Glycoconjugate Journal 16, 707–718, 1999

Asn-linked sugar chain structures of recombinant human thrombopoietin produced in Chinese hamster ovary cells

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Human thrombopoietin (TPO) that regulates the numbers of megakaryocytes and platelets is a heavily *N*- and *O*-glycosylated glycoprotein hormone with partial homology to human erythropoietin (EPO). We prepared recombinant human TPO produced in Chinese hamster ovary (CHO) cells and analyzed the sugar chain structures quantitatively using 2-aminobenzamide labeling, sequential glycosidase digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

We found bi-, tri- and tetraantennary complex-type sugar chains with one or two *N*-acetyllactosamine repeats, which are common to recombinant human EPO produced in CHO cells. On the other hand, there were triantennary sugar chains with one or two *N*-acetyllactosamine repeats that were specific to the recombinant human TPO, and their distributions of branch structures were also different. These results suggested that proximal protein structure should determine the branch structure of Asn-linked sugar chains in addition to the glycosyltransferases subset.

Keywords: **thrombopoietin, Mpl-ligand, CHO, Asn-linked sugar chain structures**

Abbreviations: **2-AB, 2-aminobenzamide; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Man, D-mannose; Glc, D-glucose; Gal, D-galactose; GalN, D-galactosamine; GlcN, D-glucosamine; GlcNAc, *N*-acetylglucosamine; Fuc, L-fucose; TPO, thrombopoietin; EPO, erythropoietin; CFU-MK, colony forming unit-megakaryocyte; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; DHB, 2,5-dihydroxybenzoic acid; 5HT, hydroxytryptamine; DMB, 1,2-diamino-4,5-methylene dioxybenzene; DP, *Diplococcus pneumoniae*; NDV, Newcastle Disease virus; AU, *Arthrobactor ureafaciens*; JB, Jack bean; AAL, *Aleuria aurantia* lectin; ConA, Concanavalin A; PHA, *Phaseolus vulgaris* agglutinin; SSA, *Sambucus sieboldiana* agglutinin; CHO, Chinese hamster ovary; BHK, baby hamster kidney**

Introduction

TPO is a glycoprotein hormone that regulates platelet production by promoting the proliferation and maturation of megakaryocytes and their precursor cells. Thus, TPO is a potentially useful pharmaceutical agent for disease- and therapy-related severe thrombocytopenia. TPO was purified from rat plasma [1] and cDNA was cloned [2]. The cDNA of human TPO was cloned based on the rat cDNA [3]. Around the same time, the cDNAs of human and murine TPO were cloned as c-Mpl ligands [4,5].

TPO, which consists of 332 amino acids, has a molecular weight of 80,000, and has 6 potential *N*-glycosylation sites. TPO has two domains, a cytokine domain and a C-terminal domain [4,5]. The C-terminal domain has Asn-linked and mucin type sugar chains, whereas the cytokine domain has only mucin type sugar chains. The cytokine domain, which includes the amino acid residues from the *N*-terminal to 153, is responsible for c-Mpl binding and stimulation of megakaryocytes *in vitro*. Human hepatocytes express TPO and bone marrow cells express the TPO receptor c-Mpl. TPO stimulates the proliferation of megakaryocytes and maturation of platelets. The sugar chains of TPO might play an important

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role in serum stability during transit from the liver to the bone marrow. Sialic acid may also play an important role in serum stability, as exemplified by erythropoietin [6]. In fact, sialidase digestion reduces the *in vivo* activity of TPO.

In this study, the sugar chain structures of recombinant human TPO produced in CHO cells were analyzed by the 2-AB labeling method with matrix-assisted laser desorption ionization time-of-flight mass spectrometry(MALDI TOF/MS), which enabled us to determine the precise structure with limited samples. The sugar chain structures of recombinant human TPO produced in baby hamster kidney(BHK) cells have already been reported [7]. The same sugar chain structures were commonly found in TPO and erythropoietin produced in BHK cells [8], and in human urinary erythropoietin [8,9]. A comparative study between human erythropoietin from CHO and human urinary erythropoietin also showed similar sugar chain moieties [9].

However, recombinant human TPO produced in CHO cells had a unique sugar chain structure that was not found in erythropoietin produced in CHO cells. This indicated that the different sugar chain structures appeared on the glycoprotein even in the same cell. Thus, natural TPO might have unique sugar chain structures.

Materials and methods

Materials

Recombinant human TPO was purified from the culture medium of CHO into which the human TPO gene had been introduced. The purified human TPO increased the uptake of [^{14}C]-labeled hydroxytryptamine (^{14}C-5HT) into rat colony forming unit-megakaryocyte(CFU-MK) cells *in vitro*. The *in vivo* activity was measured by the increase in platelet number in the mice. In normal mice, the subcutaneous injection of recombinant human TPO at a dose of 0.03–2 μg/mouse/day for 4 days increased the platelet number dose-dependently. Two days after the last injection, platelet number was increased by fivefold at a dose of 2 μg/mouse/day.

Endo-*β*-galactosidase from *Bacteroides fragilis* was purchased from Wako Pure Chemicals (Osaka, Japan). *β*-*N*-Acetylhexosaminidase from Jack bean (JB), α-mannosidase from JB, *Aleuria aurantia* lectin column (LA-AAL-column), Concanavalin A column (LA-Con A-column), *Phaseolus vulgaris* agglutinin agarose (PHA-E4-agarose) and *Sambucus sieboldiana* agglutinin column (LA-SSA-column) were purchased from Seikagaku Kogyo (Tokyo, Japan). Sialidase from Newcastle Disease virus (NDV), *β*-galactosidase from *Diplococcus pneumoniae* (DP) and *β*-*N*-acetylglucosaminidase from DP were purchased from Boehringer Mannheim (Mannheim, Germany). Sialidase from *Arthrobactor ureafaciens*(AU) and 2-aminobenzamide (2-AB) were purchased from Nacalai Tesque(Kyoto, Japan). 2, 5-Dihydroxybenzoic acid(DHB) was purchased from Aldrich Chemical (Milwaukee, WI, USA).

Sugar composition analysis

Neutral sugars were released from 1 μg of TPO in 4 M trifluoroacetic acid at 100°C for 90 min. Amino sugars were released in 6 N HCl at 100°C for 120 min. After hydrolysis, samples were immediately dried in a vacuum evaporator, dissolved in 200 μl of distilled water and analyzed with a Dionex GlycoStation^{TM} (Austin, TX, USA), equipped with a CarboPac PA1 column and a CarboPac PA1 guard column. Sialic acids were released in 4 N acetic acid at 80°C for 3 h, derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and analyzed using an ODS 120 T column (4.6 × 250 mm, Tosoh, Tokyo, Japan) at 0.5 ml/min [10].

Preparation of 2-AB-labeled oligosaccharides from recombinant human TPO

Asn-linked sugar chains were released from 0.2 mg of recombinant human TPO by hydrazinolysis at 100°C for 16 h and re-*N*-acetylated [11]. The oligosaccharides were reductively aminated with 2-AB in 20 μl of acetic acid/dimethylsulfoxide (3 : 7) solution containing 7 μmol of 2-AB and 2 mg of sodium cyanoborohydrate at 37°C for 16 h [12,13]. The 2-AB-labeled Asn-linked oligosaccharides were purified by a series of paper and gel-permeation chromatographies. An upsending chromatography was performed on No. 514 paper (Advantec Toyo, Tokyo, Japan) with 1-butanol/ethanol/distilled water (4 : 1 : 1) as a solvent. Gel permeation chromatography was performed on a Superdex Peptide HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 50 mM pyridine acetate buffer, pH 5.0. 2-AB-labeled Asn-linked oligosaccharides were collected from the flow-through fraction. 2-AB-labeled oligosaccharides were detected by fluorescence with excitation at 330 nm and emission at 420 nm.

Charge analysis of Oligosaccharides

Charge analysis of the 2-AB-labeled oligosaccharides was performed by anion-exchange chromatography on a HiTrap-Q column (1 ml, Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min, using a linear gradient of sodium acetate buffer, pH 5.0, from 2 mM to 160 mM per 30 min as described previously [13].

The 2-AB-labeled oligosaccharides were digested with 20 mU of sialidase from NDV in 20 μl of 10 mM sodium phosphate buffer, pH 7.0, at 37°C for 20 h [14]. The reaction mixture was diluted with 80 μl of distilled water and analyzed by anion-exchange chromatography.

Preparation of desialylated oligosaccharides

The 2-AB-labeled oligosaccharides were treated with 0.2 U of sialidase from AU in 200 μl of 20 mM sodium acetate buffer, pH 5.0, at 37°C for 20 h [15]. The reaction was terminated by heating the solution at 100°C for 3 min.

The following 2-AB-labeled oligosaccharides were obtained from recombinant EPO [9]. Galβ1-4GlcNAcβ1-2Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB, Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB, Galβ1-4GlcNAcβ1-2Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB and Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB were used as standard.

Glycosidase digestion was carried out under the following conditions: 20 mU of endo-β-galactosidase [16] in 200 µl of 50 mM sodium acetate buffer, pH 5.8, at 37°C for 20 h; 0.1 U of β-*N*-acetylhexosaminidase from JB [17] in 200 µl of 100 mM sodium citrate phosphate buffer, pH 6.0, at 37°C for 20 h; 1 U of α-mannosidase from JB [17] in 200 µl of 50 mM sodium acetate buffer, pH 4.5, at 37°C for 48 h; 40 mU of β-galactosidase from DP [18] in 80 µl of 10 mM sodium cacodylate buffer, pH 6.0, containing 40 µg of bovine serum albumin at 37°C for 20 h; 4 mU of β-*N*-acetylglucosaminidase from DP [19] in 100 µl of 100 mM sodium citrate phosphate buffer, pH 5.0, containing 2 µg of bovine serum albumin at 37°C for 20 h. The reactions were terminated by heating the solution at 100°C for 3 min.

Lectin affinity column chromatography of oligosaccharides

The desialylated 2-AB-labeled oligosaccharides were analyzed by lectin affinity column chromatography, using LA-AAL-column [20], LA-ConA-column [21], LA-SSA-column [22] and PHA-E4-agarose (2 ml) column [23] with 10 mM Tris-H_2SO_4 buffer, pH 7.5, as a loading buffer at 0.5 ml/min. The bound fractions were eluted with 1 mM Fuc from the LA-AAL-column, 5 mM α-methyl glucoside from the LA-ConA-column and 200 mM lactose from the LA-SSA-column.

Size analysis of oligosaccharides by gel permeation chromatography

The molecular sizes of desialylated oligosaccharides were analyzed by gel permeation chromatography on a Bio-Gel P-4 column (2 × 100 cm, 20–30 micron, Bio-Rad, Hercules, CA, USA) eluted with distilled water at 0.2 ml/min at 50°C [24].

Reverse phase chromatography

The 2-AB-labeled oligosaccharides were purified by reverse phase chromatography using a TSKgel ODS 80 Ts column (4.6 × 150 mm, Tosoh, Tokyo, Japan) at 0.5 ml/min. The mobile phase was 6.8% acetonitrile in 100 mM ammonium acetate buffer, pH 4.0.

The MALDI-TOF/MS spectra were analyzed using a Lasermat 2000 (Finnigan MAT, Hemel Hempstead, UK). Neutral oligosaccharides were positively ionized with DHB solution and observed as [M + Na] + ions. The DHB solution consisted of acetonitrile/distilled water (3 : 7) containing 1% (w/v) DHB and 0.01% (w/v) sodium dihydrogenphosphate dihydrate.

Results

Sugar composition

The sugar compositions of recombinant human TPO and its 2-AB-labeled Asn-linked oligosaccharides were analyzed. The sugar composition of TPO was 31.5 mol NeuAc, 0.3 mol NeuGc, 6.6 mol Fuc, 32.6 mol Gal, 14.8 mol Man, 32.1 mol GlcN, and 14.7 mol GalN per protein. On the other hand, 2-AB-labeled Asn-linked oligosaccharides contained 5.9 mol Fuc, 17.8 mol Gal, 14.8 mol Man and 28.6 mol GlcN per protein. GalN was not detected in 2-AB-labeled Asn-linked oligosaccharides. The sugar composition suggested that recombinant human TPO has 6 Asn-linked and about 15 mucin type sugar chains.

Charge analysis of 2-AB-labeled oligosaccharides

The 2-AB-labeled Asn-linked oligosaccharides of recombinant human TPO were separated into five peaks (N, A1, A2, A3 and A4) by anion-exchange column chromatography (Fig. 1). N, A1, A2, A3 and A4 indicate the oligosaccharide fractions with 0, 1, 2, 3 and 4 negative charges (sialic acid), respectively. Ninety-five percent of negatively charged peaks were sensitive to sialidase from NDV, which specifically released α2,3 linked sialic acids. The remaining 5% were not sensitive to the non-specific sialidase from AU, but were sensitive to solvolysis [25]. The SSA lectin has specificity for sialic acid with α2,6 linkage. The LA-SSA-column did not bind any 2-AB-labeled Asn-linked oligosaccharides. These results indicated that recombinant human TPO has only α2,3 linked sialic acids and suggested that the remaining 5% of the negatively charged oligosaccharides might have been due to a sulfate group similarly to EPO [9].

Structure analysis of 2-AB-labeled oligosaccharides

The 2-AB-labeled oligosaccharides neutralized by AU sialidase digestion were separated on the LA-AAL-column. AAL lectin has a specificity for Fuc α1,6 residues at reducing terminal GlcNAc. The amount of LA-AAL unbound oligosaccharides was less than 1% of the total. LA-AAL bound and eluted oligosaccharides were subjected to Bio-Gel P-4 column chromatography, which yielded 5 peaks corresponding to 15, 18, 21, 24 and 27 glucose units (G) (Fig. 2A). After LA-AAL-column chromatography, the LA-AAL unbound oligosaccharides showed the same pattern on the Bio-Gel P-4 column as that shown in Figure 2A (data not shown). The PHA-E-agarose column did not bind the desialylated 2-AB-labeled oligosaccharides. PHA-E lectin has a specificity for bisecting

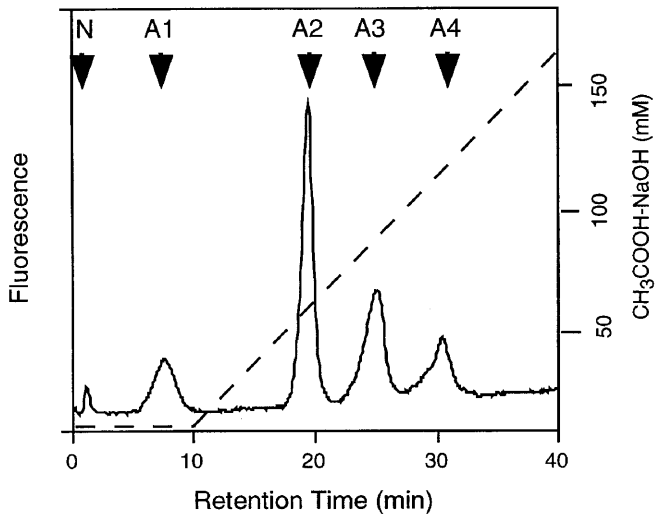


Figure 1. Anion-exchange chromatography of Asn-linked sugar chains.

2-AB-labeled Asn-linked sugar chains of recombinant human TPO were applied onto a HiTrap-Q column. N, A1, A2, A3 and A4 with arrows indicate the elution positions of 2-AB-labeled Asn-linked sugar chains obtained from recombinant human erythropoietin produced in CHO cells containing 0, 1, 2, 3 and 4 negative charges, respectively.

GlcNAc. The majority of the Asn-linked oligosaccharides of recombinant human TPO produced in CHO cells were fucosylated complex types without bisecting GlcNAc. Seventy-two percent of desialylated 2-AB-labeled oligosaccharides passed through the LA-Con A-column. The LA-Con A-column bound oligosaccharides were eluted by 5 mM α-methylglucoside. The oligosaccharides of the 15 G peak on Bio-Gel P-4 chromatography bound to the LA-Con A-column, while the other oligosaccharides (18, 21, 24 and 27 G) passed through the column. The MALDI/MS signal of the LA-Con A-column bound oligosaccharide was m/z 1930 which corresponded to the sodium adduct of 2-AB-labeled Gal_2Glc-$GlcNAc_4Man_3Fuc$. Sequential digestion with β-galactosidase from DP and β-*N*-acetylglucosaminidase from DP shifted the MALDI/MS signal to m/z 1606 and then to 1199, respectively. These results indicated that the oligosaccharide structure of the 15G fraction was Galβ1-4GlcNAcβ1-2Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB. The 18 G fraction from the Bio-Gel P-4 column was then separated into two peaks designated as 18G-1 and 18G-2 by reverse phase chromatography with a molecular ratio of 73 : 27 (18G-1 : 18G-2) as shown in Figure 3A. The MALDI/MS signals of 18G-1 and 18G-2 were both m/z 2296, which corresponded to 2-AB-labeled $Gal_3NAc_5Man_3Fuc$. The fractions of 18G-1 and 18G-2 were digested with β-galactosidase from DP and were designated 18G-1-g and 18G-2-g, respectively. They were also separated by reverse phase chromatography (Fig. 3B). The MALDI/MS signals of the two samples were both m/z 1809, which

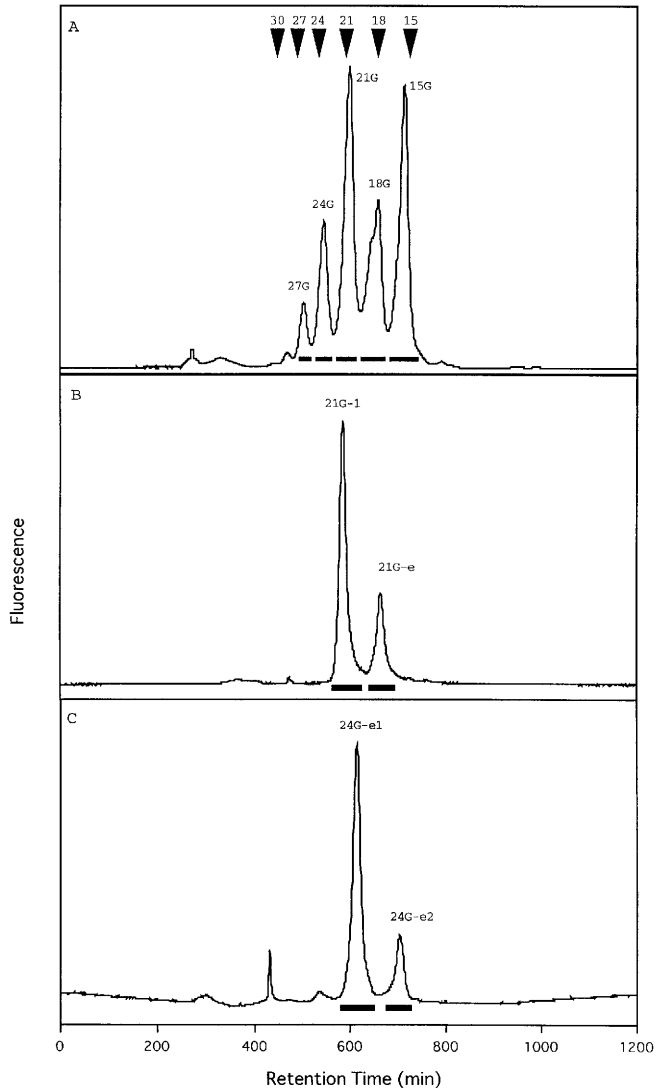


Figure 2. Size analysis of Asn-linked sugar chains on Bio-Gel P-4 gel permeation chromatography.

Panel A, neutralized LA-AAL-bound Asn-linked sugar chains of recombinant human TPO were separated on a Bio-Gel P-4 column. Arrows indicate elution positions of 2-AB-labeled glucose oligomers obtained from partial hydrolysate of dextran. The numbers are glucose units. Bars show the pooled fractions. Panel B, the 21G fractions were separated by endo-β-galactosidase digestion; Panel C, the 24G fractions were separated by endo-β-galactosidase digestion.

corresponded to 2-AB-labeled $GlcNAc_5Man_3Fuc$. The products from 18G-1-g and 18G-2-g digested with β-*N*-acetylglucosaminidase from DP were analyzed by MALDI/MS and their signals were m/z 1606 and 1403, which corresponded to 2-AB-labeled $GlcNAc_4Man_3Fuc$ and $GlcNAc_3Man_3Fuc$, respectively. β-*N*-Acetylglucosaminidase from DP can hydrolyze GlcNAcβ1-2Man, but not GlcNAcβ1-6 (GlcNAcβ1-2)Man structure [26]. This indicated that the product from 18G-2-g corresponded to Manα1-6 (GlcNAcβ1-

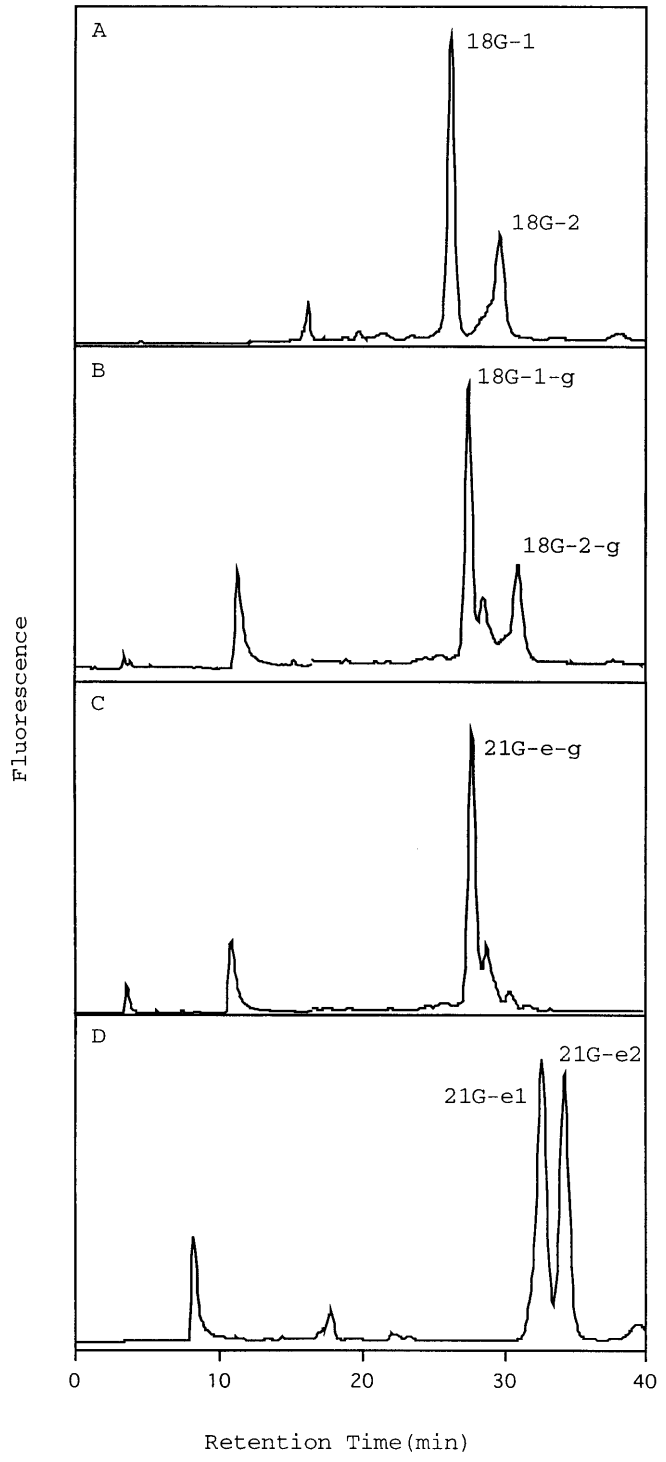


Figure 3. Reverse phase chromatography of Asn-linked sugar chains.

Panel A, the 18G fraction was separated on a TSKgel ODS 80Ts column. Panel B, the 18G fraction was digested with β-galactosidase and analyzed. Peaks were designated as 18G-1-g and 18G-2-g. Panel C, the 21G-e fraction was digested with β-galactosidase and analyzed. Panel D, the 21G-e fraction] was separated. Peaks were designated as 21G-e1 and 21G-e2.

4Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB and that from 18G-1-g corresponded to GlcNAcβ1-6 (GlcNAcβ1-2) Manα1-6 (Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB. Therefore the structures of 18G-1 and 18G-2 should be Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB and Galβ1-4GlcNAcβ1-2Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB, respectively.

The 2-AB-labeled oligosaccharides collected in the 21 G fraction appeared as a single peak on the Bio-Gel P-4 column. However, after endo-β-galactosidase digestion, an additional peak was observed beside the insensitive peak on the Bio-Gel P-4 column (Fig. 2B). The endo-β-galactosidase insensitive peak and additional peak were designated as 21G-1 and 21G-e, respectively. The MALDI/MS signal of 21G-1 was m/z 2661 corresponding to 2-AB-labeled $Gal_4GlcNAc_6Man_3Fuc$. Sequential digestion of 21G-1 with β-galactosidase from DP and β-*N*-acetylhexosaminidase from JB shifted the MALDI/MS signal to m/z 2012, which corresponded to 2-AB-labeled $GlcNAc_6Man_3Fuc$, and then to 1199, which corresponded to 2-AB-labeled $GlcNAc_2Man_3Fuc$, respectively. Therefore, 21G-1 should be Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB. 21G-e was separated into two peaks on reverse phase chromatography, designated as 21G-e1 and 21G-e2 with a molecular ratio of 54 : 46, respectively (Fig. 3D). 21G-e1 and 21G-e2 gave the same MALDI/MS signal at m/z 2133 corresponding to 2-AB-labeled $Gal_2GlcGlcNAc_5Man_3Fuc$ (Fig. 4, 21G-e). 21G-e-g produced by β-galactosidase digestion of 21G-e eluted at exactly the same retention time as 18G-1-g [GlcNAcβ1-6 (GlcNAcβ1-2) Manα1-6 (GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB] on the reverse phase column (Fig. 3C). Furthermore, JB β-*N*-acetylhexosaminidase digestion of 21G-e1 and 21G-e2 gave 21G-e1h and 21G-e2h, respectively, which gave the same MALDI/MS signal at m/z 1930 corresponding to 2-AB-labeled $Gal_2GlcNAc_4Man_3Fuc$. The structural candidates of 21G-e1h and 21G-e2h were 21G-eh-A, 21G-eh-B and 21G-eh-C as shown in Figure 4. After sequential digestion with β-galactosidase from DP and β-*N*-acetylglucosaminidase from DP, the MALDI/MS signals of products from 21G-e1h and 21G-e2h were m/z 1403 and 1199, respectively. The MALDI/MS signal at m/z 1403 corresponded to 2-AB-labeled $GlcNAc_3Man_3Fuc$ derived from 21G-eh-B and the MALDI/MS signals at m/z 1199 corresponded to 2-AB-labeled $GlcNAc_2Man_3Fuc$ derived from 21G-eh-A (Fig. 4). β-*N*-Acetylglucosaminidase from DP hydrolyzed the GlcNAcβ1-2Man but not that of GlcNAcβ1-6 (GlcNAcβ1-2) Man. These results indicated that 21G-e1h and 21G-e2h were 21G-eh-B and 21G-eh-A, respectively (Fig. 4). Therefore 21G-e1 and 21G-e2 should have been derived from triantennary oligosaccharides with an *N*-acetyllactosamine

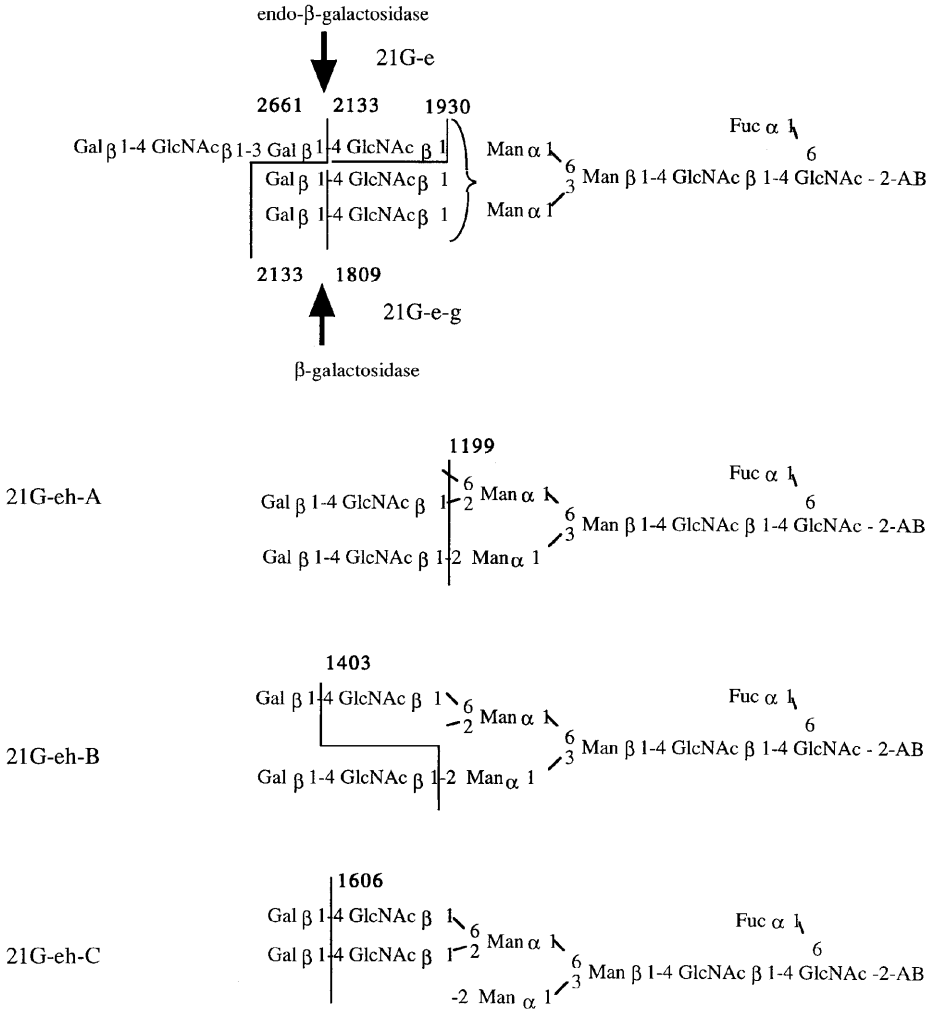


Figure 4. Glycosidase digestion pattern of 21G fraction. 21G-e was derived from the indicated sugar chain structure by endo-*β*-galactosidase digestion. A portion of 21G-e was digested with *β*-galactosidase and designated as 21G-e-g, while the remainder was digested with *β*-*N*-acetylglucosaminidase. The predicted MALDI/MS signals and structures from 21G-e are shown as 21G-eh-A, 21G-eh-B and 21G-eh-C.

repeat that were Gal*β*1-4GlcNAc*β*1-6 (Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4GlcNAc*β*1-2) Man*α*1-6 (Gal*β*1-4GlcNAc*β*1-2Man*α*1-3) Man*β*1-4GlcNAc*β*1-4 (Fuc*α*1-6) GlcNAc-2AB (21G-21) and Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4GlcNAc*β*1-6 (Gal*β*1-4GlcNAc*β*1-2) Man*α*1-6 (Gal*β*1-4GlcNAc*β*1-2Man*α*1-3) Man*β*1-4GlcNAc*β*1-4 (Fuc*α*1-6) GlcNAc-2AB (21G-22), respectively. The 2-AB-labeled oligosaccharides collected in the 24 G fraction appeared as a single peak on Bio-Gel P-4 column chromatography. However, after endo-*β*-galactosidase digestion, it was separated into two peaks on the Bio-Gel P-4 column designated as 24G-e1 and 24G-e2 (Fig. 2C). 24G-e1 was subjected to reverse phase chromatography and separated into two peaks designated as 24G-e11 and 24G-e12 with a molecular ratio of 48 : 52. Both peaks gave the same MALDI/MS signal at m/z 2499 corresponding to 2-AB-labeled $Gal_3GlcNAc_6Man_3Fuc$. This molecular mass indicated that the original structure of 24G-e1 was a tetraantennary sugar chain containing one *N*-acetyllactosamine repeat (Figure 5). After the JB *β*-*N*-acetylhexosaminidase digestion of 24G-e11 and 24G-e12, the digested products of 24G-e11 and 24G-e12 were designated as 24G-e11h and 24G-e12h, respectively. Structural candidates are shown as 24G-e1h-A, B, C and D in Figure 5. Reverse phase chromatography of 24G-e11h showed a peak identical to 18G-2 that was shown to be Gal*β*1-4GlcNAc*β*1-2Man*α*1-6 [Gal*β*1-4GlcNAc*β*1-4(Gal*β*1-4GlcNAc*β*1-2) Man*α*1-3] Man*β*1-4GlcNAc*β*1-4 (Fuc*α*1-6) GlcNAc-2AB. Additional sequential digestion with *β*-galactosidase from DP and *β*-*N*-acetylhexosaminidase from DP shifted the MALDI/MS signal of 24G-e11h to m/z 1403, which corresponded to 2-AB-labeled $GlcNAc_3Man_3Fuc$. The signal shift pattern of 24G-e11h was the same as that of 18G-2. Therefore, the structure of 24G-e11h should be 24G-e1h-A

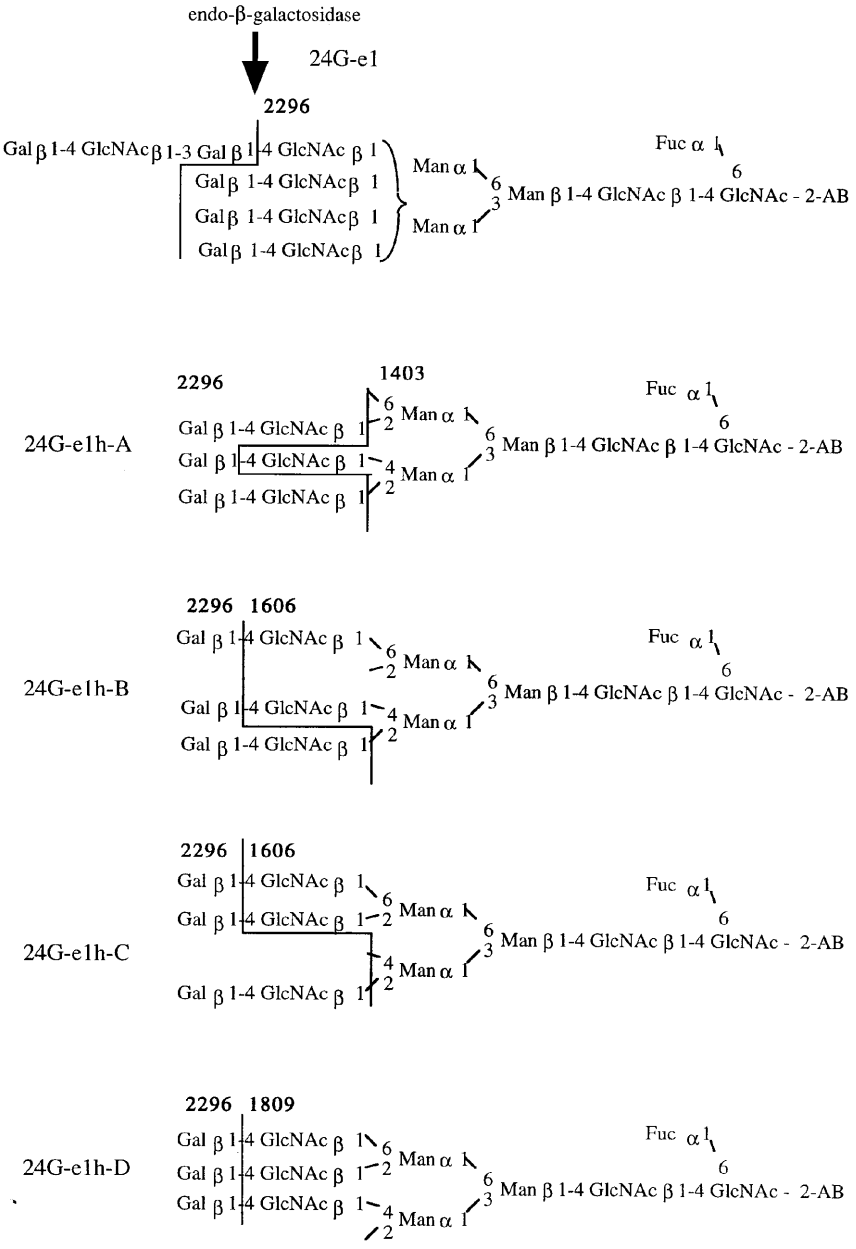


Figure 5. Glycosidase digestion pattern of 24G-e1 fraction. 24G-e1 was derived from the indicated sugar chain structure by endo-β-galactosidase digestion. The predicted MALDI/MS signals and structures from 24G-e11h and 24G-e12h are shown as 24G-e1h-A, 24G-e1h-B, 24G-e1h-C and 24G-e1h-D.

(Fig. 5). Additional sequential digestion of 24G-e12h with β-galactosidase from DP and β-*N*-acetylhexosaminidase from DP shifted the MALDI/MS signal to m/z 1606, which corresponded to 2-AB-labeled $GlcNAc_4Man_3Fuc$ (24G-e1h-B or 24G-e1h-C). 24G-e12h eluted at a different retention time from 18G-1 that was shown to be Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB on reverse phase chromatography. Therefore, the structure of 24G-e12h must not be 24G-e1h-C but should be 24G-e1h-B in Figure 5. 24G-e11 and 24G-e12 should have been derived from tetraantennary sugar chains with an *N*-acetyllactosamine repeating that were Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB (24G-11) and Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2) Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-

4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB (24G-12), respectively. The MALDI/MS signal of 24G-e2 was m/z 1971 corresponding to 2-AB-labeled $GalGlcNAc_5Man_3Fuc$. This molecular mass indicated that the original structure of 24G-e2 was a triantennary sugar chain containing two *N*-acetyllactosamine repeats, as shown in Figure 6. 24G-e2 digestion with DP β-Galactosidase produced 24G-e2-g in Figure 6. On reverse phase chromatography, 24G-e2-g showed a peak identical to 18G-1-g that was shown to be GlcNAcβ1-6 (GlcNAcβ1-2) Manα1-6 (GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB. Sequential digestion of 24G-e2 with β-*N*-acetylhexosaminidase from JB and α-mannosidase from JB shifted the MALDI/MS signal from m/z 1971 to m/z 1565 and then to m/z 1402 corresponding to 2-AB-labeled $GalGlcNAc_3Man_2Fuc$. These results indicated that two *N*-acetyllactosamine repeats linked at the same α1-6 side mannose of the core sugar chain structure, because α-mannosidase would digest the exposed mannose unit after endo-β-galactosidase digestion followed by β-*N*-acetylhexosaminidase digestion (24G-e2-m in Fig. 6). Therefore, 24G-2 should be a triantennary oligosaccharide with two *N*-acetyllactosamine repeats: Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB.

The 2-AB-labeled oligosaccharides collected in the 27 G fraction were digested with endo-β-galactosidase, which was designated as 27G-e. The MALDI/MS signal of 27G-e was m/z 2336 corresponding to 2-AB-labeled $Gal_2GlcGlcNAc_6Man_3Fuc$. Therefore, the 27G-e fraction was derived from a tetraantennary sugar chain with two *N*-acetyllactosamine repeats. The sequential digestion of 27G-e with *N*-acetylhexsaminidase from JB and α-mannosidase from JB shifted the MALDI/MS signal from m/z 2336 to m/z 1606 and then to m/z 1443 corresponding to 2-AB-labeled $Gal_2GlcNAc_4Man_2Fuc$. These results indicated that two *N*-acetyllactosamine repeats linked at the same α1-6 side mannose of the core sugar chain structure, because α-mannosidase digestion would result in mannose exposure after endo-β-galactosidase digestion followed by β-*N*-acetylhexosaminidase digestion. Therefore, the 27 G fraction obtained by Bio-Gel P-4 column chromatography should be a tetraantennary sugar chain with two *N*-acetyllactosamine repeats: Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (Galβ1-

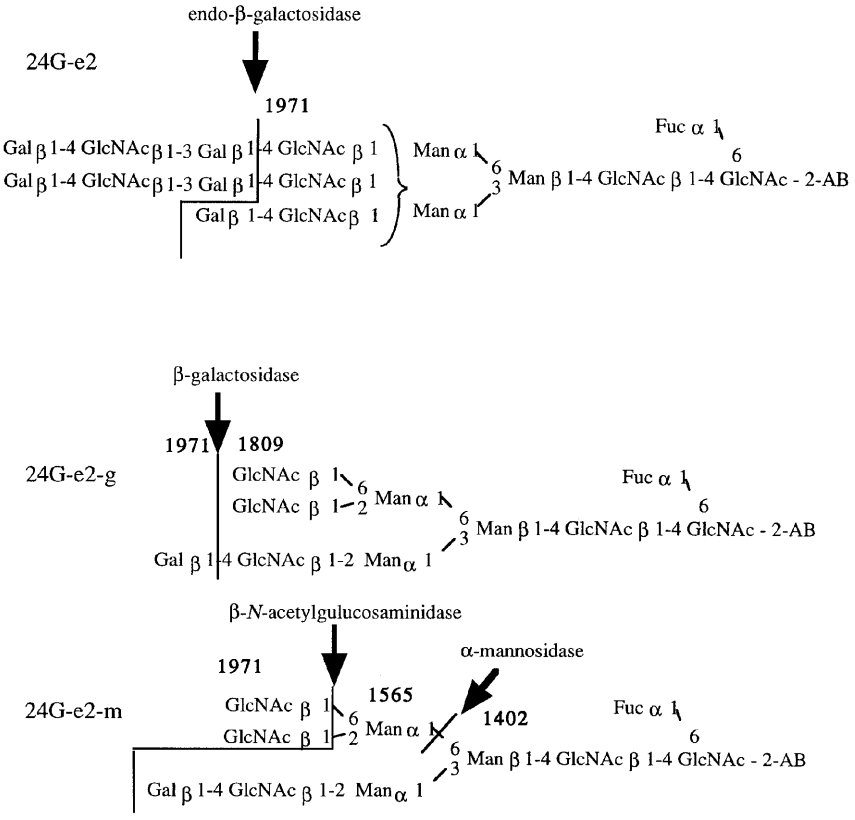
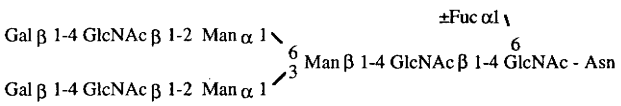
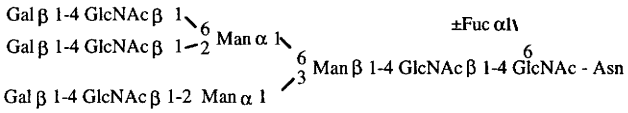
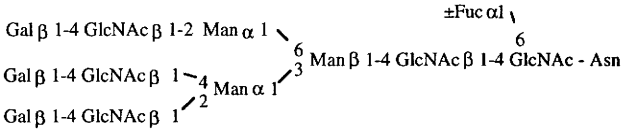
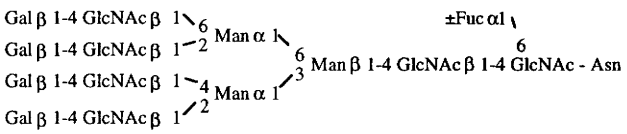
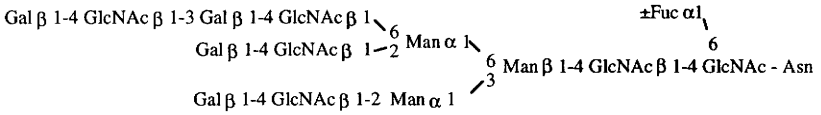
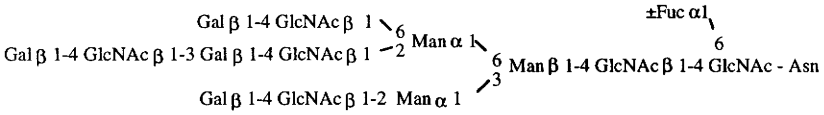
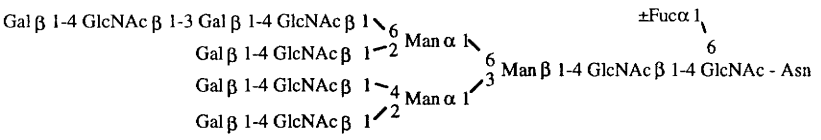
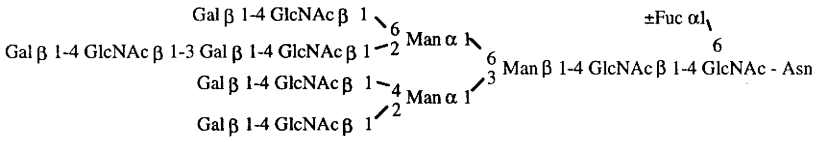
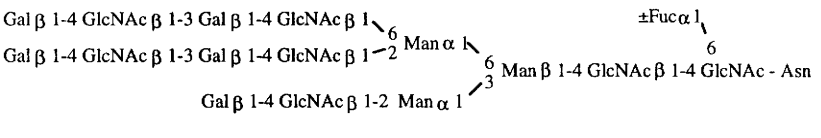
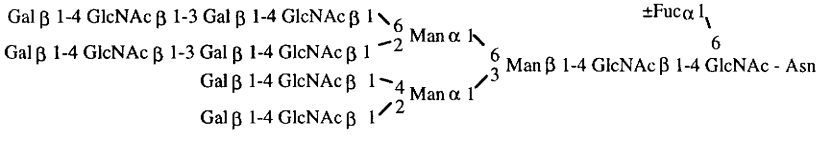


Figure 6. Glycosidase digestion pattern of 24G-e2 fraction. 24G-e2 was derived from the indicated sugar chain structure by endo-β-galactosidase digestion. A portion of 24G-e2 was digested with β-galactosidase and designated as 24G-e2-g. The remainder was digested to 24G-e2-m by β-*N*-acetylglucosaminidase and α-mannosidase.

Table 1. Structures of the Asparagine linked sugar chains of recombinant human TPO

Size	*Structures*	*Molar Ratio (%)*
15G		27.9
18G-1		16.2
18G-2		5.9
21G-1		22.6
21G-21		3.4
21G-22		4.0
24G-11		5.2
24G-12		5.7
24G2		2.7
27G		5.2

4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2) Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-2AB.

The 2-AB-labeled oligosaccharides from recombinant EPO were used as standard to determine the sugar chain structure of 15G, 18G-1, 18G-2 and 21G-1.

The structures of asialo Asn-linked oligosaccharides of recombinant human TPO produced in CHO cells are summarized in Table 1.

Discussion

By quantitative hydrazinolysis, 13.5 nmol of Asn-linked sugar chains were released from 2.5 nmol of TPO, suggesting that most of the 6 potential *N*-glycosylation sites were glycosylated. These *N*-glycosylation sites are located in the C-terminal domain. It is of particular interest to compare sugar chain structures between recombinant EPO [9] and recombinant TPO produced in CHO cells, because EPO and the cytokine domain of TPO were reported to show structural homology [4]. Ninety-eight percent of Asn-linked sugar chains released from recombinant TPO were acidic, and 95% of these negative charges came exclusively from α2,3 linked sialic acids. Most of the sialic acids were in the *N*-acetyl form, but there was a trace amount of *N*-glycolyl form. Ninety-nine percent of sugar chains had α1,6-linked fucose at GlcNAc of the reducing terminal. With regard to branching structures, bi-, tri- and tetraantennary complex-type sugar chains with *N*-acetyllactosamine repeats were detected. Hoffman *et al.* reported that recombinant TPO produced in BHK cells also had bi-, tri- and tetraantennary sugar chains with fucose as the major structure [7]. The populations of sugar chain structures were different between recombinant EPO [8] and TPO both produced in BHK cells, but the types of sugar chain structures were the same. These observations suggested that the cellular glycosylation systems are mainly responsible for the types of sugar chain structures.

There were several features specific to recombinant TPO produced in CHO cells: the major branching structure was the bi-antennary type (27.9% of the total sugar chains), which was a minor structure and acted as a factor to reduce *in vivo* biological activity in the case of EPO [6,27]. This result strongly suggested that the protein structure should determine the branching structure of sugar chains.

Recombinant EPO and TPO had tetraantennary sugar chains with *N*-acetyllactosamine repeats. The recombinant TPO also had several minor structures that recombinant EPO did not; triantennary sugar chains with one and two *N*-acetyllactosamine repeats (10.1% of total sugar chains), new isomers of triantennary sugar chains with one *N*-acetyllactosamine repeat (3.4% and 4.0%), and a trace amount of triantennary sugar chains with three *N*-acetyllactosamine repeats. *N*-Acetyllactosamine repeatings structures appeared on only Galβ1-4GlcNAcβ1-6 (Galβ1-4GlcNAcβ1-2) Manα1-6 (Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα 1-6) GlcNAc and not on Galβ1-4GlcNAcβ1-2Manα1-6 [Galβ1-4GlcNAcβ1-4 (Galβ1-4GlcNAcβ1-2)Manα1-3] Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc. It appears that the i antigen-forming GlcNAc transferase of CHO cells, which acts at the first step of *N*-acetyllactosamine repeating biosynthesis [28], prefers Galβ1-4GlcNAcβ1-6Manα1-6Man and Galβ1-4GlcNAcβ1-2Manα1-6Man of tri- and tetraantennary sugar chains.

One of our goals was to elucidate the precise sugar chain structures of human native TPO in blood and to understand their functional roles as in the case of EPO [29]. However, it is almost impossible to purify human TPO in sufficient amounts from blood, because blood TPO content was estimated to be below 0.7 amol/L [30]. Human transferrin and α1-acid glycoprotein, known to be secreted from the liver, might provide clues to the sugar chain structure of native TPO, because the liver should be the major organ producing TPO [31]. Human transferrin, 80 kDa with 2 N-glycans, exclusively has bi-antennary complex-type sugar chains with α2,6-linked sialic acids [32]. It has also been reported that the liver damaged by cancer or alcohol produced human transferrin possessing the triantennary structure of GnT-IV product type and/or α1,6Fuc to GlcNAc at the reducing terminal [19,33]. α1-Acid glycoprotein, 44 kDa with 6 N-glycans, has tri- and tetraantennary complex-type sugar chains with mainly α2,6-linked sialic acids [34] and has sialyl Lewis X structures [35]. It has also been reported that amount of sialyl Lewis X structures were increased in patients with inflammation [36]. According to our previous study, the non-reducing terminal structure of N-glycans should be largely dependent on the glycosylation system of the host cells. It appears that α2,6-linked sialic acids and a lack of α1,6Fuc are characteristic features of normal human hepatocytes. If this is the case in native human TPO, there should be qualitative differences in sugar moieties between native TPO and recombinant TPO produced in CHO cells that always yield α2,3-linked sialic acids and α1,6Fuc. Such differences in glycoforms may significantly alter the their distribution in the human body and the biochemical features of human TPO. Therefore, it is very important to elucidate the precise sugar chain structures of natural human TPO in future studies, although it is almost impossible to purify it in sufficient amounts. This work will be helpful to elucidate the sugar chain structures of natural human TPO.

Acknowledgements

The authors are grateful to Y. Sakata for technical assistance and to C. Simmons for proof reading this manuscript.

References

1 Kato T, Ogami K, Shimada Y, Iwamatsu A, Y. Sohma, Akahori H, Horie K, Kokubo A, Kudo Y, Maeda E (1995) Purification and characterization of thrombopoietin. *J Biochem (Tokyo)* **118:** 229–36.

2 Ogami K, Shimada Y, Sohma Y, Akahori H, Kato T, Kawamura K, Miyazaki H (1995) The sequence of a rat cDNA encoding thrombopoietin. *Gene* **158:** 309–10.

3 Sohma Y, Akahori H, Seki N, Hori T, Ogami K, Kato T, Shimada Y, Kawamura K, Miyazaki H (1994) Molecular cloning and chromosomal localization of the human thrombopoietin gene. *FEBS Lett* **353:** 57–61.

4 Lok S, Kaushansky K, Holly R, Kuijper J, Lofton-Day C, Oort P, Grant F, Heipel M, Burkhead S, Kramer J, Bell L, Sprecher C, Blumberg H, Johnson R, Prunkard D, Ching A, Osborn S, Evans S, Sheppard P, Presnell S, O'Hara P, Hagen F, Roth G, Foster D (1994) Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* **369:** 565–8.

5 Sauvage Fd, Hass P, Spencer S, Malloy B, Gurney A, Spencer S, Darbonne W, Henzel W, Wong S, Kuang W, Oles K, Hultgren B, Jr LS, Goeddel D, Eaton D (1994) Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* **369:** 533–8.

6 Takeuchi M, Inoue N, Strickland T, Kubota M, Wada M, Shimizu R, Hoshi S, Kozutsumi H, Takasaki S, Kobata A (1989) Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A*: 7819–22.

7 Hoffman RC, Andersen H, Walker K, Krakover JD, Patel S, Stamm MR, Osborn S (1996) Peptide, disulfide, and glycosylation mapping of recombinant human thrombopoietin from ser1 to Arg246. *Biochemistry* **35:** 14849–61.

8 Tsuda E, Goto M, Murakami A, Ueda M, Kawanishi G, Takahashi N, Sasaki R, Chiba H, Ishihara H, Mori M, Tejima S, Endo S, Arata Y (1988) Comparative structual study of N-linked oligosaccharides of urinary and recombinant erythropoietin. *Biochemistry* **27:** 5646–54.

9 Takeuchi M, Takasaki S, Miyazaki H, Kato T, Hoshi S, Kochibe N, Kobata A (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J Biol Chem* **263:** 3657–63.

10 Hara S, Yamaguchi M, Takemori Y, Furuhata K, Ogura H, Nakamura M (1989) Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Anal Biochem* **179:** 162–6.

11 Takasaki S, Mizuochi T, Kobata A (1982) Hydrazinolysis of asparagine-linked sugar chains to produce free oligosaccharides. *Methods Enzymol* **83:** 263–8.

12 Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB (1995) Nonselective and Efficient Fluorescent Labeling of Glycans Using 2-Amino Benzamide and Anthranilic Acid. *Anal. Biochem.* **230:** 229–38.

13 Watanabe T, Inoue N, Kutsukake T, Matsuki S, Takeuchi M (2000) Development of a method for quantitative analysis of Sialo-olifosaccharides using 2-aminobenzamide labeling under improved condition. *Biol. Pharm. Bull.* **23:** 269–273.

14 Nakamura K, Inagaki F, Tamai Y (1988) A novel ganglioside in dogfish brain. Occurrence of a trisialoganglioside with a sialic acid linked to N-acetylgalactosamine. *J Biol Chem* **263:** 9896–900.

15 Uchida Y, Tsukada Y, Sugimori T (1974) Production of microbial neuraminidases induced by colominic acid. *Biochim Biophys Acta* **350:** 425–31.

16 Scudder P, Hanfland P, Uemura K, Feizi T (1984) Endo-β-D-galactosidases of Bacteroides fragilis and Escherichia freundii hydrolyze linear but not branched oligosaccharide domains of glycolipids of the neolacto series. *J Biol Chem* **259:** 6586–92.

17 Li YT, Li SC (1973) α-Mannosidase, β-N-Acetylhexosaminidase, and β-galactosidase from Jack Bean Meal. *Method in Enzymology* **28:** 702–13.

18 Paulson J, Prieels J, Glasgow L, Hill R (1978) Sialyl- and fucosyltransferases in the biosynthesis of asparaginyl-linked oligosaccharides in glycoproteins. Mutually exclusive glycosylation by β-galactoside alpha2 goes to 6 sialyltransferase and N-acetylglucosaminide α1 goes to 3 fucosyltransferase. *J Biol Chem* **253:** 5617–24.

19 Yamashita K, Koide N, Endo T, Iwaki Y, Kobata A (1989) Altered glycosylation of serum transferrin of patients with hepatocellular carcinoma. *J Biol Chem* **264:** 2415–23.

20 Yamashita K, Kochibe N, Ohkura T, Ueda I, Kobata A (1985) Fractionation of L-fucose-containing oligosaccharides on immobilized Aleuria aurantia lectin. *J Biol Chem* **260:** 4688–93.

21 Krusius T, Finne J, Rauvala H (1976) The structural basis of the different affinities of two types of acidic N-glycosidic glycopeptides for concanavalin A-sepharose. *FEBS Lett* **71:** 117–20.

22 Shibuya N, Tazaki K, Song Z, Tarr G, Goldstein I, Peumans W (1989) A comparative study of bark lectins from three elderberry (Sambucus) species. *J Biochem (Tokyo)* **106:** 1098–103.

23 Cummings R, Kornfeld S (1982) Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized Phaseolus vulgaris leukoagglutinating and erythroagglutinating lectins. *J Biol Chem* **257:** 11230–4.

24 Yamashita K, Mizuochi T, Kobata A (1982) Analysis of oligosaccharides by gel filtration. *Methods Enzymol* **83:** 105–26.

25 Baenziger J, Green E (1988) Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochim Biophys Acta* **947:** 287–306.

26 Yamashita K, Ohkura T, Yoshima H, Kobata A (1981) Substrate specificity of Diplococcal β-N-acetylhexosaminidase, a useful enzyme for the structural studies of complex type asparagine-linked sugar chains. *Biochim Biophys Res Commun* **100:** 226–32.

27 Misaizu T, Matsuki S, Strickland T, Takeuchi M, Kobata A, Takasaki S (1995) Role of antennary structure of N-linked sugar chain in renal handling of recombinant human erythropoietin. *Blood* **86:** 4097–104.

28 Sasaki K, Kurata K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M (1997) Expression cloning of cDNA encoding a human β-1, 3-N-acetylglucosaminyltransfease that is essential for poly-N-acethllactosamine synthesis. *Proc Natl Acad Sci USA* **94:** 14294–9.

29 Takeuchi M, Kobata A (1991) Structures and functional roles of the sugar chains of human erythropoietin. *Glycobiology* **1:** 337–46.

30 Tahara T, Usuki K, Sato H, Ohashi H, Morita H, Tsumura H, Matsumoto A, Miyazaki H, Urabe A, Kato T (1996) A sensitive sandwich ELISA for measuring thrombopoietin in human serum: serum thrombopoietin levels in healthy volunteers and in patients with haemopoietic disorders. *Br J Haematol* **93:** 783–8.

31 Nomura S, Ogami K, Kawamura K, Tsukamoto I, Kudo Y, Kanakura Y, Kitamura Y, Miyazaki H, Kato T (1997) Cellular localization of thrombopoietin mRnA in the liver by in situ hybridization. *Exp Hematol* **25:** 565–72.

32 Spik G, Coddeville B, Montreuil J (1988) Comparative study of the primary structure of sero- lacto- and ovotransferrin glycans from different species. *Biochemie* **70:** 1459–69.

33 Spik G, Debruyne V, Montreuil J, Halbeek H, Vliegenthart J (1985) Primary structure of two sialylated triantennary glycans from human serotransferrin. *FEBS lett* **183:** 65–9.

34 Yoshima H, Matsumoto A, Mizuochi T, Kawasaki T, Kobata A (1981) Comparative study of the carbohydrate moieties of rat and human plasma alpha 1-acid glycoprotein. *J Biol Chem* **256:** 8476–84.

35 Van Dijk W, Havenaar EC, Brinkman-Van der Linden ECM (1995) α1-Acid glycoprotein (orosomucoid): pathophysiological changes in glycosylation in relation to its function. *Glycoconjugate J* **12:** 227–33.

36 Brinkman-Van der Linden ECM, de Haan PF, Havenaar EC, Van Dijk W (1998) Inflammation-induced expression of sialyl Lewis X is not restricted to α1-acid glycoprotein but also occurs to a lesser extent on α1-antichymotrypsin and haptoglobin. *Glycoconjugate J* **15:** 177–82.

Received 27 October 1999; revised 1 December 1999; accepted 29 February 2000