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THE STRUCTURES OF THE SERINE-LINKED SUGAR CHAINS ON HUMAN CHORIONIC GONADOTROPIN

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SUMMARY: The human chorionic gonadotropin beta-subunit tryptic COOH-terminal peptide (residues 123-145) which contains 3 serine-linked sugar chains was isolated. The sugar chains were cleaved by beta-elimination and then separated by gel filtration. The peaks were pooled and their compositions determined. The products of serial glycosidase digestion and periodate oxidation of the intact glycopeptide were also characterized. Of the serine-linked sugar chains, 13% were the hexasaccharide NeuAc alpha2,3 Gal beta1,3 (NeuAc alpha2,3 Gal beta1,4 GlcNAc beta1,6) GalNAc, 34% the tetrasaccharide NeuAc alpha2,3 Gal beta1,3 (NeuAc alpha2,6) GalNAc, 43% the trisaccharide NeuAc alpha2,3 Gal beta1,3 GalNAc and 10% the disaccharide NeuAc alpha2,6 GalNAc.

INTRODUCTION: Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of 2 dissimilar subunits, alpha and beta, joined non-covalently. The alpha-subunit contains 2 N-linked oligosaccharides, as does the beta-subunit, which also contains 4 O-glycosidic sugar chains (1). The hCG dimer is produced by the trophoblast in pregnancy and by trophoblastic neoplasms. In recent studies, we examined the [3H]GlcN-biosynthetically labeled hCG-beta and free alpha-subunit produced by JAr malignant trophoblastic cells (2,3). In the O-linked oligosaccharides cleaved by beta-elimination, radioactive GlcNAc was found as well as GalNAc, suggesting that the structures of these sugar

Abbreviations: hCG, human chorionic gonadotropin; hCG-alpha, alpha component of hCG; hCG-beta, beta component of hCG; beta-CTP, the COOH-terminal tryptic peptide (residues 123-145) of hCG-beta; PNA-agarose, peanut agglutinin (Arachis hypogaea lectin) linked to agarose; PBS, phosphate-buffered saline, pH7.5; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GlcN, glucosamine; GalN, galactosamine; GalNAc-ol, N-acetylgalactosaminitol; GalN-ol, galactosaminitol; Gal, galactose; NeuAc, N-acetylneuraminic acid; Man, mannose; NRT, non-reducing terminus.

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chains was different from that reported, NeuAc alpha2,3 Gal beta1,3 (NeuAc alpha2,6) GalNAc (4). This prompted the present study, in which we examine the structures of the O-linked oligosaccharides of hCG (purified from pooled pregnancy urine) and show that they are a mixture of di-, tri-, tetra- and hexasaccharides, rather than solely tetrasaccharides as previously reported (4).

MATERIALS AND METHODS: Standard urinary hCG was purified, and hCG-alpha and hCG-beta generated by dissociation as previously described (5). The hCG-beta tryptic COOH-terminal peptide (beta-CTP, residues 123-145) and the thermolytic peptide 115 - 141 were also prepared and quantitated by methods previously published (6). Both peptides were assayed for purity by amino acid analyses (6).

C. perfringens alpha-neuraminidase, and bovine testes and jack bean beta-galactosidase digestions (2 x 1 unit, 16 hr at 37deg.C in pH 5, 5, and 4.5 acetate buffers respectively) were carried out as described (2, 7, 8). By methods previously published (2, 3), gel filtration on a 1 x 110 cm column of Bio-Gel P4 (in 0.1 M ammonium bicarbonate, 4 ml/hr, 1 ml fractions) and alkaline-borohydride beta-elimination (0.1 N NaOH-1 M sodium borohydride, 45deg.C, 72 hr) were performed.

Periodate oxidations were carried out by a modification of the procedures of Kessler et al. (4). To 40 nmol samples in 0.1 ml water, 0.024 ml of 0.1 M sodium periodate was added (10-fold molar excess over total sugar). Mixtures were incubated 24 hr at 4deg.C. PBS (0.025 ml of 10X) was then added and the pH of solutions adjusted to 8.0 with NaOH. Following the addition of 0.15 ml of 2 M sodium borohydride, mixtures were incubated on ice an additional 1 hr. Samples were neutralized with 2 N acetic acid and then hydrolyzed for amino acid/sugar analyses.

Three types of acid hydrolyses were employed. Glycopeptides or oligosaccharides were incubated 1 hr at 80deg.C in 0.1 N sulfuric acid to release NeuAc residues for assay. For analyses of neutral sugars, samples were hydrolyzed in 4N trifluoroacetic acid at 100deg.C for 2 hr. Amino sugar analyses required hydrolyses in 6 N HCl at 100deg.C for 3 hr. NeuAc was quantitated by the Warren thiobarbituric acid method (9). Neutral sugars were converted to the corresponding glycamines (10). Amino sugars and glycamines were separated on a cation exchange column using a Kratos automated amino acid analyzer, reacted with an o-phthalaldehyde reagent, and then detected with a Kratos FS950 fluorometer (10).

Affinity chromatography on PNA-agarose (0.7 x 30 cm columns) was performed as previously described (2). Anion-exchange was carried out on AG1-X8 resin (1 x 30 cm columns) equilibrated with 10 mM sodium acetate buffer, pH 5. Applied samples (1 ml) were eluted with 6 column volume 10 - 500 mM sodium acetate gradients. Fractions, 2 ml, were assayed for their NeuAc content.

RESULTS AND DISCUSSION: The sugar chains on 300 nmol of beta-CTP were cleaved by beta elimination with alkali, neutralized, and then the products separated on a Bio-Gel P4 column (Fig. 1). The NeuAc content of fractions was measured and those comprising oligosaccharide peaks pooled as illustrated. Aliquots of the pools, when applied to AG1-X8 anion exchange resin eluted as single peaks, indicating homogeneous preparations (data not shown). The results of carbohydrate analyses of the pools are presented in Table 1. The sums of the sugar components

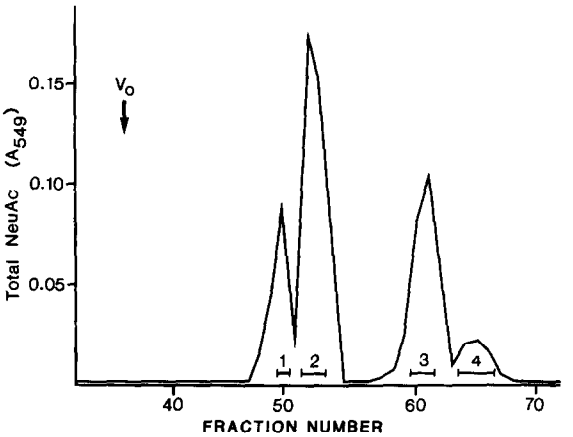

Fig. 1. Gel filtration on Bio-Gel P4 of the alkaline-borohydride beta-elimination products of beta-CTP. Fractions were detected by a colorimetric assay for NeuAc (9). Pools of fractions in peaks 1 - 4 were prepared as illustrated.

of the 4 pools (Table 1) was equal to the sugar composition of beta-CTP (Table 2, GalNAc replaces GalNAc-ol) demonstrating that all oligosaccharides were cleaved by the beta-elimination procedure, were detected and were NeuAc-containing. From the beta-elimination products a hexasaccharide (13% of total; likely composition 2 NeuAc, 2 Gal, GlcNAc, GalNAc), tetrasaccharide (34%; 2 NeuAc, Gal, GalNAc), trisaccharide (43%; NeuAc, Gal, GalNAc) and disaccharide (10%; NeuAc, GalNAc) component was recognized.

To determine sugar arrangements and linkages serial glycosidase digestion and periodate oxidations were carried out on beta-CTP (see Table 2 flow chart). From the results (Table 2), the 4 structures in Fig. 2 were formulated. Alpha-neuraminidase cleaved all the NeuAc on

Table 1. Sugar analyses of beta-CTP beta-elimination products[a]

Samples	Distribution[b]	NeuAc[c]	Gal[c]	GlcNAc[cd]	GalNAc[ce]	GalNAc-ol[cf]	Man[c]
Peaks from Fig. 1							
1	13%	2.3	2.1	1.0	0.0	1.0	0.0
2	33%	2.0	.80	0.0	0.0	1.0	0.0
3	43%	.94	.94	0.0	0.0	1.0	0.0
4	10%	1.3	0.0	0.0	0.0	1.0	0.0
Sum[g]	100%	1.5	.95	.13	0.0	1.0	0.0

a See "Materials and Methods" for details
b From integration of peaks in Fig. 1, adjusting for their NeuAc content
c Number of residues, calculated assuming GalNAc-ol as 1.0
d Assayed as GlcN
e Assayed as GalN
f Assayed as GalN-ol
g Adjusted for distribution

Table 2. Sugar analyses of beta-CTP glycosidase digestion and periodate oxidation products [a]

Samples	NeuAc [b]	Gal [b]	GlcNAc [bc]	GalNAc [bd]
Beta-CTP	1.5	.95	.13	1.0
alpha-neuraminidase digested	0(1.5 [e])	.95	.13	1.0
bovine testis beta-galactosidase digested	1.6	.98	.12	1.0
bovine testes beta-galactosidase digested		0(.99 [e])	.14	1.0
jack bean beta-galactosidase		f(.16 [e])	f	1.0
periodate oxidation	0.0	1.1	.14	1.0
periodate oxidation		0.0	.13	1.0
periodate oxidation			0.0	0.0

a See "Materials and Methods" for details
b Number of residues, calculated assuming GalNAc as 1.0
c Assayed as GlcN
d Assayed as GalN
e That released, detected in unhydrolyzed sample
f Not determined

beta-CTP indicating that its present as the alpha anomer at the NRT of all 4 structures. Bovine testes beta-galactosidase released all the Gal on beta-CTP, but only following neuraminidase treatment, indicating that its present as part of the sequence -beta Gal - alpha NeuAc at the NRT of structures. Following galactosidase digestion, only GlcNAc and GalNAc remained attached to the peptide. Since the O-linkage was through GalNAc (GalNAc-ol was the only sugar alcohol component of the

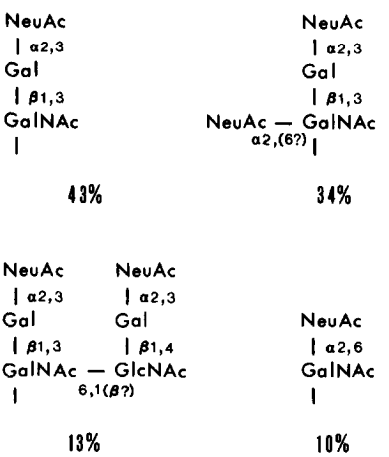

Fig. 2. Proposed structures for the O-linked oligosaccharides on hCG.

beta-eliminated oligosaccharides), it was apparent that GlcNAc was at the NRT of this asialoagalacto beta-CTP preparation.

The periodate oxidation procedure destroys sugar residues with hydroxyl groups at C-3 and C-4, or C-2 and C-3. Regarding the hexasaccharide structure (Fig. 2): Periodate oxidation of sequentially neuraminidase and galactosidase digested beta-CTP destroyed the remaining GlcNAc and GalNAc, indicating the presence of hydroxyls at C-3 and C-4 of the latter (C-2 was acetamidated) and a 1,6 linkage. However, neither GlcNAc nor GalNAc were destroyed in the periodate oxidation of the neuraminidase digested preparation, showing that the first Gal is 1,3 or 1,4 linked to the GalNAc and the second similarly linked to the GlcNAc blocking their oxidation. NeuAc, but not Gal, was destroyed by the periodate oxidation of intact beta-CTP, indicating that NeuAc was linked through C-3 on each Gal blocking destruction.

Regarding the tetra- and trisaccharides structures: GalNAc was destroyed in the periodate oxidation of sequentially neuraminidase and galactosidase digested beta-CTP but not in that of the neuraminidase treated preparation, indicating Gal attachment through a 1,3 or 1,4 linkage. NeuAc, but not Gal, was destroyed by the periodate oxidation of intact beta-CTP, indicating that a NeuAc residue was 2,3 linked to the single Gal. Since all NeuAc was destroyed by the periodate oxidation of beta-CTP, we inferred that the second residue of this sugar in the tetrasaccharide structure was not attached to the first. It seemed likely that it was 2,6 linked to GalNAc as found by several other laboratories (4, 11) for the second NeuAc on tetrasaccharides of the same composition (we were unable to rule out linkage of the second NeuAc through C-4 or C-6 on Gal).

Periodate oxidation of beta-CTP increased the ratio of Gal and GlcNAc residues to GalNAc residues by around 10%, indicating approximately 10% destruction of the latter residues. With all NeuAc destroyed, this would be consistent with the simultaneous oxidation of the GalNAc of the 10% disaccharide component, indicating a 2,6 linkage (hydroxyls present at C-3 and C-4) as commonly found on disaccharides of this composition (11).

PNA-agarose and two beta-galactosidases of differing specificities were utilized in to distinguish Gal 1,3 and 1,4 linkages to amino sugars. Bovine testes beta-galactosidase cleaves beta1,3 and beta1,4 linked Gal (2, 12). The jack bean enzyme, however, which cleaves beta1,4 linked Gal, has little or no activity with the beta1,3 linked substrate (8). PNA-agarose binds glycopeptides with the NRT sequence Gal beta1,3 GalNAc (2, 13). As previously shown (2), hCG-beta COOH-terminal fragments

containing 1, 2, or 3 O-linked oligosaccharides (residues 134 - 145, 123 - 133 and 123 - 145), when neuraminidase treated bind PNA-agarose, suggesting that a Gal in the structures is in the sequence Gal beta1,3 GalNAc. Binding of all the peptides to PNA-agarose was completely ablated by their prior digestion with the bovine testes enzyme, but not at all by their treatment with that from jack bean (data not shown), confirming their Gal 1,3 linkage to GalNAc (as indicated in Fig. 2). Treatment of neuraminidase digested beta-CTP with jack bean beta-galactosidase cleaved 0.16 Gal residues (Table 2, calculated assuming GalNAc as 1) versus 0.99 residues released by the bovine testes enzyme, consistent with the second Gal on the 13% hexasaccharide being 1,4 linked to GlcNAc. In the present study, due to the unavailability of an appropriate glycosidase, we were unable to determine the anomeric form of the GlcNAc on the hexasaccharide. Other laboratories have found the beta form of GlcNAc (as indicated in Fig. 2) in this same structure (11).

As we have previously reported (6), hCG contains a total of 4 O-linked oligosaccharides, on serine residues 121, 127, 132 and 138 of the beta-subunit. Here we examined beta-CTP (residues 123 - 145), which contains 3 of them. The 4th (attached to serine 121) is likely to contain similar structures, since asialo peptides containing 3 (Table 2) and 4 (residues 115 - 141, 0.12 GlcNAc, 0.93 Gal, 1.0 GalNAc) O-linked oligosaccharides have similar compositions. We did not determine whether the 4 types of O-linked oligosaccharide (Fig. 2) were randomly or specifically distributed among serines acceptor residues on hCG. However, that 60% of the O-linked oligosaccharides on hCG and free alpha-subunit (1 threonine attachment site) produced by cultured malignant trophoblast cells contain GlcNAc (2, 3), versus the 13% reported here, is somewhat suggestive of a random distribution of the 4 structures (Fig. 2).

Kessler et al. in their studies of the structure of the O-linked oligosaccharides of hCG (4) suggested a similar composition to ours for the beta-elimination products (1.8 NeuAc, 1.2 Gal, 0.1 GlcNAc, 0.97 GalNAc-ol), however, they concluded that the GlcNAc was a contaminant and that these figures indicated a single type of structure composed of 2 NeuAc, 1 Gal and 1 GalNAc. The finding of Mizouchi and Kobata of 3 different N-linked oligosaccharides (14) and our finding of 4 different O-linked structures on hCG, illustrates not only the complexity of this molecule but the intricacy of the synthesis of this and other glycoproteins. Several laboratories have reported significant differences in the immunoreactivities of pregnancy and cancer-derived hCG towards

carbohydrate-specific beta-CTP-directed antisera (7, 15). These findings, and ours of 13% GlcNAc-containing O-linked oligosaccharides on pregnancy urine hCG versus about 60% on that from JAr cultured cancer cells, suggest that changes in the patterns of hCG O-glycosylation accompany carcinogenesis and that specific carbohydrate structures may be tumor markers.

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