Structural Analysis of N-Linked Oligosaccharides of Equine Chorionic Gonadotropin and Lutropin β-Subunits†

Taei Matsui,*,‡ Tsuguo Mizuochi,‡,§ Koiti Titani,‡ Tatsuyuki Okinaga,⊥ Motonori Hoshi,⊥ George R. Bousfield,∥ Hiromu Sugino,○ and Darrell N. Ward#

Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11, Japan, Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuda, Yokohama 227, Japan, Department of Biological Sciences, Wichita State University, Wichita, Kansas 67208, Institute for Enzyme Research, University of Tokushima, Tokushima 770, Japan, and Department of Biochemistry and Molecular Biology, University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

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ABSTRACT: Equine chorionic gonadotropin (eCG) and lutropin (eLH) are composed of α- and β-subunits with an identical amino acid sequence but show different biological activities. To elucidate the molecular difference between these gonadotropins, the structure of the N-linked oligosaccharides of each β-subunit was determined. N-Linked sugar chains, liberated as tritium-labeled oligosaccharides by hydrazinolysis followed by N-acetylation and reduction with NaB^3H_4, were neutralized by sialidase digestion and/or methanolytic desulfation. Neutralized oligosaccharides were fractionated by sequential chromatography on serial lectin affinity columns and on a Bio-Gel P-4 column. Each oligosaccharide structure was determined by sequential exoglycosidase digestion in conjunction with elution profiles on lectin columns and methylation analysis. Each β-subunit contained a single N-glycosylation site, but a high degree of microheterogeneity was observed in the structure of its N-linked oligosaccharides. eCGβ contained mono-, bi-, tri-, and tetraantennary complex-type oligosaccharides in a ratio of 3:63:13:1. eCGβ oligosaccharides contained about 16% of the bisecting GlcNAc and about 20% of poly-*N*-acetyllactosamine structures. Elongation of *N*-acetyllactosamine units showed a preference to the Manα1→6 side rather than the Manα1→3 side. Triantennary chains had only a C-2, 4-branched structure. eLHβ contained only mono- and biantennary complex-type and hybrid-type oligosaccharides in a ratio of approximately 18:67:10. eLHβ also contained bisected structures in about 18%. Oligosaccharides derived from the sulfated fraction of eLHβ contained GalNAc residues at nonreducing termini. Oligosaccharides from the sialylated/sulfated fraction of eLHβ contained both Gal and GalNAc residues at nonreducing termini, and those GalNAc residues were preferentially distributed to the Manα1→3 side of the trimannosyl core. These results clearly indicate that eCGβ and eLHβ possess structurally distinct N-linked oligosaccharides in addition to different charge groups even though they have a protein moiety identical to each other. Our results suggest that the biological activity of these hormones might be modulated by its terminal charge groups and stem structures of carbohydrate moiety synthesized in different organs.

Gonadotropins, including pituitary lutropin (LH),[1] follicle stimulating hormone (FSH), and placental chorionic gonadotropin (CG), are a closely related family of glycoproteins. They are heterodimers composed of noncovalently associated α- and β-subunits. The α-subunits are identical in the amino acid sequence within the same animal species, whereas β-subunits are different. Therefore, the β-subunit has been considered to direct each hormone specificity (Pierce & Parsons, 1981; Stockell Hartree & Renwick, 1992). However, the complete amino acid sequences of equine CG (eCG) and equine LH (eLH) β-subunits have an identical sequence (Sugino et al., 1987; Bousfield et al., 1987), and there is only a single β gene in equine (Sherman et al., 1992). LH and CG recognize a common receptor, but eCG has a less active receptor binding activity and stimulation of steroidogenesis than eLH (Light et al., 1979; Aggarwal & Papkoff, 1981). Since they have identical protein moiety, their hormonal difference is most likely to be directed by the carbohydrate portion. In fact, the biological activity of eCG is increased by desialylation (Aggarwal & Papkoff, 1981).

Carbohydrate moiety of gonadotropins is essential for evoking the biological activity, subunit association, correct folding of protein structure, secretion, and in vivo survival but not for hormone-receptor binding (Ryan et al., 1988;

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* To whom correspondence should be addressed. Telephone: 011-81-562-93-9381; Fax: 011-81-562-93-8832

‡ Fujita Health University.

§ Present address: Department of Industrial Chemistry, Tokai University, 1117 Kitakaname, Hiratsuka 259-12, Japan.

⊥ Tokyo Institute of Technology.

∥ Wichita State University.

○ University of Tokushima.

University of Texas.

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[1] Abbreviations: CG, chorionic gonadotropin; Con A, concanavalin A; E-PHA, *Phaseolus vulgaris* agglutinin E4; FSH, follicle stimulating hormone; GS-II, *Griffonia simplicifolia* agglutinin II; HexNAc, *N*-acetylhexosamine; LH, lutropin (luteinizing hormone); RCA120, *Ricinus communis* agglutinin 120; WFA, *Wistaria floribunda* agglutinin. Subscript OT denotes NaB^3H_4-reduced oligosaccharides. All sugars described in this paper were of D-configuration except for fucose with L-configuration.

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Drickamer, 1991; Kobata, 1992; Stockell Hartree & Renwick, 1992). The structure of oligosaccharides of bovine, ovine, and human gonadotropins have been already reported (Baenziger & Green, 1988; Green & Baenziger, 1988; Stockell Hartree & Renwick, 1992; Kobata, 1992). In addition to a remarkable heterogeneity, distinct differences in N-linked oligosaccharide structure exist among those gonadotropins.

Both eCGβ and eLHβ possess a single N-glycosylation site and several O-glycosylation sites (Sugino et al., 1987; Bousfield et al., 1987). Structural analysis of sugar chains of eCG and eLH has been performed by several groups (Anumula & Bahl, 1986; Damm et al., 1990; Matsui et al., 1991; Smith et al., 1993). In our previous paper (Matsui et al., 1991), we showed differences in the charge group and size of the N-linked oligosaccharides of each β-subunit. Oligosaccharides of both β-subunits were highly acidic, but eCGβ contained only sialylated oligosaccharides, whereas eLHβ contained both sialylated and sulfated carbohydrate moieties. Oligosaccharides of eCGβ were relatively larger than those of eLHβ. The major structure of N-linked oligosaccharides of eCG and eLH released by peptide-*N*-glycosidase F has been recently reported (Damm et al., 1990; Smith et al., 1993). In the present study, we determined in detail the structure of N-linked oligosaccharides liberated from each β-subunit by hydrazinolysis and found that both β-subunits possess distinct oligosaccharide structures with a highly complex microheterogeneity more than that previously reported.

EXPERIMENTAL PROCEDURES

Materials and Chemicals. Purification of eCGβ and eLHβ and preparation of tritium-labeled N-linked oligosaccharides (Takasaki et al., 1982) of β-subunits have been previously described (Sugino et al., 1987; Bousfield et al., 1987; Matsui et al., 1991). Radioactive oligosaccharide mixtures were neutralized by digestion with sialidase or methanolytic desulfation (Yamashita et al., 1983a) and isolated by paper electrophoresis as described earlier (Matsui et al., 1991). Concanavalin A (Con A)−Sepharose and Sephadex LH-20 were purchased from Pharmacia (Uppsala). Agarose beads coupled with *Wistaria floribunda* agglutinin (WFA), *Griffonia simplicifolia* agglutinin II (GS-II), and *Phaseolus vulgaris* agglutinin E_4 (E-PHA) were obtained from E-Y Laboratories (San Mateo, CA). The *Ricinus communis* agglutinin 120 (RCA120) HPLC column was purchased from Honen (Tokyo). The following glycosidases were from Seikagaku Kogyo (Tokyo): jack bean β-galactosidase, β-*N*-acetylhexosaminidase, α-mannosidase, and snail β-mannosidase. *Diplococcus pneumoniae* β-galactosidase and β-*N*-acetylhexoaminidase were purified from the culture fluid as described by Glasgow et al. (1977) or purchased from Boehringer Mannheim. α-Fucosidase from bovine epididymis was obtained from Sigma (St. Louis, MO). Sialidase purified from *Arthrobacter ureafaciens* was from Nacalai Tesque (Kyoto). *Aspergillus saitoi* α-mannosidase, which specifically cleaves the Manα1→2Man linkage (Yamashita et al., 1980a), was purified from Morushin as described previously (Ichishima et al., 1991).

Glycosidase Digestion. Radioactive oligosaccharides [(0.3−10) × 10^4 cpm] were digested with glycosidases as described previously by our group (Matsui et al., 1992) unless otherwise described. Digestion with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase was performed in 80 μL of 0.25 M citrate phosphate buffer (pH 6.0) containing 90 mM mannose. The enzyme mixture showed 2 mU of β-galactosidase and 5 mU of β-*N*-acetylglucosaminidase activities, respectively, when it was assayed with *p*-nitrophenyl (PNP) sugars as substrates (1 U represents enzyme activity which hydrolyzes 1 μmol of each PNP substrate in 1 min at pH 5.0 and 37 °C). Under conditions of the experiment, jack bean β-*N*-acetylhexosaminidase contained 0.7 U of β-*N*-acetylglucosaminidase and 0.5 U of β-*N*-acetylgalactosaminidase activities in 60 μL of 0.2 M citrate phosphate buffer (pH 5.0), whereas diplococcal β-*N*-acetylhexosaminidase showed 5 mU and 0.2 mU of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities, respectively. In the conditions used, jack bean β-*N*-acetylhexosaminidase effectively hydrolyzes both terminal GlcNAc and GalNAc residues, while the diplococcal enzyme hydrolyzes only the GlcNAc residue. Diplococcal β-galactosidase specifically hydrolyzes the Galβ1→4 linkage but not the Galβ1→3 or Galβ1→6 linkage (Paulson et al., 1978). Diplococcal β-*N*-acetylhexosaminidase hydrolyzes the GlcNAcβ1→2 or GlcNAcβ1→3 linkage but not the GlcNAcβ1→4 or GlcNAcβ1→6 linkage (Yamashita et al., 1981). It hydrolyzes non-bisected triantennary complex-type oligosaccharide derivatives of [GlcNAcβ1→6-(GlcNAcβ1→2)Manα1→6](GlcNAcβ1→2Manα1→3)-Manβ1→R(C-2,6-branched) and GlcNAcβ1→2Manα1→6-[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3]Manβ1→R(C-2,4-branched) differently and converts them into GlcNAcβ1→2-(GlcNAcβ1→6)Manα1→6(Manα1→3)Manβ1→R and Man-α1→6(GlcNAcβ1→4Manα1→3)Manβ1→R (authentic structure VI) where R is →4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OT}$ by releasing one and two GlcNAc residues, respectively (Yamashita et al., 1981). It releases only one GlcNAcβ1→2 residue linked to the Manα1→3 branch from degalactosylated bi- and triantennary derivatives with bisecting GlcNAc and tetraantennary derivatives of oligosaccharides (Yamashita et al., 1981; Renwick et al., 1987). Jack bean α-mannosidase hydrolyzes one Man residue from the Galβ1→4Glc-NAcβ1→2Manα1→6(Manα1→3)Man structure but not from the Galβ1→4GlcNAcβ1→2Manα1→3(Manα1→6)Man structure under the conditions used (Yamashita et al., 1980b). The distribution of *N*-acetyllactosamine side chains either on the Manα1→3 or the Manα1→6 branch was determined using this specificity as described previously (Mizuochi et al., 1987; Matsui et al., 1992).

Methylation Analysis. The intact or the desialylated oligosaccharides (20 nmol each) from eCGβ and the intact or the desulfated oligosaccharides (15 nmol each) from eLHβ were methylated as described by Hakomori (1964). Methylated oligosaccharides were purified on a Sephadex LH-20 column and processed as described (Kubo et al., 1988). Partially methylated alditol acetate derivatives were analyzed with a QP-2000A (Shimadzu) gas chromatogram−mass spectrometer using a capillary column of ULBON HR-1 (0.25 mm × 25 m, Chromato Packings Center, Kyoto).

Affinity Chromatography. Affinity chromatography on various lectin columns was performed as described previously (Mizuochi et al., 1987; Matsui et al., 1992). Briefly, a Con A−Sepharose column [0.7 × 13 cm or minicolumn (0.5 mL)] was equilibrated with TBS (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) and eluted with TBS containing 100 mM α-methylmannoside. RCA120 HPLC was performed at 30 °C using PBS (10 mM sodium

phosphate buffer, pH 7.2, containing 150 mM NaCl) containing 50 mM lactose for elution. An E-PHA−agarose column (0.7 × 15 cm) was equilibrated and eluted with PBS at 25 °C. A WFA−agarose column (0.5 × 10 cm) was equilibrated with PBS and eluted with PBS containing 100 mM GalNAc at 4 °C. A GS-II−agarose column (0.5 × 10 cm) was equilibrated with PBS containing 0.5 mM $CaCl_2$ and eluted with PBS containing 100 mM GlcNAc at 4 °C. Elution was monitored by measuring the radioactivity of the eluate.

Other Methods. Bio-Gel P-4 (Bio-Rad, Richmond, VA) column chromatography was performed as described by Kobata et al. (1987) using authentic radioactive oligosaccharides as the standard (Matsui et al., 1992). Sugar composition and reducing-terminal sugar analyses were performed according to Takasaki and Kobata (1978). Sulfate was estimated using the sodium rhodizonate method (Terho & Hartiala, 1971).

RESULTS

Methylation Analysis of Oligosaccharides from eCG and eLH β-Subunits. Composition analysis of N-linked oligosaccharides released from eCG and eLH β-subunits by hydrazinolysis indicated the presence of NeuAc, Gal, GlcNAc, Man, and Fuc residues. The GalNAc residue was detected in eLH. About 0.9 nmol of sulfate was also identified in 1 nmol of N-linked oligosaccharides of eLHβ but not in eCGβ. Only *N*-acetylglucosaminitol was detected as the reducing-terminal sugar of both tritium-labeled oligosaccharides, indicating that contamination of O-linked sugar chains is minimum, if any.

The intact and desialylated oligosaccharides of eCGβ were subjected to methylation analysis without further fractionation. As shown in Table 1, 2,3,4,6-tetra-*O*-methylgalactitol, 2,3,4-tri-*O*-methylfucitol, and 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol were detected as derivatives of nonreducing-terminal sugars, and 1,3,5-tri-*O*-methyl and a trace amount of 1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitols were detected as reducing-terminal sugars. These results indicate that oligosaccharides of eCGβ contain Gal, Fuc, and GlcNAc as nonreducing-terminal and C-4,6-substituted GlcNAc as the major reducing-terminal sugar. Detection of 3,4,6-tri-, 2,4-di-, 3,6-di-, 3,4-di-, and 2-mono-*O*-methylmannitols indicated that those Man residues were substituted at positions of C-2; C-3,6; C-2,4; C-2,6; and C-3,4,6 by other sugars. The inner GlcNAc residues were all substituted at the C-4 position. 2,3,4,6-Tetra-*O*-methylgalactitol increased upon desialylation whereas 2,4,6-tri- and 2,3,4-tri-*O*-methylgalactitols decreased, but no significant change in the molar ratio of other sugar derivatives was observed. These results indicate that sialic acid residues were linked to the C-6 and C-3 positions of Gal residues in the intact oligosaccharides of eCGβ.

The intact and desulfated oligosaccharide fractions of eLHβ were subjected to methylation analysis (Table 1). In eLHβ, 2,3,4,6-tetra-*O*-methylmannitol and 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol were also detected as derivatives of nonreducing-terminal sugars, indicating that eLHβ contains Man and GalNAc residues at the nonreducing terminal in addition to Gal, Fuc, and GlcNAc residues. The inner GlcNAc residues were all substituted only at the C-4 position, and Man residues were substituted at C-2; C-3; C-3,6; and C-3,4,6 positions by other sugars. An increase in 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol and a decrease in 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol after desulfation indicate that sulfate was linked to the C-4 position of nonreducing-terminal GalNAc residues.

Table 1: Methylation Analysis of N-Linked Oligosaccharides of eCG and eLH β-Subunits

Methylated sugars	Molar ratio [a] eCG β		eLH β	
	Intact	Desialylated	Intact	Desulfated
Fucitol				
2,3,4-tri-*O*-methyl (1,5-di-*o*-acetyl)	0.8	0.8	0.8	0.8
Galactitol				
2,3,4,6-tetra-*O*-methyl (1,5-di-*o*-acetyl)	0.1	2.4	0.1	0.1
2,4,6-tri-*O*-methyl (1,3,5-tri-*o*-acetyl)	1.0	0.5	0.5	0.4
2,3,4-tri-*O*-methyl (1,5,6-tri-*o*-acetyl)	0.9	0	0	0
Mannitol				
2,3,4,6-tetra-*O*-methyl (1,5,-di-*o*-acetyl)	0	0	0.4	0.3
3,4,6-tri-*O*-methyl (1,2,5-tri-*o*-acetyl)	1.7	1.7	1.8	1.8
2,4,6-tri-*O*-methyl (1,3,5-tri-*o*-acetyl)	0	0	0.1	0.1
2,4-di-*O*-methyl (1,3,5,6-tetra-*o*-acetyl)	1.0	1.0	1.0	1.0
3,4-di-*O*-methyl (1,2,5,6-tetra-*o*-acetyl)	trace [b]	trace	0	0
3,6-di-*O*-methyl (1,2,4,5-tetra-*o*-acetyl)	0.2	0.2	0	0
2-mono-*O*-methyl (1,3,4,5,6-penta-*o*-acetyl)	0.2	0.2	0.3	0.2
2-*N*-Methylacetamido-2-deoxyglucitol				
1,3,5,6-tetra-*O*-methyl (4-mono-*o*-acetyl)	trace	trace	trace	trace
1,3,5-tri-*O*-methyl (4,6-di-*o*-acetyl)	1.1	1.1	1.1	1.1
3,4,6-tri-*O*-methyl (1,5-di-*o*-acetyl)	0.2	0.2	0.3	0.2
3,6-di-*O*-methyl (1,4,5-tri-*o*-acetyl)	4.5	4.8	2.7	2.6
2-*N*-Methylacetamido-2-deoxygalactitol				
3,4,6-tri-*O*-methyl (1,5-di-*o*-acetyl)	0	0	trace	0.9
3,6-di-*O*-methyl (1,4,5-tri-*o*-acetyl)	0	0	0.5	0

[a] Numbers were calculated by taking the value of 2,4-di-*O*-methylmannitol as 1.0. [b] Less than 0.05.

Structural Analysis of N-Linked Oligosaccharides of eCGβ. (a) Fractionation of Desialooligosaccharides from eCGβ by Lectin Column Chromatography. Radioactive oligosaccharides released from eCGβ were separated by paper electrophoresis into neutral, mono-, di-, and trisialylated fractions in a molar ratio of approximately 19:44:30:7 as calculated from radioactivities. Acidic fractions were neutralized by exhaustive sialidase digestion. A mixture of the desialooligosaccharides was sequentially fractionated on Con A−Sepharose and E-PHA−agarose columns into fractions I, II, and III in a molar ratio of 60:18:22 (Table 2).

(b) Structural Studies of Fraction I Bound to Con A Column. Affinity of fraction I to a Con A column (Table 2) indicated that the oligosaccharides in fraction I must contain at least two nonsubstituted α-mannosyl residues at C-3, C-4, and C-6 positions (Ogata et al., 1975; Matsui et al., 1992). Fraction I contained multiple components (Figure 1a). However, upon digestion of fraction I with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase, about 90% of the radioactivity was eluted on a Bio-Gel P-4 column at approximately 8 and 7 glucose units (reference markers) in a ratio of about 90:10. This is the same position as authentic trimannosyl core structures with (oligosaccharide II) or without Fuc residue (oligosaccharide

Table 2: Fractionation of Neutralized Oligosaccharides of eCG and eLH β-Subunits by Serial Lectin Chromatography

Neutralized fractions		Lectin column					Molar ratio (%)	Designation
		WFA	GS-II	ConA	E-PHA	RCA120		
eCG	Desialo oligosaccharides			+[a]			60	I
				-	ret		18	II
				-	-		22	III
eLH	Fraction S2	+	-	+		-	27	S2
	Fraction S1	+	-	-		-	11	S1-A
		+	-	+		-	14	S1-B
		+	-	+		ret	3.0	S1-C
	Fraction NS	+	-	+	-	ret	8.3	NS-A
		+	-	+	-	-	2.7	NS-B
	Fraction N	-			-	-	7.4	N-A
		-			-	ret	4.6	N-B
		-			-	ret	2.4	N-C
		-			-	ret	1.4	N-D
		-			-	+	1.4	N-E
		-			ret	ret	2.2	N-F
		-			ret	+	10.2	N-G
		+					4.4	N-H

[a] (+) bound; (−) breakthrough; (ret) retarded.

III), respectively. The remaining 10% was eluted at about 14 glucose units (Figure 1b). Trimannosyl core structures were confirmed by further sequential digestions. These results suggest that the majority of fraction I was bi- or monoantennary complex-type sugar chains with or without *N*-acetyllactosamine (Galβ1→4GlcNAcβ1→) repeats.

Fraction I was separated into five fractions, I-1 to I-5, accounting for 3, 35, 14, 5, and 3% of the total desialo-oligosaccharides, respectively, on a Bio-Gel P-4 column as shown in Figure 1a. Sequential exoglycosidase digestion of these five fractions is summarized in Table 3, and the deduced structures are shown in Table 5.

(c) Structural Studies on Fraction II Retarded on E-PHA Column. Fraction II showed no affinity to a Con A column but was retarded on an E-PHA column (Table 2), suggesting that it contained bisected oligosaccharides with a structure of Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)[R_1→4-(R_2→4GlcNAcβ1→2)Manα1→3]Manβ1→4, where R_1 and R_2 are either hydrogens or sugars (Yamashita et al., 1983b). Fraction II was separated into one major (II-1) and two minor (II-2 and II-3) fractions corresponding to 14, 3, and 1% of the total oligosaccharides, respectively, on a Bio-Gel P-4 column as shown in Figure 1c. Fraction II-1 was eluted at the same position as authentic bisected biantennary complex-type oligosaccharide IV. Two Gal and three GlcNAc residues were released from fraction II-1 by digestion with jack bean β-galactosidase followed by β-*N*-acetylhexosaminidase, and the products were eluted at the trimannosyl core structures with and without a Fuc residue at a ratio of approximately 95:5 (Figure 1d, solid line). On the other hand, two Gal and only one GlcNAc residues were removed by diplococcal enzymes (Table 4), and the products were eluted at the position of authentic oligosaccharide V with 11 glucose units (Figure 1d, dotted line). From the specificities of diplococcal enzymes described above, methylation data and its affinity to lectin columns, fraction II-1 was deduced to have the bisected biantennary complex-type oligosaccharide structure as shown in Table 5. Sequential digestion of other fractions is also summarized in Table 4. As shown in Table 5, fraction II was a mixture of bisected, biantennary, and C-2,4-branched triantennary complex-type

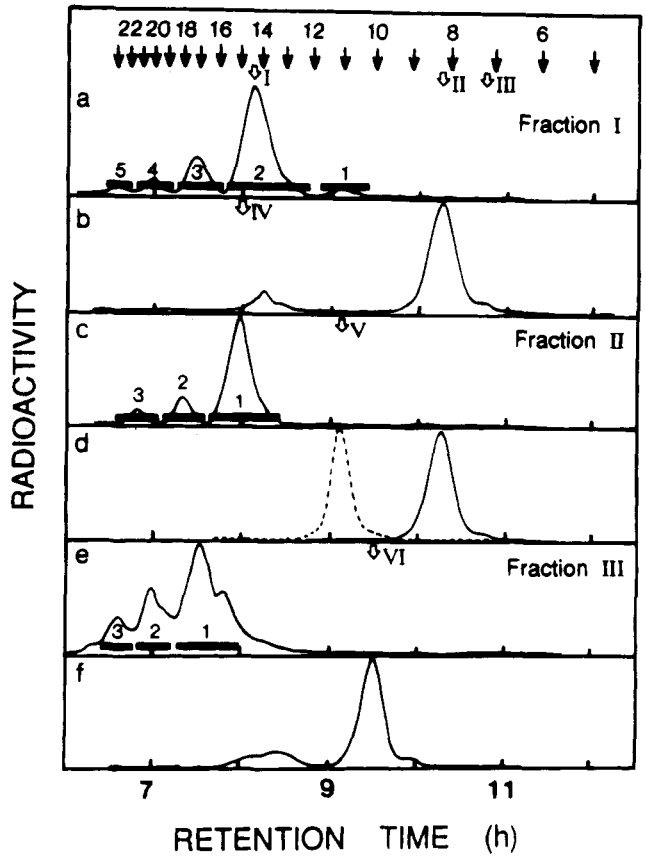


FIGURE 1: Separation on a Bio-Gel P-4 column of fractions I, II, and III of eCGβ before and after digestion with exoglycosidases as described in the text. Solid arrows indicate elution positions of glucose oligomers (numbers indicate the glucose units) added as internal standards. Open arrows indicate elution positions of authentic oligosaccharides: I, Galβ1→4GlcNAcβ1→2Manα1→6-(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4R; II, Manα1→6-(Manα1→3)Manβ1→4R; III, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$; IV, Galβ1→4GlcNAcβ1→2Manα1→6-(GlcNAcβ1→4)(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4R; V, GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Manα1→3)-Manβ1→4R; VI, Manα1→6(GlcNAcβ1→4Manα1→3)Manβ1→4R [where R is GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$].

Table 3: Sequential Exoglycosidase Digestion of Oligosaccharide Fractions from Fraction I of eCG β-Subunit

Fraction (% molar ratio)	Number of residues released by each digestion[a] 1st *DP-Gal'ase*	2nd *DP-HN'ase*	3rd *DP-Gal'ase*	4th *DP-HN'ase*	5th *DP-Gal'ase*	6th *DP-HN'ase*	Total residues released[b] Gal	GlcNAc
I-1 (3%)	1 ⟶	1					1	1
I-2 (35%)	2 ⟶	2					2	2
I-3 (14%)	2 ⟶	[2 (60%) → 1 ⟶ 1 [N.D.[c] (40%)					3	3
I-4 (5%)	[2 (80%) → [2 (80%) → [1 (90%) → 1 ⟶ 1 ⟶ 1 (I-4a) [N.D. (20%) [N.D. (20%) [2 (10%) → 2 (I-4b)						4 4	4 4
I-5 (3%)	[2 (70%) → 2 ⟶ (*DP-Mix* Gal 3, GlcNAc 3) [N.D. (30%)						5	5

[a] Oligosaccharides of each fraction were sequentially digested with diplococcal β-galactosidase (*DP-Gal'ase*) and β-*N*-acetylhexosaminidase (*DP-HN'ase*) or a mixture of them (*DP-Mix*). Number of residues released by each digestion and % molar ratio (parentheses) were expressed. [b] Total residues released by sequential digestion until it becomes trimannosyl core structures of Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. [c] Not determined.

Table 4: Sequential Exoglycosidase Digestion of Oligosaccharide Fractions from Fractions II and III of eCG β-Subunit

Fraction (% molar ratio)	Number of residues released by digestion[a] 1st *DP-Gal'ase*	2nd *DP-HN'ase*	3rd *JB-HN'ase*	Total residues released[b] Gal	GlcNAc
II-1 (14%)	2 ⟶	1 ⟶	2	2	3
II-2 (3%)	[3 (40%) ⟶	1 ⟶	3	3	4
	[N.D.[c] (60%)				
II-3 (1%)	[3 (60%) ⟶ (*DP-Mix* Gal 1, GlcNAc 2) → 3			4	5
	[N.D. (40%)				
III-1 (11%)	[3 (75%) ⟶ [2 (70%) ⟶ 1			3	3
	[N.D. (25%) [N.D. (30%)				
III-2 (5%)	[3 (65%) ⟶ (*DP-Mix* Gal 1, GlcNAc 3) → 1 (III-2a)			4	4
	[4 (10%) ⟶ 1 ⟶ 3 (III-2b)			4	4
	[N.D. (25%)				
III-3 (4%)	[3 (45%) ⟶ (*DP-Mix* Gal 2, GlcNAc 4) → 1 (III-3a)			5	5
	[4 (20%) → (*DP-Mix* Gal 1, GlcNAc 2) → 3 (III-3b)			5	5
	[N.D. (35%) (70%)				
	[N.D. (30%)				

[a] Oligosaccharides of each fraction were sequentially digested with *DP-Gal'ase*, *DP-HN'ase*, and jack bean β-*N*-acetylhexosaminidase (*JB-HN'ase*) or a mixture of dipolococcal enzymes (*DP-Mix*). Number of residues released by each digestion and % molar ratio (parentheses) were expressed. [b] Total residues released by sequential digestion until each becomes trimannosyl core structures of Manα1→6(Manα1→3)-Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. [c] Not determined.

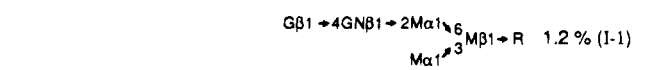
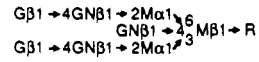
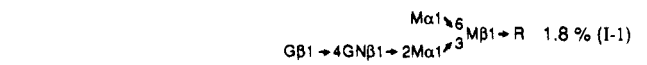
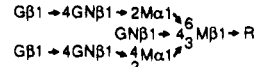
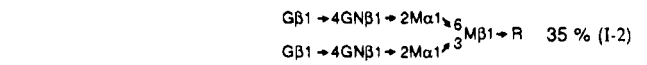
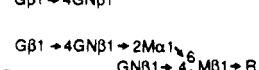
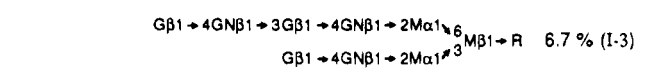
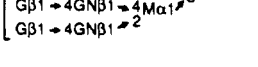
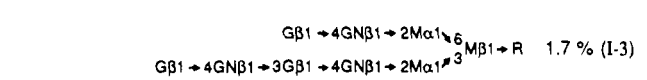
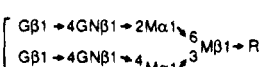
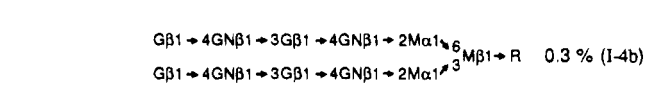
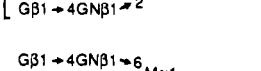
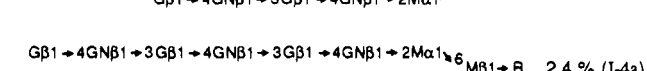
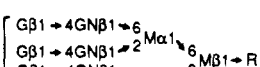
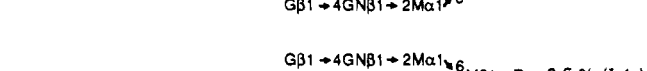
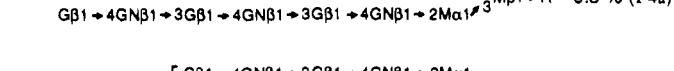
oligosaccharides. The presence of a bisected C-2,6-branched structure in this fraction can be eliminated because it does not interact with an E-PHA column (Yamashita et al., 1983b).

(d) Structural Studies on Fraction III with No Affinity to Con A and E-PHA Columns. Fraction III passed through both Con A and E-PHA columns and appeared to contain various components ranging from 14 to 24 glucose units as analyzed with a Bio-Gel P-4 column (Figure 1e). Upon digestion with a diplococcal enzyme mixture, approximately 70% of fraction III was converted to a peak at about 10 glucose units (Figure 1f), the same position as authentic oligosaccharide VI, which is the product of C-2,4-branched triantennary complex-type oligosaccharides with the fucosylated trimannosyl core structure obtained by digestion with a diplococcal enzyme mixture (Renwick et al., 1987; Matsui et al., 1992). The remaining 30% of fraction III was eluted as a mixture of minor broad peaks around 13 glucose units. The peak at 10 glucose units was converted to the trimannosyl core position after releasing one GlcNAc residue by digestion with jack bean β-*N*-acetylhexosaminidase. These results indicate that fraction III contains C-2,4-branched triantennary chains as the major backbone structure. Fraction III was separated into three major fractions (III-1, III-2, and III-3) accounting for 11, 5, and 4% of the total oligosaccharides, respectively, as shown in Figure 1e, and other minor components (2%) were not analyzed. On the basis of sequential exoglycosidase digestions (Table 4) and methylation data, about 60% of fraction III was elucidated to be a mixture of triantennary (C-2,4-branched) and tetraantennary complex-type oligosaccharides with or without a *N*-acetyllactosamine repeat(s) (Table 5).

Structural Analysis of N-Linked Oligosaccharides of eLHβ. (a) Fractionation of Radioactive Oligosaccharides of eLHβ. Radioactive oligosaccharides of eLHβ were fractionated into neutral, monosialylated, disialylated, monosulfated, disulfated, and monosialylated/monosulfated fractions by paper electrophoresis in combination with sialidase digestion and methanolytic desulfation as previously described by our laboratory (Matsui et al., 1991). Desialylated oligosaccharides from monosialylated and disialylated fractions were combined with the neutral fraction and designated as fraction N. Other neutralized oligosaccharides originating from monosulfated, disulfated, and monosialylated/monosulfated fractions were designated as fractions S1, S2, and NS, respectively. The molar ratio of fractions N, S1, S2, and NS calculated from radioactivity incorporated was 34:28:27:11. Each fraction was further fractionated by serial lectin affinity chromatographies (Table 2) and subjected to sequential exoglycosidase digestions.

(b) Structural Studies of Fraction S2 Originating from Disulfated Fraction. Fraction S2 was bound to WFA and Con A columns, but neither retardation nor binding was observed when it was applied to GS-II or RCA120 columns (Table 2). Since WFA (Smith & Torres, 1989; Yamashita

Table 5: Proposed Structures for Desialooligosaccharides Contained in Fractions I, II, and III Derived from eCG β-Subunit

Proposed structures and % molar ratio[a] (derived fraction)	R= →4 GNβ1→ 4 (±Fα1→ 6) GN_{OT}[b]
 1.2 % (I-1)	 14 % (II-1)
 1.8 % (I-1)	 1.2 % (II-2)
 35 % (I-2)	 0.6% (II-3)
 6.7 % (I-3)	 5.8% (III-1)
 1.7 % (I-3)	 n = 1, 3.3% (III-2a) n = 2, 1.8% (III-3a)
 0.3 % (I-4b)	 0.5% (III-2b)
 2.4 % (I-4a)	 0.6% (III-3b)
 0.5 % (I-4a)	
 2.1 % (I-5)	

not determined : 20.5 % (Fraction I, 8.3%; Fraction II, 2.2%; Fraction III, 10%)

[a] G, galactose; F, fucose; GN, *N*-acetylglucosamine; M, mannose; GN_{OT}, [*N*-^{3}H]acetylglucosaminitol. [b] More than 90% of each oligosaccharide has a fucosylated core structure.

et al., 1989), GS-II (Ebisu et al., 1978), and RCA120 (Mizuochi et al., 1987) show a high affinity to oligosaccharides possessing nonreducing-terminal GalNAC, GlcNAc, and Gal residues, respectively, the oligosaccharides in fraction S2 were throught to contain the terminal GalNAc residue. Binding to a Con A column indicated the presence of at least two Man residues nonsubstituted at the C-3, C-4, and C-6 positions (Ogata et al., 1975). Fraction S2 was eluted on a Bio-Gel P-4 column as a single peak at about 16 glucose units (Figure 2a). It was insusceptible to digestion by diplococcal β-*N*-acetylhexosaminidase, jack bean β-galactosidase, and α-mannosidase but converted with jack bean β-*N*-acetylhexosaminidase to the trimannosyl core structures after releasing four *N*-acetylhexosamine residues (Figure 2b, solid line). The product further released two Man residues on digestion with jack bean α-mannosidase and was eluted at the position of authentic oligosaccharide VII (Figure 2b, dotted line). By paper electrophoresis after acid hydrolysis (3 N HCl, 100 °C, 3h) (Takasaki & Kobata, 1978), the ratio of GlcNAc and GalNAc residues in fraction S2 was determined to be approximately 1:0.6, indicating that it is composed of $GalNAc_2GlcNAc_2Man_3GlcNAc(\pm Fuc)GlcNAc_{OT}$. Methylation data indicated that all GalNAc residues existed at the nonreducing terminal in desulfated oligosaccharides and that the inner GlcNAc residues were substituted only at the C-4 position (Table 1). Based on these results, it was assumed that oligosaccharide structures of fraction S2 were (GalNAcβ1→4GlcNAcβ1→2Manα1→6)(GalNAcβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4-(±Fucα1→6)$GlcNAc_{OT}$ (Table 8).

(c) Structural Studies of Fraction S1 Derived from Monosulfated Fraction. All oligosaccharides in fraction S1 were adsorbed to a WFA−agarose column and passed through a GS-II column without any retardation (Table 2), suggesting the presence of a terminal GalNAc residue in this fraction. Fraction S1 showing multiple peaks (Figure 2c) was separated into three subfractions (S1-A, S1-B, and S1-C) in a molar ratio of 11:14:3 on RCA120 and Con A affinity columns.

Fraction S1-A with no affinity to either the RCA120 or the Con A column was further separated on a Bio-Gel P-4 column into major (S1-A2) and minor (S1-A1) components in a ratio of about 70:30 eluting at 13 and 11 glucose units, respectively (Figure 2d). Fraction S1-B, which was retained on a Con A column but showed no affinity to a RCA120 column, was separated into three fractions (S1-B1, S1-B2, and S1-B3) in a ratio of 65:26:9 by Bio-Gel P-4 column chromatography (Figure 2e). Results of sequential digestion (Table 6), methylation, sugar composition (data not shown), and affinity on lectin columns indicate that fraction S1 was a mixture of complex and high-mannose-type oligosaccharides with GalNAc-GlcNAc chain(s) as shown in Table 8. Oligosaccharides in fraction S1-B3 and S1-C have the same structure as those found in fractions S2 and NS-A (see below), respectively.

(d) Structural Studies of Fraction NS Derived from Monosialylated/Monosulfated Fraction. Fraction NS (Figure 2g) was bound to WFA− and Con A−agarose columns but passed through GS-II and E-PHA columns. Fraction NS was separated on a RCA120 column into two subfractions [NS-

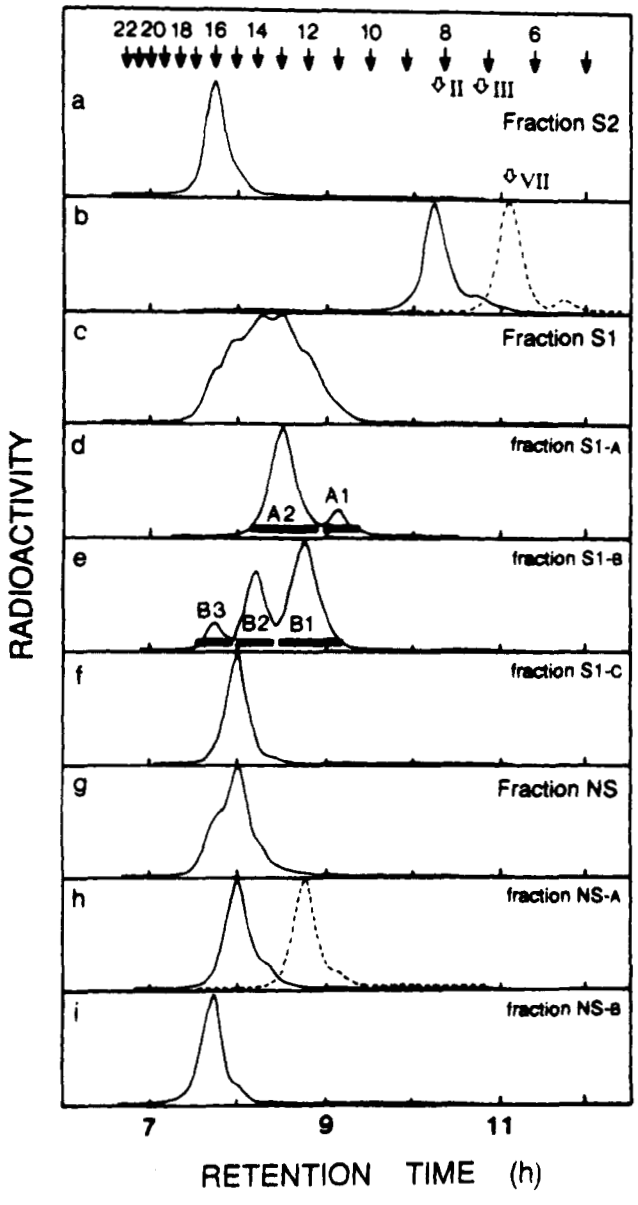


FIGURE 2: Separation on a Bio-Gel P-4 column of fractions S2, S1, and NS of eLHβ and their subfractions obtained by lectin columns. Fraction S2 before (a) and after (b, solid line) digestion with jack bean β-*N*-acetylhexosaminidase followed by α-mannosidase (b, dotted line). Fraction S1 before (c) and after (d–f) fractionation on lectin columns (Table 2). Fractions S1-A and S1-B were further fractionated into two (d) and three (e) fractions, respectively. Fraction NS before (g) and after (h, i) separation on lectin columns (Table 2) and after digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase (h, dotted line). Open arrows point to elution positions of authentic oligosaccharides: II and III, see Figure 1; VII, Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$.

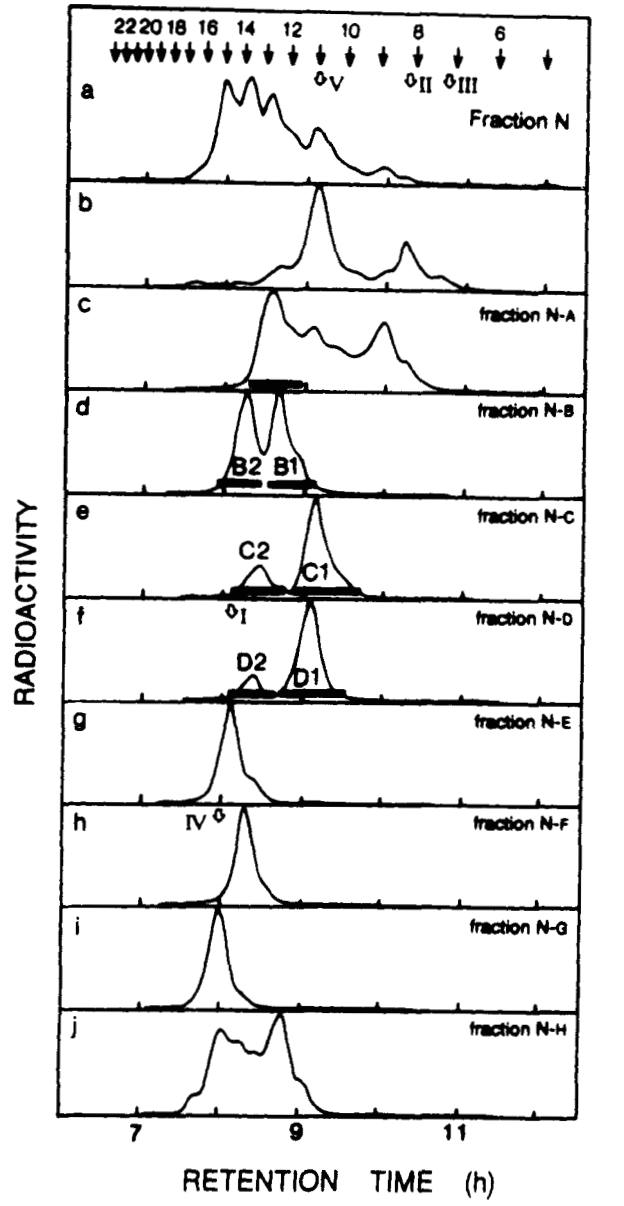


FIGURE 3: Separation on a Bio-Gel P-4 column of fraction N of eLHβ and its subfractions obtained by lectin columns. Fraction N before (a) and after (b) digestion with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase and after separation on lectin columns as shown in Table 2 (c–j). Fractions N-A, N-B, N-C, and N-D were further fractionated into one or two fractions, respectively. For authentic oligosaccharides, see Figure 1.

B (pass-through fraction) and NS-A (retarded fraction)] in a ratio of 25:75 (Table 2). These data indicate that fraction NS-A contained both nonreducing-terminal Gal and GalNAc residues. Fraction NS-A was eluted on a Bio-Gel P-4 column as a single peak at about 15 glucose units (Figure 2h, solid line). One Gal residue was removed upon digestion with diplococcal β1→4 galactosidase. When the product was further digested with diplococcal β-*N*-acetylhexosaminidase, one GlcNAc residue was released and eluted at the same position as subfraction S1-B1 from fraction S1 (Figure 2h, dotted line). In a separate experiment, upon incubation with jack bean β-*N*-acetylhexosaminidase but not with diplococcal enzyme, fraction NS-B released two HexNAc residues and was eluted at the same position as fraction I-1 of eCGβ. This product further released one Man residue upon digestion with jack bean α-mannosidase, indicating that the Galβ1→4GlcNAcβ1→2 chain is linked to the Manα1→6 side of core structures. On the basis of results of exoglycosidase digestion and methylation, fraction NS-A was deduced to have the structure shown in Table 8. On the other hand, fraction NS-B was deduced to have the same structure as Fraction S2. Since fractions NS and S2 were separated from the same acidic fraction after extensive sialidase digestion (Matsui et al., 1991), it is likely that oligosaccharides in fraction NS-B may be originally present in the disulfated fraction and separated by spontaneous desulfation during preparation.

(e) Structural Studies of Fraction N. Fraction N, a mixture of neutral and desialylated oligosaccharide fractions, was eluted on a Bio-Gel P-4 column as multiple peaks between 8 and 16 glucose units (Figure 3a). Preliminary studies using a mixture of diplococcal β-galactosidase and β-*N*-acetyl-

Table 6: Sequential Exoglycosidase Digestion of Oligosaccharide Fractions from Fraction S1 of eLH β-Subunit

Fraction (% molar ratio)	Number of residues released by each digestion[a]				Total residues released[b]		
	1st *DP-HN'ase*	2nd *JB-HN'ase*	3rd *JB-Man'ase*	1st *JB-Man'ase*	GalNAc	GlcNAc	α-Man
S1-A1 (3.3%)	0	2	1	0	1	1	1
S1-A2 (7.7%)	0	2	3	1	1	1	3
S1-B1 (9.1%)	0	2	2	0	1	1	2
S1-B2 (3.6%)	0 (50%)	2	4 (S1-B2a)	2	1	1	4
	1 (50%)	2	2 (S1-B2b)	0	1	2	2
S1-B3 (1.3%)	0	4	2	0	2	2	2

[a] Oligosaccharides of each fraction were sequentially digested with *DP-HN'ase*, *JB-HN'ase*, and jack bean α-mannosidase (*JB-Man'ase*). Separately, each fraction was digested with JB-Man'ase. Number of residues released by each digestion and % molar ratio (parentheses) were expressed. [b] Total residues released by sequential digestion until each becomes core structures of Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. Man residues were not released by digestion with *A. saitoi* α1→2 mannosidase.

Table 7: Sequential Exoglycosidase Digestion of Oligosaccharide Fractions from Fraction N of eLH β-Subunit

Fraction (% molar ratio)	Number of residues released by each digestion[a]					Total residues released[b]	
	1st *DP-Gal'ase*	2nd *DP-HN'ase*	3rd *JB-HN'ase*	1st *DP-HN'ase*	1st *JB-Man'ase*	Gal	GlcNAc
N-A (7.4%)	0	1 (18%)	2 (N-A1)			0	3
		2 (12%)	(N-A2)			0	2
		N.D.[c] (70%)					
N-B1 (2.3%)	1	1	1		0	1	2
N-B2 (2.3%)	1	1	2	0		1	3
N-C1 (1.7%)	1	1			1	1	1
N-C2 (0.7%)	1	2		1		1	2
N-D1 (1.0%)	1	1			0	1	1
N-D2 (0.4%)	1	2		1		1	2
N-E (1.4%)	2	2				2	2
N-F (2.2%)	1	1	2	1		1	3
N-G (10.2%)	2	1	2			2	3

[a] Oligosaccharides of each fraction were sequentially digested with *DP-Gal'ase*, *DP-HN'ase*, and *JB-HN'ase*. Separately, some fractions were digested with DP-HN'ase or *JB-Man'ase*. Number of residues released by each digestion and % molar ratio (parentheses) were expressed. [b] Total residues released by sequential digestion until each becomes trimannosyl core structures of Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. [c] Not determined.

hexosaminidase demonstrated that most peaks were converted to two major peaks in a ratio of approximately 70:30 at 11 and 8 glucose units where authentic oligosaccharide V and II eluted, respectively (Figure 3b). Subsequent digestion with jack bean β-*N*-acetylhexosaminidase revealed that almost all radioactivity was eluted at the core position. These results suggest that neutral or sialylated oligosaccharides of eLHβ are structurally of the biantennary or monoantennary complex-type oligosaccharides with or without bisecting GlcNAc residue. Fraction N was fractionated by serial lectin affinity chromatography into eight fractions (N-A to N-H) as shown in Table 2. Fractions N-B, N-C, and N-D passed through WFA and E-PHA columns but were retarded on a RCA120 HPLC column, indicating the presence of monogalactosylated structures. About 42% of fraction N was retarded on an E-PHA−agarose column and separated into two fractions (N-F and N-G) on a RCA120 HPLC column, indicating the presence of a bisected structure in these fractions. Fractions N-A to N-D were further fractionated on a Bio-Gel P-4 column (Figure 3) and subjected to exoglycosidase digestion as summarized in Table 7. The structure of these fractions, deduced by sequential digestions, methylation data, and affinity to lectin columns, is shown in Table 8. Fraction N-H, which was bound to a WFA−agarose column and accounted for about 13% of fraction N, contained multiple components as shown in Figure 3j. It was further fractionated on RCA120 and Con A columns and deduced to be a mixture of oligosaccharides found in fractions S1, S2, and NS in a molar ratio described in Table 8.

DISCUSSION

Proposed structures of neutralized N-linked oligosaccharides of eCGβ and eLHβ are shown in Tables 5 and 8, respectively. About 80% of the total N-linked oligosaccharides from eCGβ and 95% of those from eLHβ have been elucidated. In this study, we clearly demonstrated that both the hormones have structurally distinct and characteristic oligosaccharides with a highly complex microheterogeneity more than previously reported (Damm et al., 1990; Smith et al., 1993) even though they have only a single N-glycosylation site in the identical amino acid sequence (Sugino et al., 1987; Bousfield et al., 1987).

eCGβ contains mono-, bi-, tri-, and tetraantennary complex-type N-linked oligosaccharides (Table 5). This is in contrast to hCGβ which contains only biantennary oligosaccharides (Mizuochi & Kobata, 1980), resembling N-linked oligosaccharides of hFSH (Renwick et al., 1987). Interestingly, all identified triantennary complex-type oligosaccharides were

Table 8: Proposed Structures of Desialylated and Desulfated Oligosaccharides Contained in Fractions S1, S2, NS, and N from eLH β-Subunit

Proposed structure [a] [R= → 4 GNβ1→4 (±Fα1→6) GNoT] [b]	% Molar ratio (derived fraction) Sulfated [c]	Sialylated & Sulfated [c]	Neutral or Sialylated [c]
GaNβ1→4GNβ1→2Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	27 % (S2) 1.3 % (S1-B3)	2.7 % (NS-B)	0.2 % (N-H)
GNβ1→2Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	1.8 % (S1-B2b)		0.1 % (N-H)
Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	9.1 % (S1-B1)		1.8 % (N-H)
GaNβ1→4GNβ1→2Mα1↗3Mβ1→R	3.3 % (S1-A1)		0.6 % (N-H)
Mα1→3Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	7.7 % (S1-A2)		
(Mα1→)2Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	1.8 % (S1-B2a)		0.2 % (N-H)
Gβ1→4GNβ1→2Mα1↘6 / GaNβ1→4GNβ1→2Mα1↗3 Mβ1→R	3.0 % (S1-C)	8.3 % (NS-A)	1.5 % (N-H)
Gβ1→4GNβ1→2Mα1↘6 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			1.4 % (N-E)
Gβ1→4GNβ1→2Mα1↘6 / GNβ1→2Mα1↗3 Mβ1→R			0.7 % (N-C2)
GNβ1→2Mα1↘6 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			0.4 % (N-D2)
Gβ1→4GNβ1→2Mα1↘6 / Mα1↗3 Mβ1→R			1.7 % (N-C1)
Mα1↘6 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			1.0 % (N-D1)
GNβ1→2Mα1↘6 / GNβ1→2Mα1↗3 Mβ1→R			0.9 % (N-A2)
Gβ1→4GNβ1→2Mα1↘6 GNβ1→4↗3 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			10.2 % (N-G)
Gβ1→4GNβ1→2Mα1↘6 GNβ1→4↗3 / GNβ1→2Mα1↗3 Mβ1→R			2.2 % (N-F)
GNβ1→2Mα1↘6 GNβ1→4↗3 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			2.3 % (N-B2)
Mα1↘6 GNβ1→4↗3 / Gβ1→4GNβ1→2Mα1↗3 Mβ1→R			2.3 % (N-B1)
GNβ1→2Mα1↘6 GNβ1→4↗3 / GNβ1→2Mα1↗3 Mβ1→R			1.3 % (N-A1)
not determined	0 %	0 %	5.2 %
Total Contents	55 %	11 %	34 %

[a] G, galactose; F, fucose; GN, *N*-acetylglucosamine; GaN, *N*-acetylgalactosamine; M, mannose; GN_{OT}, [*N*-^{3}H]acetylglucosaminitol. [b] More than 80% of the oligosaccharides have a fucosylated core structure. [c] Initial fractions.

the C-2,4-branched type. Since tetraantennary complex-type oligosaccharides with both C-2,4- and C-2,6-branched structures were present in small amounts, we cannot exclude the possibility that C-2,6-branched triantennary complex-type oligosaccharides are also present in small amounts. This implies that GlcNAcβ1→4 transferase (IV) activity is stronger than GlcNAcβ1→6 transferase (V) activity in equine placenta.

eCGβ oligosaccharides uniquely contain about 20% of poly-*N*-acetyllactosamine structures. The major components are biantennary complex-type oligosaccharides with one to three *N*-acetyllactosamine repeats. Elongation of the *N*-acetyllactosamine units showed a preference to the Manα1→6 side rather than the Manα1→3 side. The poly-*N*-acetyllactosamine structure has been also identified in *O*-linked oligosaccharides of eCGβ (Anumula & Bahl, 1986; Damm et al., 1990; Matsui, unpublished observation). Although half of the *N*-linked oligosaccharides of eCGβ are biantennary complex-type with or without a bisecting GlcNAc residue, it seems likely that these oligosaccharides tend to elongate by making either poly-*N*-acetyllactosamine structures or branching structures.

eLHβ basically contains mono- and biantennary and hybrid-type oligosaccharides (Table 8). Neither poly-*N*-acetyllactosamine nor tri- and tetraantennary structures were detected in eLHβ in contrast to eCGβ. The side chain of mono- and biantennary oligosaccharides was GalNAcβ1→4GlcNAc in addition to Galβ1→4GlcNAc like ovine, bovine, and human LHs (Stockell Hartree & Renwick, 1992; Baenziger & Green, 1988). Smith et al. (1993) showed that the GalNAc-GlcNAc chain distributes to both Manα1→3 and Manα1→6 sides in the fraction NS of equine LH. However, we found that the GalNAc-GlcNAc side chain is strictly located at the Manα1→3 side of core structures in hybrid structures containing Man_4 or Man_5 residue, monoantennary chains, and in the mixed biantennary structure like hLH (Weisshaar et al., 1991). Contrary, Gal-GlcNAc side chains are located at both Manα1→3 and Manα1→6 sides. These results suggest that GalNAc transferase is more accessible to the Manα1→3 side. Both GalNAc and Gal transferases compete for common oligosaccharides as an acceptor, but the accessibility may be different with each structure in addition to the amino acid sequence motif (Smith & Baenziger, 1988; Dharmesh & Baenziger, 1993).

The proportion of bisected structure in eLHβ is similar to that in eCGβ, but the former contains rather a small amount of biantennary complex-type oligosaccharides that are dominant in eCGβ. We could not identify bisected structures in sulfated fractions of eLHβ, suggesting either that GlcNAc transferase (III), which synthesizes the bisecting GlcNAc structure, is inaccessible after the transfer of GalNAc or that GalNAc transferase cannot transfer it after transferring the bisecting GlcNAc residue.

hCG contains only the NeuAcα2→3Gal group, whereas sialic acid residues in eCGβ are mostly linked to the terminal Gal residue with a α2→6 linkage and partially with a α2→3 linkage in agreement with the finding of Damm et al. (1990). eLHβ also contains about 30% of sialylated oligosaccharides (Matsui et al., 1991). Although we have no methylation data on desialylated oligosaccharides of eLHβ due to limited amounts of the sample, the presence of C-3 substituted Gal but the absence of C-6 substituted Gal (Table 1), and the presence of all Gal residues as nonreducing terminal after neutralizing indicate that sialic acid residues in eLHβ are linked to the terminal Gal with a α2→3 linkage.

The biological significance of sugar chains in gonadotropins has not yet been fully understood. The presence of sialic acid residues, independent of sialyl linkage, is essential for the full expression of hormonal activity of hCG (Goverman et al., 1982; Keutmann et al., 1985; Ryan et al., 1988; Amano et al., 1989). Amano and Kobata (1993) recently demonstrated that sialic acid residue in hLH is also necessary for the full expression of hormonal activity. However, it was reported that testosterone production was rather stimulated in a bioassay system using rat Leydig cells after desialylation of eCG or eLH contrary to hCG (Aggarwal &

Papkoff, 1981). Although carbohydrate structures of α-subunits of eCG and eLH and O-linked oligosaccharides of eLH have not yet been analyzed in detail, these results suggest that the difference in hormonal activity between eCG and eLH observed in vitro is likely due to the extent of sialylation. Sialic acid residues linked to the elongated or expanded N-linked sugar chains in eCGβ may influence hormone assignment to the receptor and signal transduction of the hormone.

Baenziger et al. (1992) recently reported that modification of terminal residues in N-linked oligosaccharides of bLH does not significantly alter the binding to LH/CG receptor or the production of cAMP and progesterone but altered clearance from circulation and metabolic clearance rates. Furthermore, Smith et al. (1993) also demonstrated that eLH is rapidly recognized by the SO_4-GalNAc receptor in rat liver, resulting in a shorter turnover ratio of eLH. These investigators suggested that the terminal carbohydrates (SO_4-GalNAc or sialic acid) regulate the transient or periodic reaction of the hormone.

It seems necessary to further study these relationships using a homogeneous bioassay system. These accumulating results indicate, however, that terminal residues in N-linked oligosaccharides of glycoprotein hormones play a role in controlling the signal transduction and the life span of hormones like serum glycoproteins. To build up functionally significant oligosaccharide structures, each organ specifically expresses a subset of glycosyltransferase or sulfotransferase, resulting in the production of eLH and eCG with characteristic oligosaccharides to the individual hormone. Production of "serum-type" oligosaccharides on the same protein moiety in placenta may induce a more sustained effect of the hormone that may be important for maintaining pregnancy.

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