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Determination of glycan structures and molecular masses of the glycovariants of serum transferrin from a patient with carbohydrate deficient syndrome type II

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Serum transferrin from a child with carbohydrate deficient syndrome type II was isolated by immunoaffinity chromatography and separated into minor and major fractions by fast protein liquid chromatography. The structure of the glycans released from the major fraction by hydrazinolysis was established by application of methanolysis and ^{1}H-NMR spectroscopy. The results led to the identification of an *N*-acetyllactosamininic type monosialylated, monoantennary Man(α1-3) linked glycan. By electrospray-mass spectrometry analysis, the whole serum transferrin was separated into at least seven species (I to VII) with molecular masses ranging from 77 958 to 79 130 Da. On the basis of a polypeptide chain molecular mass of 75 143 Da, it was calculated that the major transferrin species III (78 247 Da) contains two monosialylated monoantennary glycans. The molecular mass of transferrin species V and VI (78 678 and 78 971 Da) suggests that one of their two glycans contains an additional *N*-acetyllactosamine and a sialylated *N*-acetyllactosamine units, respectively. Transferrin species I and V were found to correspond to the desialylated forms of species III and VI. The abnormal glycan structures can be explained by a defect in the *N*-acetylglucosaminyltransferase II activity [Charuk *et al.* (1995) *Eur J Biochem* 230: 797–805].

***Keywords*: carbohydrate deficiency glycoprotein syndrome, electrospray, glycan structure, glycoforms, transferrin**

***Abbreviations*: CDG, carbohydrate deficient glycoprotein; Tf, transferrin; FPLC, fast protein liquid chromatography**

Introduction

Human transferrin is an abundant serum glycoprotein involved in iron metabolism, whose structure is well defined. In fact, the polypeptide chain has been sequenced [1, 2] and its molecular mass of 75 143 Da has been determined by mass spectrometry and confirmed by calculation [3]. The two glycosylation sites Asn^{413} and Asn^{611} located in the C-terminal part are occupied by either biantennary *N*-acetyllactosamine type glycans [4, 5] or triantennary 3 and 3′ *N*-acetyllactosamine type glycans [6]. In addition to the bi and triantennary structures, a very low amount of tetraantennary glycans has been detected [7]. Due to the heterogeneity of the glycan structures and their specific location on the polypeptide chain [8] whole serum transferrin includes a pool of glycovariants. Several methods have been used to characterize these glycovariants. In particular, based on the difference of affinity of biantennary and triantennary glycans for concanavalin A, glycovariants Tf-I (less than 1%), Tf-II (17 $\pm$ 2%) and Tf-III (82 $\pm$ 3%) containing two triantennary, one triantennary and one biantennary and two biantennary glycans respectively have been isolated [9]. Based on the electronegative charges of *N*-acetylneuraminic acid residues and the immune reaction of the polypeptide chain with monospecific anti-transferrin antibodies, several glycovariants have been detected by immune isoelectric focusing [10] or crossed immuno-isoelectric focusing [11]. Applying this last method, the authors have detected in the iron saturated serum of healthy male individuals, transferrin glycovariants containing di (2.6%); tri (7.4%); tetra (66.1%); penta (18.9%); hexa (4.9%); and hepta (0.1%) *N*-acetylneuraminic acid residues. These methods have been used to detect abnormal glycosylation modifications of glycoproteins in hereditary or non-physiopathological conditions. Until now, identification of four different types of transferrin glycovariant profiles by isoelectric focusing has led to the report of four different types of CDG syndromes with different clinical symptoms

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and glycoprotein abnormalities [12]. The most abundant studies concern CDG types I and II. CDG syndrome type I is characterized by transferrin glycovariants with one or two N-linked glycans deleted [13]. This deletion being explained by a phosphomannomutase deficiency [14]. In patients with CDG syndrome type II, the isoelectric focusing profile of serum transferrin glycovariants shows disialotransferrin as the major fraction. On the basis of preliminary biochemical and enzymatical results showing a profound deficient activity of *N*-acetylglucosaminyl-transferase II, the presence in one transferrin glycovariant of a truncated monoantennary glycan has been proposed [15].

In the present data, the glycan structure of the most abundant CDG syndrome type II transferrin glycovariant was determined on the basis of ^{1}H-NMR spectroscopy parameters, we also analysed by electrospray-mass spectrometry the molecular masses of the different CDG syndrome type II transferrin glycovariants and deduced the presence of either two monoantennary or one monoantennary and one truncated biantennary glycans per polypeptide chain.

Materials and methods

Materials

The following materials were obtained from the companies indicated: CNBr-activated Sepharose 4B, Mono Q HR 10/10 column and Phast gel gradient 10/15%, Pharmacia Fine Chemicals (Uppsala, Sweden); specific anti-human transferrin IgG, Dako Corp. (Carpinteria CA, USA); Centriprep concentrators, Amicon Div. (Grace Industrial Chemicals, Inc. Pully, Switzerland); neuraminidase *Clostridium perfringens* type VI-A, Sigma Chemical Co. (St Louis, MO, USA); Bio-Gel P-2, Bio-Rad Laboratories (Richmond, CA, USA); human transferrin, Behringwerke (Marburg, Germany); D_2O (99.95 atom %D), Commissariat à l'Energie Atomique (Saclay, France); hydrazine, acetic anhydride, sodium acetate, copper acetate and N + O glycans column set (Dowex AG 50×12, Chelex 100), Oxford GlycoSystems (Abingdon, UK). All other reagents were of the highest purity.

Source of serum

Blood was withdrawn from a patient, an 11-year-old boy, who from birth share floppiness, dysmorphic features, severely retarded psychomotor development and coagulation abnormalities. On the basis of the symptoms and pathology and the distribution of serum transferrin by isoelectrofocusing this patient was recognized as suffering from CDG syndrome type II [15]. The serum from CDG syndrome type I was obtained from a 15-year-old girl and was a gift from Dr Jaeken. The two serum samples were stored at $-20\,^{\circ}$C until analysis.

Transferrin concentration

Concentration of transferrin was determined by single radial immunodiffusion, according to [16] using monospecific rabbit anti-human transferrin.

Isolation of transferrin

Serum from the patient (15 ml) was loaded onto the specific anti-human transferrin IgG Sepharose 4 B column (2.4 cm × 9 cm, 5 mg IgG per ml beads [17]. The column was washed with Tris/HCl buffer, 50 mM, pH 8.0, containing 150 mM NaCl, followed by the same buffer containing 1 M NaCl and eluted with 0.2 M glycine/HCl, pH 2.8. To the solution containing the eluted transferrin, 1 M sodium citrate and 1 M sodium hydrogen carbonate were added to give a final concentration of 0.1 M. The transferrin was saturated with iron by adding 5 µl $FeCl_3$ solution (400 µg $FeCl_3$/ml) of 0.1 M sodium citrate and sodium hydrogen carbonate solution, pH 8.2) [18]. The iron saturated solution was concentrated to 1 ml by filtration on a dialysis membrane (Centriprep concentrator). Salts were removed by further dilution with distilled water and concentrated to 1 ml.

Fast protein liquid chromatography separation of the transferrin glycovariants

The iron saturated transferrin isolated by affinity chromatography was subjected to ion-exchange chromatography on a mono Q HR 10/10 column using a fast protein liquid chromatography (FPLC) system [19]. The column was equilibrated with 50 mM Tris/HCl, pH 8.6 (buffer A) and the chromatography was developed with increased concentrations of NaCl (0–1 M) obtained by mixing buffer A with buffer B. Buffer B was obtained by adding 1 M NaCl to buffer A. Transferrin was eluted at a flow rate of 1 ml min^{-1}. Each fraction collected was concentrated to 1 ml on Centriprep concentrator and desalted on an HR 10/10 column using an FPLC system.

Electrophoretic assays

Polyacrylamide gel electrophoresis, with or without detergents, was performed in Phast-gel (gradient 10–15%) using a Phast System under conditions recommended by Pharmacia Biotech (separation technique file no. 120). Isoelectric focusing PAGE was performed as previously described [20].

Ionspray-mass spectrometry

Samples of standard transferrin and transferrin isolated from serum of the patient by immuno-affinity were dissolved in acetonitrile 20% in water, formic acid 0.1% (20 pmol µl^{-1}). Ionspray-mass spectra were recorded on a simple-quadrupole mass spectrometer API I

(Perkin-Elmer Sciex) equipped with an ionspray (nebulizer-assisted electrospray) source (Sciex, Toronto, Canada). The solutions were continuously infused with a medical infusion pump (model 11, Harward apparatus, South Natick, USA) at a flow rate of 5 µl min^{-1}. Polypropylene glycol (PPG) was used to calibrate the quadrupole mass spectrometer. Ionspray-mass spectra were acquired at unit resolution by scanning from m/z 1800 and 2400 with a step size of 0.1 Da and a dwell time of 2 ms. Five to 10 spectra were summed. The potential of the spray needle was held at + 5.5 kV. Spectra were recorded at an orifice voltage of + 120 V. Mac BIO Spec was the computer program used for calculation of the molecular masses of the samples.

Neuraminidase treatment

CDG syndrome type II transferrin and standard transferrin were desialylated by recycling on a *Clostridium perfringens* type VI-A immobilized neuraminidase column equilibrated with 0.1 M sodium citrate, pH 5.0, at 37 °C for 24 h [21]. Sialic acid was eliminated by gel filtration on a Bio-Gel P-2 column equilibrated with water.

Release and isolation of oligosaccharides

The oligosaccharides from fraction I of CDG syndrome type II transferrin were released by hydrazinolysis procedure using an automated process. Transferrin sample (2 mg) was introduced into the GlycoPrep 1000 (Oxford GlycoSystem) and the hydrazinolysis reaction was performed under controlled conditions of temperature and time and under argon atmosphere [22]. After excess hydrazine removal, *N*-acetylation was achieved by the addition of acetic anhydride in saturated $NaHCO_3$. Sodium ions and peptide material were removed from the sample by passage through a column of Dowex AG 50 × 12. The acetohydrazone derivatives were converted to unreduced glycans by the addition of 1 mM copper acetate for 30 min at 27 °C [23]. After passage through a mixed bed column of Chelex 100/Dowex AG 50 × 12, the oligosaccharides were collected and freeze-dried for further analysis.

Carbohydrate composition

Percentage composition in total neutral sugars, hexosamines and sialic acids and monosaccharides molar ratios were determined and methanolysis, re-*N*-acetylation [24] and gas-liquid chromatography of their trimethylsilylated methyl glycosides [25] on a capillary CP SIL 5 CB column (0.2 mm × 25 m).

^{1}H-NMR spectroscopy

For ^{1}H-NMR spectroscopic analysis, the oligosaccharides were repeatedly exchanged in D_2O at room temperature and at pD 7 with intermediate lyophilization [26]. The 400 MHz ^{1}H-NMR spectra were recorded on a Bruker AM 400-WB spectrometer operating in the pulsed-Fourier transform mode and equipped with a Bruker Aspect 3000 computer at a probe temperature of 300 K (Centre Commun de Mesures, Université des Sciences et Technologies de Lille). Chemical shifts (δ) are given relative to sodium 4-4-dimethyl-4-silapentane-sulphonate, but were actually measured by reference to internal acetone in D_2O: $\delta = 2.225$ ppm with an accuracy of $\pm$ 0.002 ppm.

Results

Transferrin concentration

The transferrin concentration (1 g l^{-1}) in the serum of the patient with CDG syndrome type II was decreased compared to control sera from children 5–10 years old. Decreased values were also previously obtained for a large number of serum glycoproteins including haptoglobin, coagulation factors IX, XI, XII, antithrombin III and IgG [15].

Electrophoretic migration of transferrin glycovariants

Transferrin was easily prepared from the patient serum by a single-step procedure using immunoaffinity chromatography on immobilized rabbit anti-human transferrin IgG column with a recovery of about 90%. The purity of the eluted transferrin after iron saturation was checked by SDS/PAGE. This fraction was revealed as a single protein band whose electrophoretic migration was similar to that of the standard transferrin. Polyacrylamide gel electrophoresis performed in the absence of detergent (Figure 1A) showed that the CDG syndrome type II transferrin was separated into a major and a minor components. These two components possessed an electrophoretic mobility lower than the major standard transferrin glycovariant containing two disialylated biantennary glycans. CDG syndrome type I transferrin showed a markedly different pattern. In fact, three glycovariants were detected, one possessing the same electrophoretic mobility as the standard one, and the two others were characterized by a lower electrophoretic mobility. A better resolution of the transferrins was obtained by isoelectric focusing (Figure 1B). Compared with standard transferrin, tetrasialo-transferrin was nearly absent in the CDG type II transferrin sample and a large increase of disialylated transferrin was detected. A band was present at the trisialylated transferrin position and a minor band at the monosialylated transferrin position. In comparison with standard transferrin, CDG syndrome type I transferrin showed the presence of tetrasialotransferrin, an increase of disialotransferrin and the presence of asialotransferrin.

Fast protein liquid chromatography

As shown in Figure 2A, the standard human transferrin was resolved into one major and three minor components by

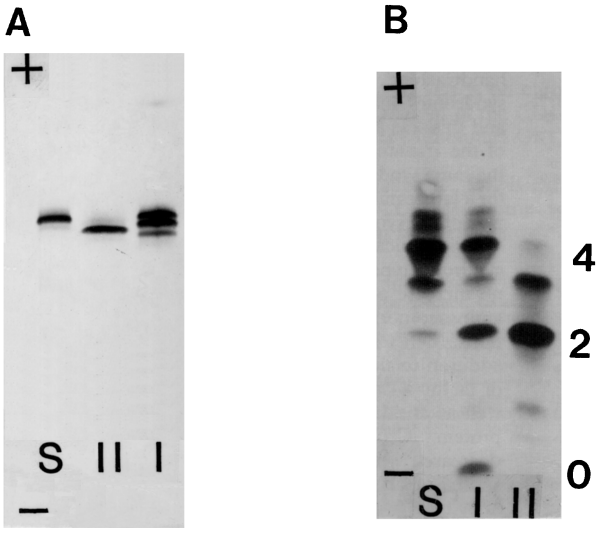

Figure 1. (A) Native PAGE pattern of standard serum transferrin (S), CDG syndrome type I (I) and CDG syndrome type II (II) transferrins. (B) Isoelectric focusing pattern of standard serum transferrin (S), CDG syndrome type I (I) and in CDG syndrome type II (II) transferrins. 0, 2, 4 indicate the number of *N*-acetylneuraminic acid residues.

fast protein liquid chromatography. The major one (fraction II) corresponds to a tetrasialylated transferrin glycovariant. The minor components correspond to di (fraction I), penta (fraction III) and hexa (fraction IV) sialylated transferrin glycovariants. Fractionation of the CDG syndrome type I transferrin (Figure 2B) led to the elution of three fractions I, II and III corresponding respectively to an asialo, a di and a tetrasialylated transferrin glycovariant.

Fractionation of the affinity-purified CDG syndrome type II transferrin led to the elution of a major fraction I (95% ± 3%) and a minor fraction II (4% ± 1%) (Figure 2C). The electrophoretic mobilities of purified fractions I and II correspond to a di and trisialylated transferrin glycovariant, respectively. After complete removal of sialic acid residues, the electrophoretic mobilities of these two fractions were similar to the migration of standard asialotransferrin. These results indicate that CDG syndrome type II transferrin must differ from the standard transferrin by the number of *N*-acetylneuraminic acid residues. Because of the very low amount of transferrin fraction II, the determination of the glycan structures was performed only on the transferrin fraction I.

Carbohydrate composition

The carbohydrate component representing 4% of the CDG syndrome type II transferrin fraction I consisted of 2.0% neutral sugars, 1.5% hexosamines and 0.5% sialic acids. The sugars were identified as galactose, mannose, *N*-acetylglucosamine and *N*-acetylneuraminic acid by gas liquid chromatography of their trimethylsilyl derivatives. The molar ratio of these monosaccharides (Table 1) was similar to

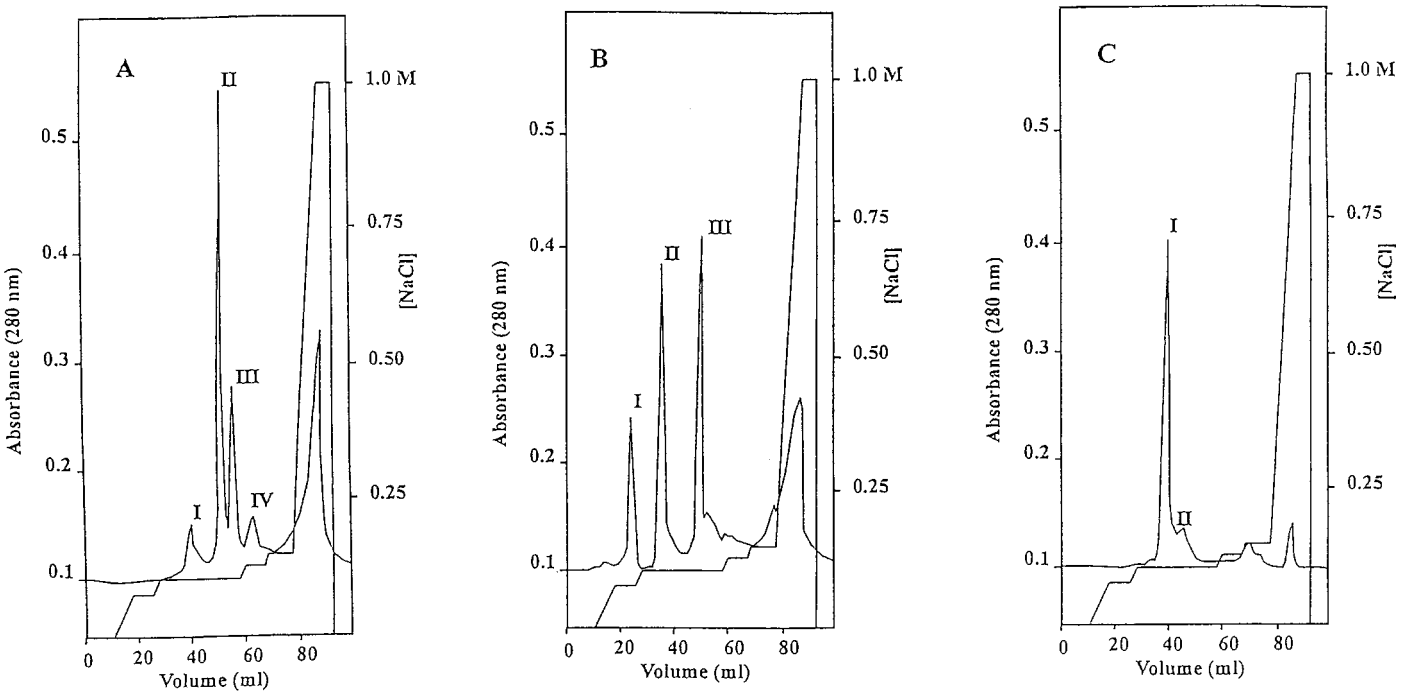

Figure 2. Elution profiles on a mono Q HR 10/10 column using the FPLC system. (A) standard transferrin; (B) CDG syndrome type I transferrin; (C) CDG syndrome type II transferrin.

Table 1. Comparison of the molar carbohydrate compositions of transferrin standard with the oligosaccharides released by hydrazinolysis and CDG syndrome type II transferrin (fraction I).

Sample	*Sugar*			
	Gal	*Man*[a]	*GlcNAc*	*NeuAc*
Transferrin (standard)	2.1	3.0	3.9	1.8
Transferrin fraction I (CDG type II)	1.1	3.0	3.3	0.8
Released oligosaccharides	1.2	3.0	3.2	0.7

[a] Molar ratios were calculated on the basis of three mannose residues.

that of the oligosaccharides released by hydrazinolysis. Compared to standard human transferrin, both molar carbohydrate compositions were characterized by a lower content in galactose, *N*-acetylglucosamine and *N*-acetylneuraminic acid, suggesting the presence of incomplete glycan structure in the CDG syndrome type II transferrin.

Preparation of the oligosaccharides

Oligosaccharides from the CDG syndrome type II transferrin fraction I were released by hydrazinolysis. The procedure used allowed the liberation of a good yield (80%) of oligosaccharides from a small amount of transferrin. Technically, the released unreduced glycans possess a free terminal GlcNAc without any acetohydrazone derivatives – these derivatives were removed by the action of copper acetate. The presence of the reducing terminal GlcNAc residue has been confirmed by ^{1}H-NMR spectroscopy.

^{1}H-NMR analysis of the CDG syndrome type II transferrin fraction I

In order to establish the complete primary structure, the oligosaccharides released by hydrazinolysis were submitted to 400 MHz ^{1}H-NMR spectroscopy. From the 400 MHz ^{1}H-NMR spectrum (Figure 3), we can deduce that the oligosaccharide released from the transferrin fraction I is of the *N*-acetyllactosamine type. On the two parts of the spectrum, the scale (*ie* amplitudes and shifts) are the same. The anomeric proton of the GlcNAc-1 ($\delta_{\text{H-1}\alpha} = 5.190$) corresponded to a totally released glycan. However due to the low amount of material, it has not been possible to further purify the reaction end product after hydrazinolysis. This could explain the poor shape of the baseline on the NMR spectrum. The presence of the Man-4 and Man-4′ anomeric protons ($\delta = 5.137$ and 4.918) respectively and also the H-2 of the three mannose residues ($\delta_{\text{H-2}}$ Man-3 = 4.252, $\delta_{\text{H-2}}$ Man-4 = 4.192 and $\delta_{\text{H-2}}$ Man-4′ = 3.975) are in favour of a biantennary structure with a Man-4′ in the non-reducing terminal position. The antenna in (α1-3) linked to Man-3, is constituted by a classical NeuAc(α2-6) Gal(β1-4)GlcNAc(β1-2)-structural element ($\delta_{\text{H-3ax}} = 1.715$, $\delta_{\text{H-3eq}} = 2.669$ for NeuAc; $\delta_{\text{H-1}}$ Gal-6 = 4.444; $\delta_{\text{H-1}}$ GlcNAc-5 = 4.603 respectively) [27]. From all these observations, we can conclude without ambiguity that the glycan of the CDG syndrome type II transferrin fraction I possesses

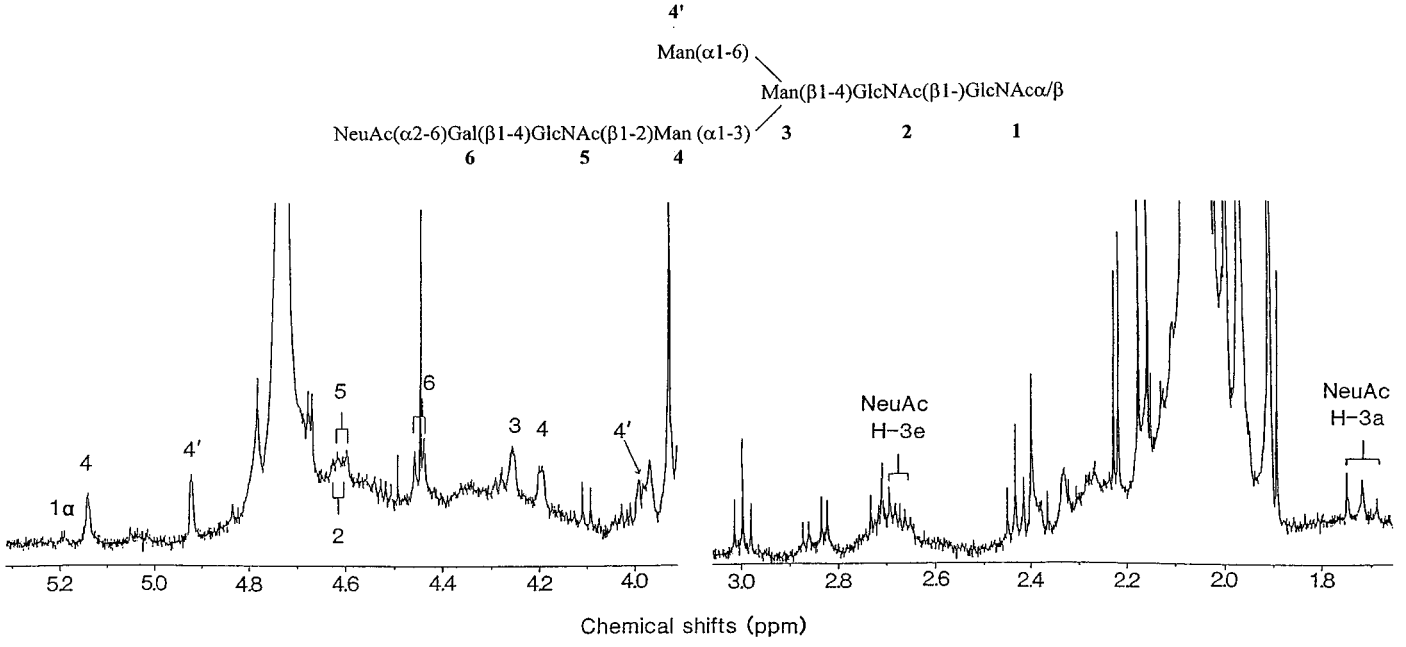

Figure 3. Resolution-enhanced 400 MHz ^{1}H-NMR spectrum (D_2O, 27 °C) of the oligosaccharides isolated from CDG syndrome type II transferrin.

Figure 4. Structures of the oligosaccharides from CDG syndrome type II transferrin. (A) Monosialylated monoantennary glycan as determined by NMR and electrospray mass spectrum analysis. (B) to (D) glycan structures proposed on the basis of the electrospray mass spectrum analysis and the results previously obtained for normal transferrin glycans [6]: (B) asialomonoantennary glycan; (C) monosialylated biantennary truncated glycan; (D) disialylated biantennary truncated glycan.

a monoantennary monosialylated structure (Figure 4A). The molecular mass of this glycan was evaluated at 1548 Da. In order to determine the number of glycans linked to the polypeptide chain, the molecular mass of patient transferrin was determined and compared to that of standard transferrin.

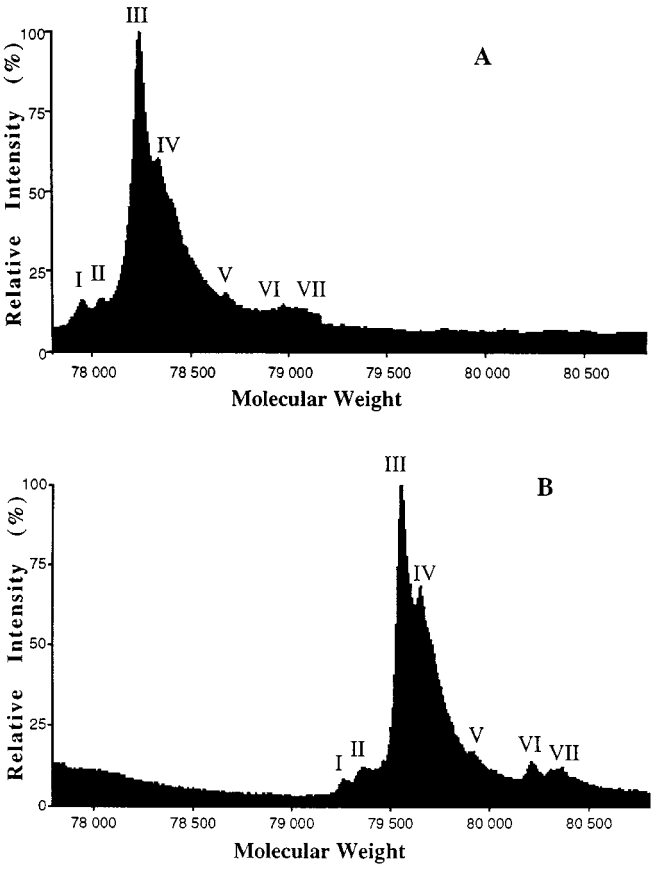

Figure 5. Transformed electrospray mass spectra of transferrin. (A) CDG syndrome type II serum transferrin; (B) standard human serum transferrin.

Ionspray-mass spectrometry

The molecular masses of glycovariants from standard transferrin and the whole CDG syndrome type II transferrin

Table 2. Molecular mass (Da) of transferrin species isolated from the serum of standard transferrin and carbohydrate deficient glycoprotein syndrome type II transferrin.

	Species characterized by ion mass spectrometry						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Standard transferrin							
Experimental molecular mass	79 265	79 366	79 557	79 656	79 921	80 215	80 360
Experimental glycan molecular mass[a]	4122	4223	4414	4513	4778	5072	5226
Theoretical glycan molecular mass	4117	4117	4408	4408	4773	5064	–
CDG type II transferrin							
Experimental molecular mass	77 958	ND	78 247	78 340	78 678	78 971	79 130
Experimental glycan molecular mass*	2815	–	3104	3197	3535	3828	3987
Theoretical glycan molecular mass	2805	–	3096	3096	3461	3752	–

[a] The experimental glycan molecular mass is derived from the experimental mass of each species characterized by ionspray-mass spectrometry minus the molecular mass of 75 143 Da corresponding to the polypeptide chain of the protein [3].
ND, not determined

isolated by immunoaffinity chromatography were determined by ionspray-mass spectrometry. Both transferrin samples gave a complex pattern, showing the presence of at least seven types of species (peaks I to VII, Figure 5). In both samples, a large molecular mass peak (peak III) with a shoulder and small lower and higher molecular mass peaks were characterized. The molecular mass range (77 900 Da to 79 400 Da) of patient transferrin species differs from that of standard transferrin (79 200–80 600 Da) confirming that in the patient transferrin, the glycans are incompletely processed. The calculated molecular mass of the glycan moiety was derived from the values of the experimental mass, obtained for each species characterized by ionspray-mass spectrometry, minus the molecular mass of 75 143 Da corresponding to the polypeptide chain of the protein. From the results described in Table 2, it appears that the value of 4414 Da calculated for glycans of a standard transferrin species III, corresponds to the mass of two disialylated biantennary *N*-acetyllactosaminic glycans. The mass value of species I glycan of 4122 Da which differs from the last one by a value of 292 seems to indicate the absence of one *N*-acetylneuraminic acid residue in this glycan. The addition of a *N*-acetyllactosamine unit or a sialylated *N*-acetyllactosamine unit to a disialylated biantennary *N*-acetylactosamine glycan may explain the increase of 364 and 658 in the values determined for the molecular mass of species V and VI respectively. Molecular species IV and II of standard transferrin correspond most probably to the molecular species III and I with a sulfated adduct moiety (at mass (+ 99) and (+ 101) respectively) [3].

Similar modifications are noticeable in the molecular mass species identified in the patient transferrin sample. In particular, the molecular mass of the glycans from the major transferrin species III corresponds to a value of 3104, this value indicates that two glycans possessing the structure described in Figure 4A are linked to the polypeptide chain of the transferrin. The molecular mass of the two glycans present in species I differs from that of species III by a value of 289 corresponding most probably to the loss of one *N*-acetylneuraminic acid residue (Figure 4B). The molecular masses of the glycans present in species V and VI, which differ from that of the species III by a value of 431 and 724, seem to indicate the presence of an additional *N*-acetylglucosamine unit (364) and sialyl *N*-acetyllactosamine unit (658), respectively (Figure 4C and D). In both species, an adduct moiety at mass 66 was present. The increase in the molecular mass in species VII of 883 in patient transferrin and of 812 in standard transferrin has not been defined.

The results obtained by ^{1}H-NMR spectroscopy and ionspray-mass spectrometry indicate the presence in the serum transferrin from the patient with CDG syndrome II of at least four glycovariants in which the two *N*-glycosylation sites are occupied by truncated glycans (Figure 6A). The molecular masses of these glycovariants differ from those of standard transferrin (Figure 6B).

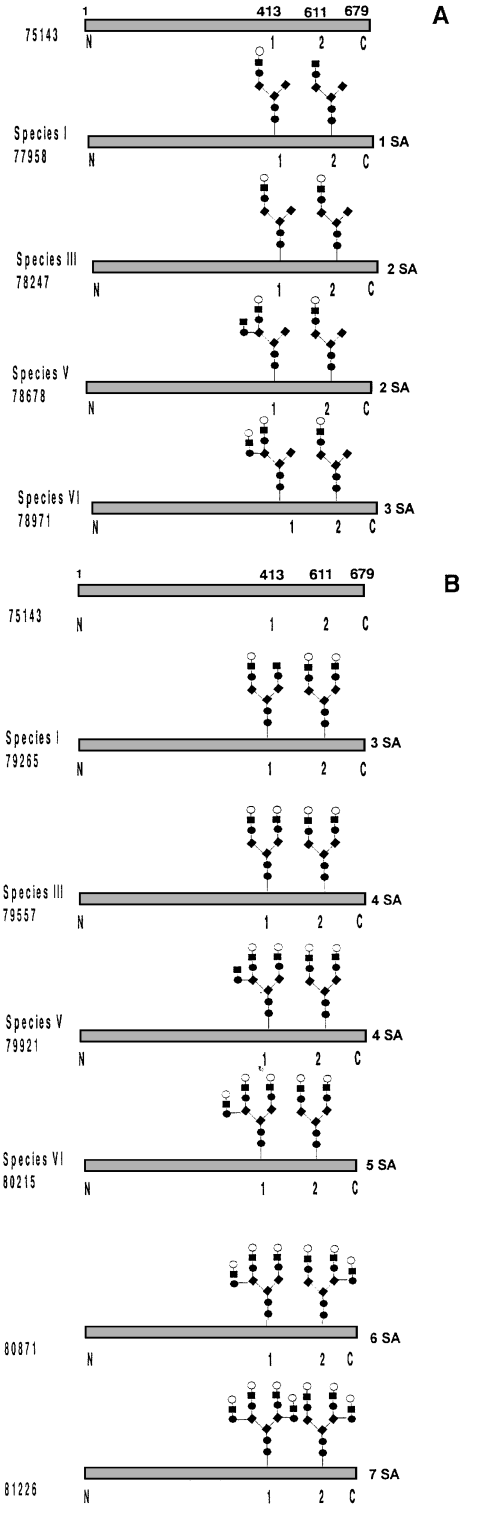

Figure 6. Proposed structures for human transferrin glycovariants. (A) CDG syndrome type II transferrin; (B) standard transferrin. ○ *N*-acetylneuraminic acid, ■ galactose; ● *N*-acetylglucosamine; ◆ mannose; SA, sialic acid. 1 and 2 indicated the glycosylation sites (Asn: 413 and 611 respectively). The glycans containing an additional antenna linked to Man-4 or -4′ might be linked either to glycosylation site 1 or 2. Transferrin species with a molecular mass of 80 871 Da and 81 226 Da which exist in very low amounts have not been characterized by electrospray-mass spectrometry.

Discussion

Symptoms and pathology as well as glycoprotein abnormalities in carbohydrate deficient glycoprotein syndromes have been described in several reviews [28–34]. The structures of some glycans present in different glycovariants of serum transferrin used as a model, were determined. For instance in CDG type I, abnormal molecular transferrin species with the defect of one or two disialylated glycans were identified by chemical methods and by physical methods such as matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF mass spectrometry) or electrospray ionization-mass spectrometry [35–38]. Recently, the transferrin glycovariants from a patient with CDG type I were separated by capillary zone electrophoresis [39]. In addition to the asialo, di and tetrasialylated transferrin glycovariants, the presence of low amounts of components migrating as penta and hexasialylated transferrin was detected. Characterization of these penta and hexasialylated glycovariants in the CDG type I transferrin suggests that the biosynthesis of these glycovariants is realized in cells in which the activity of the phosphomannomutase is still present.

In the present data, human transferrin was isolated by immunoaffinity chromatography from the serum of a patient suffering of CDG syndrome type II. This sample was resolved into minor and major fractions by native/PAGE and FPLC. The glycan structure present in the major fraction was identified as: Man(α1-6)[NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc. This incomplete glycan structure possessing a molecular mass of 1548 Da is therefore different from the disialylated biantennary *N*-acetylactosaminic glycan structure identified in transferrin from patients with CDG syndrome type I. Owing to the electrospray ionization-mass spectrometry analysis of the whole standard and CDG syndrome type II transferrin, it was found that the two glycosylation sites of the four characterized glycovariants are occupied by truncated glycans. Glycovariants containing a monoantennary monosialylated glycan with an additional *N*-acetyllactosamine unit have not been identified before.

Isoelectric focusing represents a rapid method to detect glycoprotein isoforms, however, it may be noticed that inappropriate isoelectric focusing conditions may induce iron release from serum transferrin and modify its electrophoretic migration and therefore perturb identification of the transferrin glycovariants [40]. Moreover by isoelectric focusing, it is not possible to separate patient transferrin species III from species V since both contain two *N*-acetylneuraminic acid residues. Allelic D transferrin glycovariants which differ from the common allelic C transferrin glycovariants, by the nature of one amino acid are also able to generate false identification when separated by isoelectrofocusing [41].

Electrospray-ionization mass spectrometry therefore represents an interesting method to detect alterations in glycosylation of glycoproteins from patients with CDG syndromes. Minor glycovariants must, however, be present in sufficient amount to be identified (higher than 10% in relative intensity versus the major glycovariant).

The major truncated glycan present in CDG type II transferrin was previously characterized in erythrocyte Band 3 glycoprotein from patients with an heriditary erythroblastic multinuclearity with positive acidified-serum-lysis test (HEMPAS) [42–44]. In this congenital dyserythropoietic anaemia type II, a defect in the gene encoding the Golgi *N*-acetylglucosaminyltransferase (GlcNAc-T II) as well as α-mannosidase II, has been detected. In the CDG type II patient, an enzymatic defect of GlcNAc-T II has also been shown [15]. Moreover, Charuk *et al.* [45] have shown that the mononuclear cell extracts from this CDG syndrome type II patient have no detectable GlcNAc-T II activity, while his parents have a GlcNAc-T II level averaging 50%, indicating autosomal recessive inheritance of this disease. Patients lacking GlcNAc-T II will be unable to add GlcNAc residues to the Man α1-6 antenna of the glycan core, which explains the presence of truncated structures in transferrin from the CDG syndrome type II patient and in lactosaminoglycan proteins from HEMPAS patients. However, patients with HEMPAS disease have a different clinical presentation and the erythrocytes do not exhibit the same morphological features suggesting that genetic lesions responsible for these two diseases are possibly different.

Hypoglycosylation of serum transferrin has also been detected in non-hereditary diseases, for instance, the appearance of microheterogeneity in serum transferrin in alcoholics was first reported by Stibler *et al.* [46]. This heterogeneity concerns either the presence of truncated oligosaccharides without sialic acid and galactose residues [47] or the lack of one [48, 49] or two [49] complete glycans. Other diseases, particularly some forms of liver disease may also lead to hypoglycosylation of transferrin molecules. Therefore, the isoelectric focusing of transferrin glycovariants is not sufficient to establish the type of disease, a whole set of analysis is required to definitively identify the disease. This includes: determination of glycovariants molecular mass and complete carbohydrate structures as well as analysis of the defect in enzymatic activities.

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References

1 MacGillivray RTA, Mendez E, Schewale JG, Sinha SK, Lineback-Zins J, Brew K, (1993) *J Biol Chem* **258**: 3543–53.
2 Yang F, Lum JB, McGill JR, Moore CM, Naylor SL, van Bragt PH, Baldwin WD, Bowman BH (1984) *Proc Natl Acad Sci USA* **81**: 2752–6.
3 Mason AB, Miller MK, Funk WD, Banfield DK, Savage J, Oliver RWA, Green BN, MacGillivray RTA, Woodworth RC (1993) *Biochemistry* **32**: 5472–9.
4 Spik G, Bayard B, Fournet B, Strecker G, Bouquelet S, Montreuil J (1975) *FEBS Lett* **50**: 296–9.
5 Dorland L, Haverkamp J, Schut BB, Vliegenthart JFG, Spik G, Strecker G, Fournet B, Montreuil J (1977) *FEBS Lett* **77**: 15–20.
6 Spik G, Debruyne V, Montreuil J, van Halbeek H, Vliegenthart JFG (1985) *FEBS Lett* **183**: 65–9.
7 März L, Hatton MCW, Berry LR, Regoeczi E (1982) *Can J Biochem* **60**: 624–630.
8 Fu D, van Halbeek H (1992) *Anal Biochem* **206**: 53–63.
9 Léger D, Campion B, Decottignies JP, Montreuil J, Spik G (1989) *Biochem J* **257**: 231–8.
10. Stibler H, Sydow O, Borg S (1980) *Pharmacol Biochem Behaviour* **13 suppl 1**: 47–51.
11 de Jong G, van Noort WL, Feelders RA, de Jeu-Jaspars CMH, van Eijk HG (1992) *Clin Chim Acta* **212**: 27–45.
12 Stibler H, Stephani U, Kutsch U (1995) *Neuropediatrics* **26**: 235–7.
13 Yamashita K, Ideo H, Ohkura T, Fukushima K, Yuasa I, Ohno K, Takeshita K (1993) *J Biol Chem* **268**: 5783–9.
14 van Schaftingen E, Jaeken J (1995) *FEBS Lett* **377**: 318–20.
15 Jaeken J, Schachter H, Carchon H, De Cock P, Coddeville B, Spik G (1994) *Arch Dis Child* **71**: 123–7.
16 Mancini G, Carbonara AO, Heremans JF (1965) *Immunochemistry* **2**: 235–54.
17 March SC, Parikh I, Cuatrecasas P (1974) *Anal Biochem* **60**: 149–62.
18 Mazurier J, Spik G (1980) *Biochim Biophys Acta* **629**: 399–408.
19 Campion B, Léger D, Wieruszeski JM, Montreuil J, Spik G (1989) *Eur J Biochem* **184**: 405–13.
20 Jaeken J, van Eijk HG, van der Heul C, Corbeel L, Eeckels R, Eggermont E (1984) *Clin Chim Acta* **144**: 245–7.
21 Corfield AP, Beau JM, Schauer R (1978) *Hoppe-Seyler's Z Physiol Chem* **359**: 1335–42.
22 Parekh RB, Warren CE, Merry A, Bruce J (1990) *Glycoconjugate J*, **7**: 382.
23 Patel T, Bruce J, Merry A, Bigge C, Wormald M, Jacques A, Parekh R (1993) *Biochemistry* **32**: 679–93.
24 Zanetta JP, Breckenridge SC, Vincendon G (1972) *J Chromatog* **69**: 291–304.
25 Kamerling JP, Gerwig, GJ, Vliegenthart JFG, Clamp JR (1975) *Biochem J* **15**: 491–5.
26 Vliegenthart JFG, Dorland L, van Halbeek H (1983) *Adv Carbohydr Chem Biochem* **41**: 309–74.
27 Dorland L, Haverkamp J, Vliegenthart JFG, Strecker G, Michalski JC, Fournet B, Spik G, Montreuil J (1978) *Eur J Biochem* **87**: 323–9.
28 Stibler H, Jaeken J (1990) *Arch Dis Child* **65**: 107–11.
29 Jaeken J, Stibler H, Hagberg B (1991) *Acta Paediatr Scand* **375 Suppl 1**: 1–71.
30 Jaeken J, Carchon H (1993) *J Inherit Metab Dis* **16**: 813–20.
31 Jaeken J, Carchon H, Stibler H (1993) *Glycobiology* **5**: 423–8.
32 Hagberg BA, Blennow G, Kristiansson B, Stibler H (1993) *Pediatr Neurol* **9**: 255–62.
33 Yamashita K, Ohno K (1996) in *Glycoproteins and Disease* (Montreuil J, Vliegenthart JFG, Schachter H, eds) New Comprehensive Biochemistry Vol 30, pp 445–55. Elsevier.
34 Jaeken J, Spik G, Schachter H (1996) in *Glycoproteins and Disease* (Montreuil J, Vliegenthart JFG, Schachter H, eds) New Comprehensive Biochemistry Vol 30, pp 457–67. Elsevier.
35 Wada Y, Nishikawa A, Okamoto N, Inui K, Tsukamoto H, Okada S, Taniguchi N (1992) *Biochem Biophys Res Commun* **189**: 832–6.
36 Yamashita K, Ideo H, Okhura T, Fukushima K, Yuasa I, Ohno K, Takeshita K (1993) *J Biol Chem* **268**: 5783–9.
37 Yamashita K, Okhura T, Ideo H, Ohno K, Kanai M (1993) *J Biochem* **114**: 766–9.
38 Wada Y, Gu J, Okamoto N, Inui K, (1994) *Biol Mass Spectrom* **23**: 108–9.
39 Iourin O, Mattu TS, Mian N, Keir G, Winchester B, Dwek RA, Rudd PM (1996) *Glycoconjugate J* **13**: 1031–42.
40 Hackler R, Arndt T, Kleine TO, Gressner AM (1995) *Anal Biochem* **230**: 281–9.
41 Bean P, Peter JB (1994) *Clin Chem* **40**: 2078–83.
42 Fukuda MN, Dell A, Scartezzini P (1987) *J Biol Chem* **262**: 7195–206.
43 Fukuda MN, Masri KA, Dell A, Luzzatto I, Moremen KW (1990) *Proc Natl Acad Sci* **87**: 7443–7.
44 Fukuda MN (1990) *Glycobiology* **1**: 9–15.
45 Charuk JHM, Tan J, Bernardini M, Haddad S, Reithmeier RAF, Jaeken J, Schachter H (1995) *Eur J Biochem* **230**: 797–805.
46 Stibler H, Borg S, Allgulander C (1979) *Acta Med Scand* **206**: 275–81.
47 Stibler H, Borg S (1986) *Alcoholism Clin Exp Res* **10**: 61–4.
48 Nakanishi T, Shimizu A, Okamoto N, Ingendoh A, Kanai M (1995) *J Am Soc Mass Spectrom* **6**: 854–9.
49 Landberg E, Påhlsson P, Lundblad A, Arnetorp A, Jeppsson JO (1995) *Biochem Biophys Res Commun* **210**: 267–74.

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