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Location and characterization of the three carbohydrate prosthetic groups of human protein HC

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Three different carbohydrate prosthetic groups associated to three chymotryptic peptides, Q1, Q2 and Q3, were isolated from the reduced and carboxymethylated human protein HC. The first oligosaccharide forms an *O*-glycosidic linkage with a threonine residue at position 5 in the polypeptide chain of protein HC. The second and third carbohydrate prosthetic groups form N-linkages with asparagine residues at positions 17 and 96. Oligosaccharides present in Q1 contain 1 residue of NANA, 2 of GalNAc and 1 of Gal corresponding to the following structure: -O-GalNAc-GalNAc-Gal-NANA. Q2 contains 3 NANA, 9 GlcNAc, 2 Gal and 3 Man, and Q3 contains 2 NANA, 5 GlcNAc, 1 Gal and 2 Man. The sugar compositions of Q2 and Q3 oligosaccharides are compatible with that of the complex kind. The amount of oligosaccharides present in Q1, Q2 and Q3 corresponded respectively to 3.0%, 12.2% and 7.3% of the weight of protein HC. No difference was found between the carbohydrate composition of urinary and plasma protein HC.

Protein HC; α_1-Microglobulin; Glycoprotein; Carbohydrate

1. INTRODUCTION

Human protein HC (α_1-microglobulin) is a glycoprotein widely distributed in body fluids as free monomer and as a complex with IgA [1-3]. The free form consists of a polypeptide chain of 183 amino acids [4-6] which carries carbohydrates and retinol [7] as well as several unidentified yellow-brown fluorescent chromophoric groups [1-9]. In the HC-IgA complex, which has antibody activity [10] and lacks the chromophores [9], protein HC and IgA are linked by a reduction-insensitive linkage of unknown nature [3].

Protein HC has been included in the lipocalin superfamily [11] which is comprised of a diverse group of distantly related animal proteins (retinol binding protein, β-lactoglobulin, etc.), which transport small lipophilic biomolecules (retinol, odorants, steroids). This superfamily presents amino acid sequence homologies, being the human complement component C8γ the lipocalin member which displays the highest degree of homology with protein HC [12]. More recently, a mRNA has been isolated from human liver, which codes for the 30 kDa fragment HI-30 (a serine protease inhibitor) and for protein HC [6]. The reason why these two apparently unrelated proteins are expressed together, separated only by two arginine residues and processed into two separated functional molecules, is still unknown. Although protein HC acts in vitro as an inhibitor of neutrophil chemotaxis and has immunoregulatory properties [13], the biological function of this protein is still unknown. In this paper we study the carbohydrate prosthetic groups of human protein HC. Our data demonstrate the location of the oligosaccharide chain, the nature of their linkages to the polypeptide chains of protein HC and their monosaccharide compositions.

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Abbreviations: Glc, glucose, Gal, galactose; Man, mannose; Fuc, fucose; NANA, *N*-acetylneuraminic acid; GlcNac, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; RP-HPLC, reversed-phase high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Isolation*

Protein HC was isolated from plasma or from a pool of urine of different individuals as earlier described [1].

2.2. *Reduction and carboxymethylation*

Native protein HC (10 mg/ml) in 1 M Tris-HCl buffer, pH 8.5, containing 2 mM EDTA and 6 M guanidinium hydrochloride, was reduced with 0.06 M DTT for 120 min at 37°C. Alkylation was achieved by addition of iodoacetic acid to a final concentration of 89 mM. After incubation for 15 min at room temperature, in the absence of light, the excess of reagents was removed bi size-exclusion HPLC on a TSK-G 3000 SWG column (21.5 mm × 30 cm), by isocratic elution with 0.1 M ammonium acetate buffer, pH 6.9.

2.3. *Chymotryptic digestion*

4.5 mg of reduced and carboxymethylated protein HC were

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digested with α-chymotrypsin at an enzyme/substrate ratio of 1:100 (w/w) in 0.2 M *N*-methylmorpholine acetate buffer, pH 8.2, for 4 h at 37°C.

2.4. *Peptide fractionation*

Chymotryptic peptides were fractionated by RP-HPLC on a C-18 Nova-Pak column (3.9 mm × 15 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid. The elution of peptides was performed with a linear gradient of acetonitrile from 0% to 32%, containing 0.1% (v/v) trifluoroacetic acid, at a flow rate of 0.5 ml/min at room temperature.

2.5. *Amino acid analysis*

Peptides were hydrolysed with 0.1 ml of 5.7 M HCl containing 0.05% (v/v) 2-mercaptoethanol at 110°C for 20 h and the amino acid analyses performed using a Beckman 121 MB amino acid analyzer.

2.6. *Carbohydrate analysis*

Aliquots of protein HC obtained from both urine and plasma as well as aliquots of the three chymotryptic peptides Q1-3 were hydrolysed with 8 M HCl at 96°C for 3 h. The hydrolysates were dansylated and the obtained derivatives were purified on SepPac C-18. Glucosamine and galactosamine derivatives were then separated by means of RP-HPLC with fluorophotometric detection [4]. Another aliquot was hydrolysed in 25 mM HCl (2 h at 80°C) for subsequent analysis of sialic acids. These hydrolysates were per-*O*-benzoylated, and following purification on SepPac C-18, the obtained derivatives of *N*-acetyl- and *N*-glycolyl-neuraminic acids were separated by means of RP-HPLC with UV detection [15]. A third aliquot was lyophylised and taken for methanolysis (0.5 M HCl in dry methanol, 100°C for 30 h). The obtained neutral monosaccharides were analysed by means of RP-HPLC with refractive index detection [16]. The obtained peaks were quantitated by comparing with monosaccharides that were methylated under the same methanolytic conditions. The total content of neutral sugars was determined colorimetrically, using an automated version of the anthron procedure [17], and with 1:1 mixtures of Man and Gal as standards. The possibility of uronic acids being present in the preparations was also considered, taking a fourth aliquot for this analysis [18].

3. RESULTS AND DISCUSSION

The complete sugar composition of the urinary protein HC is shown in Table I. A total of 16 residues of GlcNAc, 2 of GalNAc, 5 of Gal, 6 of Man and 6 of NANA corresponding to the relative number of monosaccharides of 8:1:2.5:3:3, respectively, were found. *N*-Glycolylneuraminic acid derivatives were not detected, nor was any uronic acid. The sugar composition of plasma protein HC was also nearly identical to that of urinary protein HC (Table I), which is in agreement with the partial sugar composition previously reported for both urinary and plasma protein HC [3].

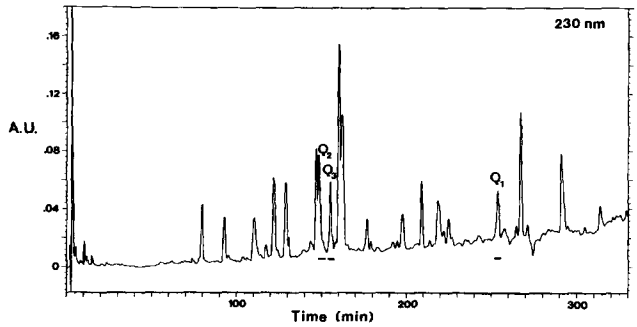

Fig. 1. Fractionation by RP-HPLC of chemotryptic peptides from reduced and carboxymethylated protein HC. The digest, 30 nmol in 220 μl of 0.1% trifluoroacetic acid, was injected into a C-18 Nova Pak column and eluted with a gradient of acetonitrile as indicated in section 2. The flow-rate was 0.5 ml/min and 0.5 ml fractions were collected. The eluent was pooled as indicated by bars. Q1, Q2 and Q3 indicate the peaks containing glycopeptides.

In order to determine the precise number of carbohydrate prosthetic groups and their location in the polypeptide chain of protein HC, the carboxymethylated protein was digested with chymotrypsin and fractionated by RP-HPLC. The chromatogram obtained is shown in Fig. 1. The amino acid composition of all chymotryptic peptides revealed that only three of them, denominated Q1, Q2 and Q3, contained amino sugars and allowed their location in the polypeptide chain of protein HC (Table II). Q1 and Q2 were located in the N-terminal region, between residues 1–16 and 17–22, respectively, while Q3 was located in the middle part of the molecule between residues 96 and 102 (Fig. 2).

The fact that in the process of sequential degradation of these peptides the corresponding anilinothiazolinone derivatives of residues Thr in Q1, Asn in Q2 and Q3

Table I

Carbohydrate composition of carbohydrate-containing chymotryptic peptides of protein HC and urinary and plasma protein HC

	Chymotryptic peptides				Protein HC	
	Q1	Q2	Q3	Q1 + Q2 + Q3	Urine	Plasma
NANA	1.5[a](1)[b]	3.1 (3)	2.0 (2)	6.6 (6)	6.1 (6)	ND
GlcNAc	0.2 (0)	9.4 (9)	5.3 (5)	14.9 (14)	15.7 (16)	14.2 (14)
GalNAc	2.5 (2)	0.2 (0)	0.2 (0)	2.9 (2)	2.0 (2)	1.7 (2)
Gal	1.0 (1)	2.0 (2)	1.2 (1)	4.3 (4)	5.3 (5)	5.4 (5)
Man	0.0 (0)	3.4 (3)	2.3 (2)	5.7 (5)	5.8 (6)	4.9 (5)
Fuc	0.0 (0)	0.2 (0)	0.1 (0)	0.3 (0)	0.2	0.6
M.W.[c]	931	3819	2265	7015	7819	ND

[a] Residues per mole of peptide

[b] The theoretical number of monosaccharides is given within parentheses

[c] Molecular weights are calculated on the basis of theoretical monosaccharide number indicated within parentheses

ND = not determined

Table II

Amino acid composition of carbohydrate containing chymotryptic peptides from the reduced and carboxymethylated protein HC

	Q1	Q2	Q3
Asp	2.7[a](3)[b]	0.9 (1)	1.0 (1)
Thr	1.3 (1)		1.0 (1)
Ser		0.9 (1)	0.9 (1)
Glu	3.0 (3)		1.0 (1)
Pro	3.8 (4)		
Gly	1.0 (1)		
Val	2.0 (2)		
Met			0.5 (1)
Ile	1.1 (1)	2.1 (2)	1.2 (1)
Tyr		0.9 (1)	0.8 (1)
Phe	0.9 (1)		
Arg		1.0 (1)	
Yield (nmol)	20.8	24.1	22.2
	(1-16)*	(17-22)*	(96-102)*

[a] Residues per mole of peptide

[b] The number of residues as calculated from the known sequence (3,4) is given within parentheses

* Location of peptides in the polypeptide chain

could not be found in the chlorobutane extract, remaining in the spinning cup, could be due to the presence of carbohydrate prosthetic groups attached to these amino acid derivatives. These results suggest the presence of sugar prosthetic groups linked to the above residues of Thr and Asn, respectively, which correspond to positions 5, 17 and 96 in the polypeptide chain of protein HC (Fig. 2).

The sugar composition of the three chymotryptic peptides shows that Q1 contains 1 NANA, 2 GalNAc and 1 Gal, Q2 contains 3 NANA, 9 GlcNAc, 2 Gal and 3 Man and Q3 contains 2 NANA, 5 GlcNAc, 1 Gal and 2 Man (Table I). The total number of monosaccharides calculated from these three peptides: 6 NANA, 14 GlcNac, 2 GalNAc, 4 Gal and 5 Man is in agreement with the value obtained for the intact molecule (Table I).

The presence of GalNAc in Q1 corroborates the existence of an *O*-glycosidic linkage between this sugar with the only residue of Thr in peptide Q1, while the presence of GlcNAc in Q2 and Q3 ratifies the existence of an *N*-glycosidic linkage between this sugar and also the only residue of Asn present in peptides Q2 and Q3. The sugar composition of Q2 and Q3 is typical of that of complex kind *N*-glycosidic linked oligosaccharides. The sequence Asn-X-Thr/Ser, necessary for the attachment of N-linked oligosaccharides, is present in both glycopeptides.

The results obtained in this paper, which display the presence in protein HC of one *O*-glycosidic linkage and two *N*-glycosidic linkages, differ from those previously reported for the α_1-microglobulin, which indicated the presence of three identical *N*-glycosidic linked carbohydrate chains without specifying their location in the polypeptide chain [19]. The main reason for this discrepancy may reside in the fact that structural studies on the carbohydrate portion of α_1-microglobulin were performed with the complete molecule rather than with individual carbohydrate containing peptides as in the present work. The present analysis of the carbohydrate content in protein HC demonstrates some significant differences between the three oligosaccharide containing peptides, Firstly, the Q1 peptide carries a quite small oligosaccharide with NANA, GalNAc and Gal in the relative molar proportions 1:2:1. This composition indicates an unbranched chain with NANA in the nonreducing end and a GalNAc-GalNAc disaccharide binding to the threonine, with the tentative sequence: -O-GalNAc-GalNAc-Gal-NANA, similar to what has been shown in epithelial glycoproteins [20]. This Q1 carbohydrate moiety is O-linked to the protein backbone, and was therefore probably not detected when fluoroacetolysis was used in the previous α_1-microglobulin study. The Q2 and Q3 peptide both have oligosaccharides with GlcNAc and Man as dominating constituents. The relative proportions of monosaccharides in these N-linked oligosaccharides are

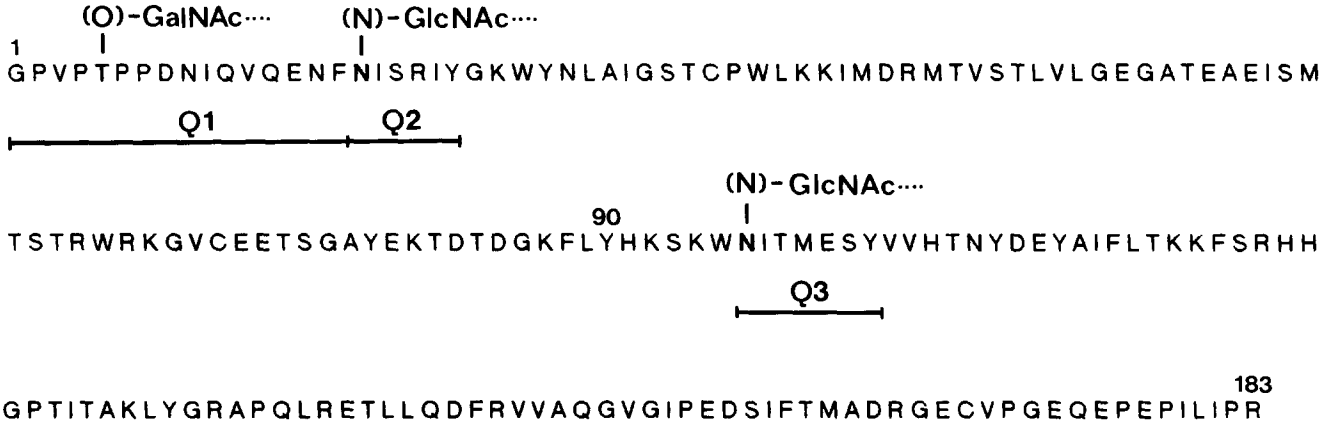

Fig. 2. Location of the three carbohydrate-containing chymotryptic peptides Q1, Q2 and Q3 in the polypeptide chain of protein HC. The first sugar of each oligosaccharide chain is indicated over the amino acid to which it is linked.

similar, but the amounts of sugars as well as the proportions of GlcNAc and NANA are considerably higher in Q2. These oligosaccharides may well be branched through a Man triplet, and with a NANA terminating each antenna. The higher contents of GlcNAc and NANA both indicate that the much larger Q2 oligosaccharide has three or more antennas, while the smaller Q3 group only carries two. The complete accurate sequential composition of these three oligosaccharides remains, however, to be elucidated.

The molecular masses of the oligosaccharides present in Q1, Q2 and Q3, as well as the total carbohydrate in protein HC, calculated on the basis of a theoretical number of monosaccharides, obtained from their carbohydrate compositions, were: 931, 3819, 2265 and 7015, respectively (Table I). Thus the whole carbohydrate content represents 23% of the molecular mass of protein HC. Oligosaccharides in Q1, Q2 and Q3 correspond to 3.0%, 12.2% and 7.3% of the molecular mass of protein HC including its chromophores.

As in other glycoproteins, the oligosaccharides of protein HC can play an active role in its physicochemical properties, turnover regulations, interaction with macromolecules and cellular surfaces. The possibility that in the case of protein HC the carbohydrates could be also involved in the linkages between the polypeptide chain and the associated chromophores is intriguint and remains to be investigated.

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