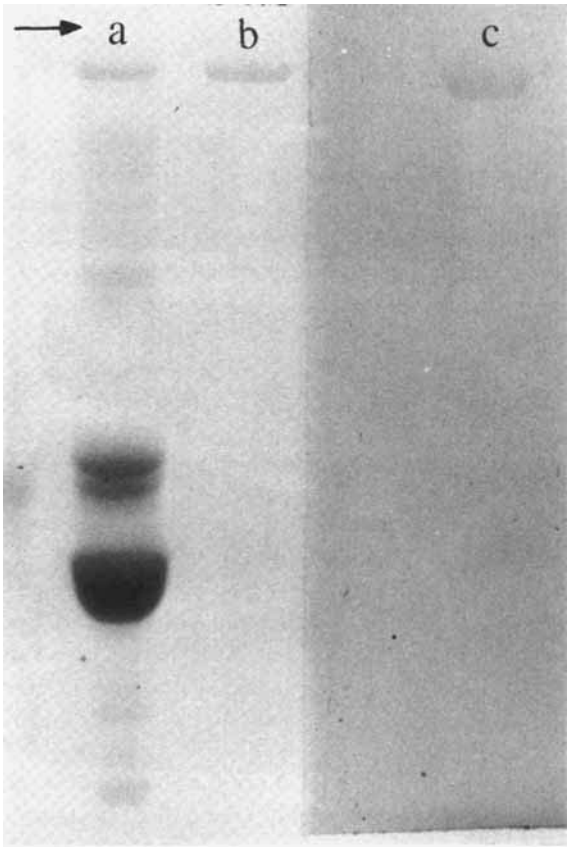

Fig. 1. SDS-polyacrylamide gel electrophoresis (arrow indicates origin) a: 10 μg CIg complexed with fibrinogen; b: 10 μg purified CIg stained for protein with Coomassie blue; c: 25 μg purified CIg stained for carbohydrate with PAS.

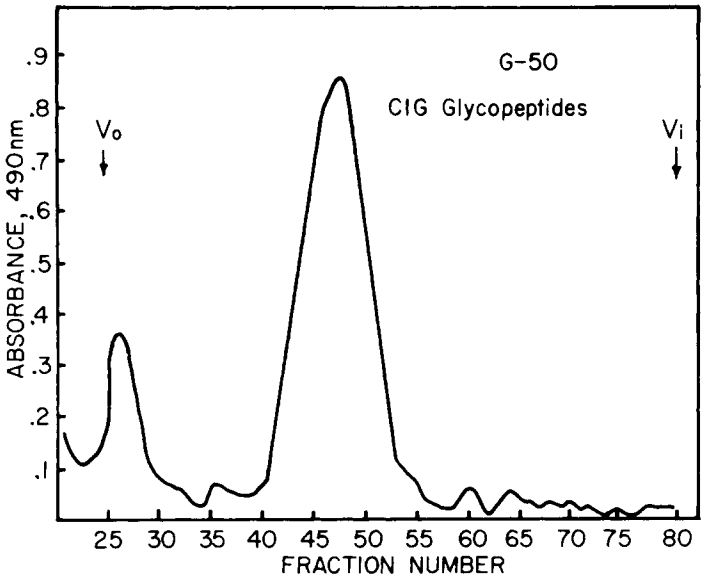

Fig. 2. Sephadex G-50 chromatography of CIg glycopeptides. Fractions 40–55 were pooled for further analysis. Vo = void volume; Vi = inclusion volume.

22.3 V; extractor, 25.6 V. Data acquisition was in the mass fragmentography mode using m/e 264 for terminal hexoses, m/e 292 for monosubstituted hexoses, m/e 320 for branched hexoses, and m/e 393 for monosubstituted amino sugars (MH-60 ions). Mass chromatograms for these ions were summed and a composite chromatogram plotted. All possible ions resulting from common sugars were searched.

RESULTS

SDS-polyacrylamide gel electrophoresis of the eluate from the gelatin-Sepharose column gave a high-molecular-weight band which appears as a diffuse doublet under close scrutiny but photographs as a single band as stained with Coomassie blue or PAS and which migrated with a CIg standard (Fig. 1). No other bands could be visualized by either method of staining. Chromatography of the total pronase-solubilized CIg glycopeptides on Sephadex G-50 (Fig. 2) gave a small peak at the void volume which could not be degraded by further protease treatment and a broad major peak corresponding to an approximate molecular weight of 2,800. The small void peak has not been further characterized.

Fractionation of the labeled transferrin, fetuin, and the major G-50 peak of the CIg glycopeptides by affinity chromatography on Con A-Sepharose (Fig. 3) yielded the following patterns. From fetuin, the glycopeptides (panel A) eluted entirely in the unbound fraction [30, 31]. From transferrin, one-fourth of the glycopeptides eluted in the unbound fraction and three-fourths in the weakly bound (WB) fraction. The transferrin glycopeptides which were not bound to the affinity column are probably of the "fetuin," triantennary type [32]. Fractionation of the major G-50 peak of the CIg glycopeptides on Con A-Sepharose (panel C) gave a pattern which closely resembled that obtained for the transferrin glycopeptides. One-third of the CIg glycopeptides passed through the col-

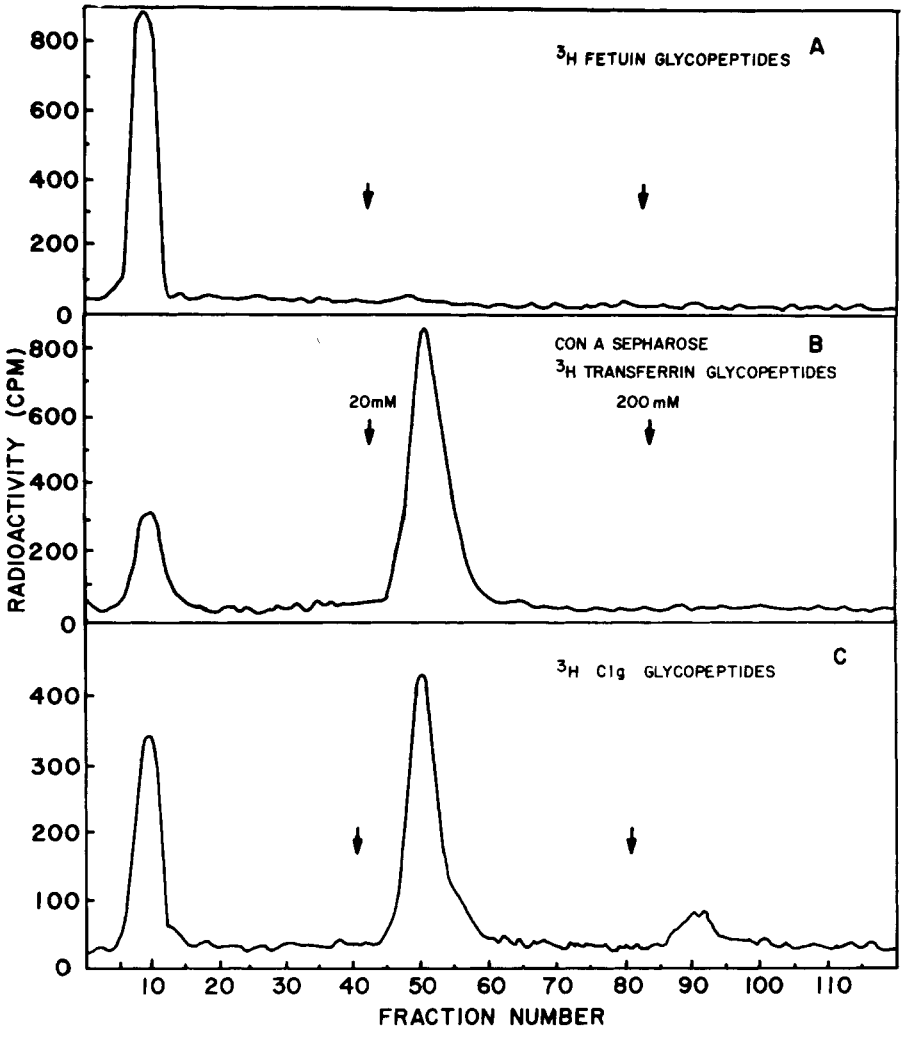

Fig. 3. Con A-Sepharose chromatography. Fractions were eluted with 20 mM methyl α-D-mannopyranoside and 200 mM methyl α-D-mannopyranoside.

umn and two-thirds were weakly bound, indicating that the single peak obtained on Sephadex G-50 (Fig. 2) could be a mixture of the biantennary and the triantennary glycopeptides.

The major fraction of CIg glycopeptides, weakly bound to Con A-Sepharose, was isolated for initial structural characterization. Based on three mannose residues, the molar ratios of galactose, N-acetylglucosamine, and N-acetylneuraminic acid were found to be 3.0, 4.9, and 2.5, respectively, for the unbound fraction and 2.0, 4.1, and 1.7 for the WB glycopeptides. Since the average chain contains three mannose residues and asparagine, the CIg glycopeptides probably have an N-glycosyl-linked core structure. The transferrin biantennary glycopeptides which were weakly bound to the affinity column and which cochromatographed on Sephadex G-50 with the WB CIg glycopeptides (Fig. 4, panels A

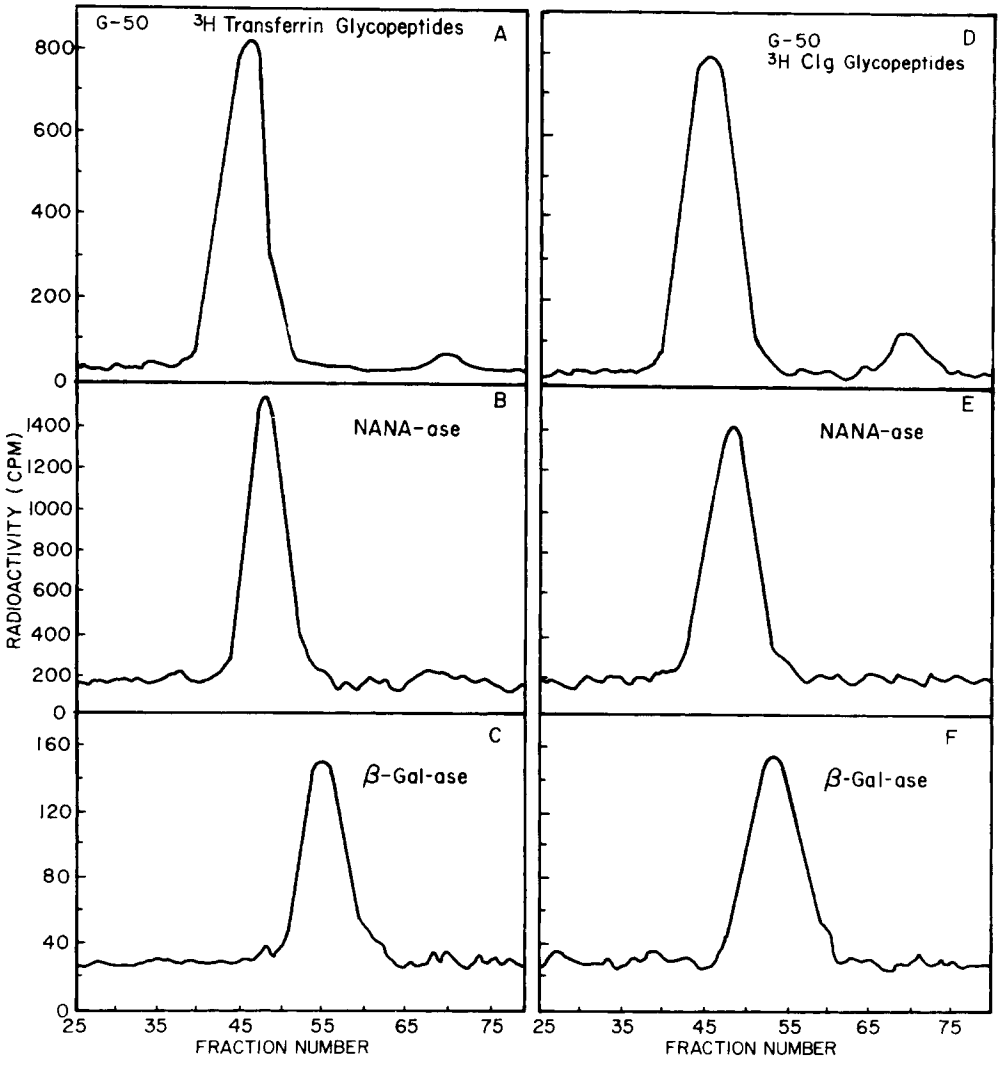

Fig. 4. Sephadex G-50 chromatography of intact and partially degraded WB transferrin (A–C) and CIg (D–F) glycopeptides. NANA-ase = neuraminidase degraded; β-Gal-ase = sequential degradation with neuraminidase and β-galactosidase.

and D), served as a standard compound for the remaining experiments. After partial degradation with neuraminidase the WB CIg and transferrin glycopeptides cochromatographed (Fig. 4, panels B and E) but with reduced apparent molecular weights, corresponding to the loss of two sialic acid residues. Subsequently to treatment with β-galactosidase the WB glycopeptides again cochromatographed (Fig. 4, panels C and F) with correspondingly lower molecular weights.

Comparison of the methylation analysis of the WB CIg and transferrin glycopeptides showed the linkage and the position of corresponding residues to be identical, both in the intact and in the partially degraded glycopeptides. Intact transferrin (Fig. 5, panel

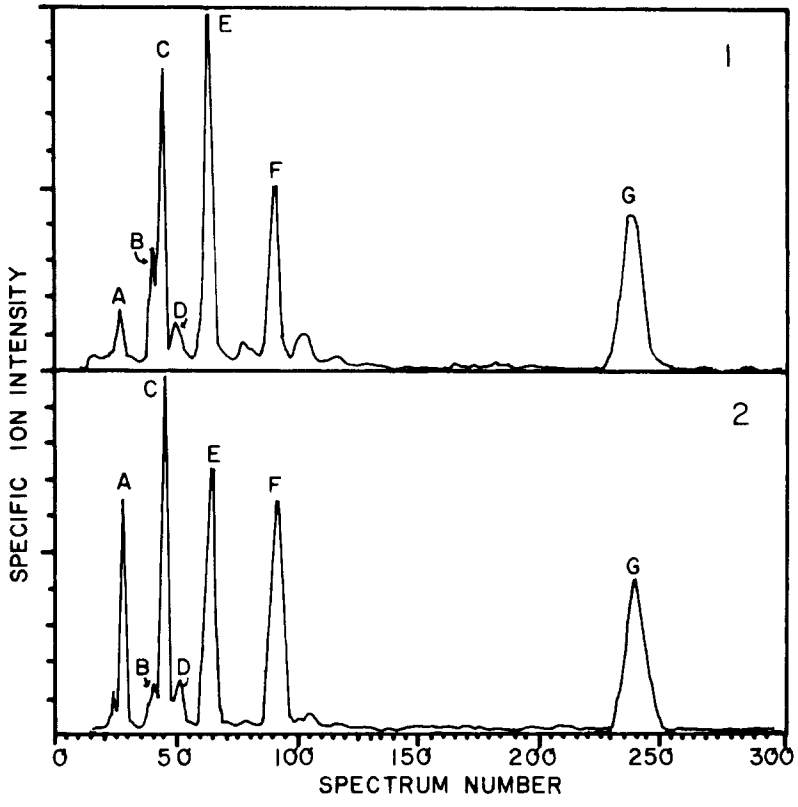

Fig. 5. Reconstructed multiple ion gas chromatogram of the partially methylated alditol acetates of the WB transferrin (panel 1) and CIg (panel 2) glycopeptides. Graphs are composite tracings of the following MH-60 ions: m/e 264 = terminal hexose; 292 = internal hexose; 320 = disubstituted hexose; 393 = mono-substituted amino hexose. A peak consisting of phthalate plasticizer at scan number 210, which gave signal at m/e 393, has been omitted. A = terminal hexose; B = 3-galactose; C = 2-mannose; D = 4-glucose; E = 6-galactose; F = 3,6-mannose; G = 4-N-acetylglucosamine.

TABLE I. Summary of Methylation Linkage Analysis of Sequentially Degraded Biantennary CIg and Transferrin Glycopeptides

Treatment	Branched residues	Internal residues	Terminal residues
NANA-ase	3,6-Mannose	2-Mannose, 4-Glucosamine	Galactose
NANA-ase, β-Gal-ase	3,6-Mannose	2-Mannose, 4-Glucosamine	Glucosamine
NANA-ase, β-Gal-ase, β-Hex-ase	3,6-Mannose	4-Glucosamine	Mannose

NANA-ase = neuraminidase; β-Gal-ase = β-galactosidase; β-Hex-ase = β-hexosaminidase.

1) and CIg glycopeptides (Fig. 5, panel 2) contained 3,6-substituted mannose, 2-mannose, 6-galactose, 4-glucosamine, and minor amounts of terminal- and 3-galactose. Methylation linkage analysis of the partially degraded glycopeptides is summarized in Table I. Following neuraminidase treatment there was a decrease in internal galactose and a concomitant increase in terminal galactose. After sequential degradation with neuraminidase and β-galactosidase the galactose residues were sharply reduced and half the glucosamine appeared in the terminal position. The terminal glucosamine residues were removed by degradation with β-hexosaminidase, after which approximately two-thirds of the mannose residues appeared in the terminal position. A trace amount of 4-glucose (less than 0.1 mole/3 mannose residues) was present in all WB transferrin and CIg preparations.

DISCUSSION

Two major classes of CIg glycopeptides were distinguished by affinity chromatography on Con A-Sepharose. 1) Two-thirds of the CIg glycopeptides were weakly bound (WB) to the lectin column. Consistent with the previously established structural requirements for Con A binding [31], the WB CIg glycopeptides contained three mannose residues, two of which were 2-linked. The structure of this glycopeptide was shown to be identical to that previously determined for the major biantennary transferrin glycopeptide [32] which also binds weakly to Con A [30]. Except for the presence of a small amount of terminal and 3-galactose residues, indicating that some of the WB CIg chains are either unsialylated or sialylated on the 3 position of galactose, other structural heterogeneity was not detected in this fraction. 2) A smaller portion (one-third) of the CIg glycopeptides passed through the Con A-Sepharose. Methylation analysis of this fraction (data not shown) indicated that the unbound CIg glycopeptides contain both 3,6- and 2,4-linked mannose, as does the fetuin triantennary glycopeptide [33]. It is probable that the 2,4-linked mannose residues, which prevent fetuin glycopeptides from binding to Con A [30, 31], also give rise to the unbound CIg fraction. The possibility that this fraction is a mixture of tri- and tetraantennary glycopeptides has not been excluded.

In close agreement with earlier observations [34, 35], human CIg was found to have a carbohydrate content of approximately 4%. From the Con A binding data each subunit of CIg, of molecular mass 210,000, may contain an average of two biantennary and one triantennary carbohydrate chains, which give a combined molecular mass of 7,277, corresponding to a carbohydrate content of 3.5%. The small void peak obtained from chromatography of the glycopeptides on Sephadex G-50 may constitute the remaining 0.5% of the calculated CIg carbohydrate content of 4%. Although the carbohydrate composition of human CIg corresponds to previously published results [36], neither type of chain contains 4-linked galactose or 4-linked mannose, which were recently reported by Wrann [36] to be components of human CIg. Galactosamine and fucose, which were shown by Carter and Hakomori to be components of LETS glycopeptides isolated from hamster embryo fibroblasts [37], were not detected in the human preparation. Stoichiometric amounts of glucose, reported in LETS glycopeptides from chick embryo fibroblasts [38], were not present in the CIg glycopeptides.

Since the carbohydrate structures of CIg resemble those carried by many other proteins, it seems likely that the specialized functions which have been proposed for this glycoprotein do not reside in the carbohydrate portion of the molecule. Instead, glycosylation may in this case be related to a more generalized function such as stabilization of the molecule against proteolytic digestion [39], maintenance of protein solubility, or clearance from the plasma [40].

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