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RESEARCH COMMUNICATION

Congenital disorders of glycosylation type I leads to altered processing of N-linked glycans, as well as underglycosylation

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The N-linked glycans on transferrin and α_1-antitrypsin from patients with congenital disorders of glycosylation type I have increased fucosylation and branching relative to normal controls. The elevated levels of monofucosylated biantennary glycans are probably due to increased α-(1 $\rightarrow$ 6) fucosylation. The presence of bi- and trifucosylated triantennary and tetra-antennary glycans indicated that peripheral α-(1 $\rightarrow$ 3), as well as core α-(1 $\rightarrow$ 6), fucosylation is increased. Altered processing was observed on both the fully and underglycosylated glycoforms.

Key words: α_1-antitrypsin, branching, fucosylation, glycoproteins, transferrin.

INTRODUCTION

The congenital disorders of glycosylation (CDG) are genetic multisystemic disorders resulting from the aberrant N-glycosylation of proteins [1,2]. CDG are classified as type I or II on the basis of the position of the defect in the glycosylation pathway. Type I consists of defects in the assembly of dolicholpyrophosphate-linked oligosaccharide and/or its transfer to the polypeptide chain, whereas type II consists of defects in the processing of the protein-bound N-linked glycans. There is underglycosylation of serum and other glycoproteins in CDG-I, because of the decreased supply of the common oligosaccharide precursor. This creates a useful biochemical diagnostic marker, because serum glycoproteins contain less sialic acid and have a characteristic cathodal shift on isoelectric focusing or electrophoresis [3]. To date, five different enzymic defects, designated CDG-Ia–e, have been identified in CDG-I patients [1]. Although there are marked differences in the range and severity of the clinical symptoms, all subtypes have the same characteristic isoelectric focusing pattern for serum transferrin. During the detailed analysis of the glycosylation of two serum glycoproteins, transferrin and α_1-antitrypsin, from CDG-Ia, -Ib and -Ic patients, we noticed that, as well as being underglycosylated, the glycoproteins were processed differently from the normal control samples. To establish the nature of this altered glycosylation, glycans were released from purified transferrin and α_1-antitrypsin from CDG-I patients using peptide N-glycosidase F (PNGase F) and analysed by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS).

MATERIALS AND METHODS

Materials

All chemical reagents were of research grade from Sigma–Aldrich (Poole, Dorset, U.K.) unless stated otherwise. Ultra-pure electrophoretic grade acrylamide [30% (w/v)] was obtained from National Diagnostics (Hull, Humberside, U.K.). Immobiline Dry Strip isoelectric focusing strips (180 mm × 3 mm × 0.5 cm, pH 4.5–5.5) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Piperazine diacrylamide cross-linker was obtained from Bio-Rad (Hemel Hempstead, Herts., U.K.). All the proteases were of sequence grade from Promega (Southampton, Hants., U.K.). PNGase F and Glyco H graphite columns were obtained from Glyko (Oxford, Oxfordshire, U.K.). Microcon YM-30 centrifugal filter devices were from Millipore (Watford, Herts., U.K.).

Sources of plasma

Plasma was obtained from three CDG-Ia, one CDG-Ib and one CDG-Ic genetically defined patients, from three CDG-Ix and three CDG-IIx patients of unknown genetic basis, and from a patient heterozygous for the $PIZ_{bristol}$ mutation in α_1-antitrypsin [4]. Normal control plasma was from age-matched volunteers.

Purification of plasma transferrin with rivanol and release of N-linked glycans

Transferrin was purified from 50–100 μl of plasma by rivanol precipitation as described previously [5], except that following ammonium sulphate precipitation the supernatant was desalted on a Microcon YM-30 centrifugal filter prior to in-solution digestion. Purified native transferrin (100 μg) was treated with 2.5 munits of PNGase F in 20 mM sodium phosphate buffer, pH 7.5, at 37 °C overnight. The released N-glycans were purified using graphite Glyco H columns before analysis by MS.

Separation of α_1-antitrypsin isoforms and release of N-linked glycans

The isoforms of α_1-antitrypsin were separated by two-dimensional PAGE as described previously [6], essentially according to standard protocols [7–9]. In brief, plasma samples for both analytical (2.5 μl) and preparative gels (10 μl for protein identification and 30 μl for glycan analysis) were focused for a minimum of 75 kV·h and a maximum of 100 kV·h. Analytical

Abbreviations used: CDG, congenital disorders of glycosylation; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; NeuAc, *N*-acetylneuraminic acid; PNGase F, peptide N-glycosidase F.

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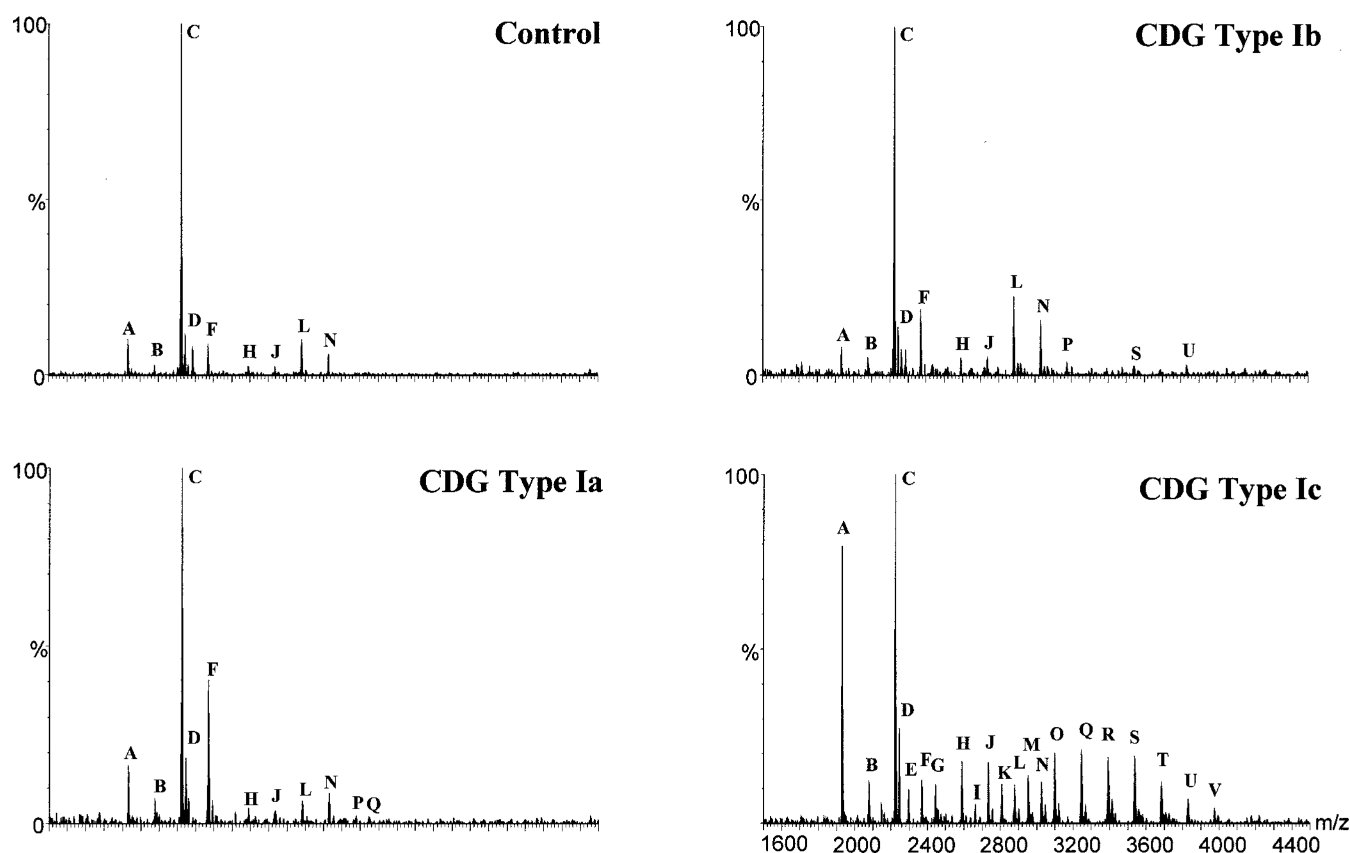

Figure 1 Representative MALDI–TOF-MS spectra of the glycans of transferrin from CDG-I patients

Glycans were released from rivanol-purified transferrin from control and CDG-Ia–Ic patients by PNGase F digestion and were analysed by MALDI–TOF-MS, as described in the Materials and methods section. Individual ions have been labelled A–V. Proposed structures for these ions are depicted in Figure 2.

gels consisted of 10% (w/v) acrylamide with 1.6% (w/v) piperazine diacrylamide cross-linker. Preparative gels for in-gel proteolytic digestions were prepared using 10% (w/v) acrylamide with 0.1% or 1.3% bis-acrylamide depending on the protease used for protein identification. Analytical gels were silver-stained using an automatic gel stainer (Amersham Pharmacia Biotech) [7,8], and the preparative gels were silver-stained manually [10]. In-gel tryptic digestions were performed [10] with some minor modifications [6]. Preparative gels for glycan analysis were stained with Coomassie Blue and glycans were released by in-gel digestion with PNGase F according to Kuster et al. [11]. The released glycans were desalted using graphite Glyco H columns in accordance with the manufacturer's instructions before analysis by MS.

MALDI–TOF-MS

MS was carried out using a MALDI–TOF instrument, fitted with a reflectron and a 337 nm UV laser (TOF Spec E; MicroMass, Manchester, U.K.). Peptide analyses were performed in positive ion mode with the following voltages: source, 20 kV; extraction, 19.95 kV; focus, 16.5 kV; and reflectron, 25 kV. Data were acquired in reflectron mode, operating over a mass range of 6000 m/z with matrix suppression set at 650 Da. Peptides were analysed using an α-cyano-4-hydroxycinnamic acid/fucose co-matrix [12].

Glycan analyses were performed in negative ion mode with voltages of 20 kV for the source, 19.95 kV for the extraction, 16.5 kV for the focus and a pulse voltage of 900 V. Data were acquired in negative linear mode, operating over a mass range of 5000 m/z with matrix suppression set at 800 Da. Glycans were analysed using a trihydroxyacetophenone/20 mM ammonium citrate co-matrix [13].

RESULTS

Characterization of N-linked glycans of human serum transferrin

N-linked glycans were released by PNGase F from rivanol-purified transferrin from control and CDG patient sera, and analysed by MALDI–TOF-MS (Figures 1 and 2). Only bi-antennary and triantennary glycans and their monofucosylated derivatives were found on transferrin from control sera. These results agreed closely with previously published glycan compositions for transferrin [14–16]. Transferrin has two N-glycosylation sites at asparagine residues 413 and 611, which normally bear complex-type biantennary (85%) glycans, with α-(2 $\rightarrow$ 6)-linked *N*-acetylneuraminic acid (NeuAc), and tri-antennary glycans (15%), with α-(2 $\rightarrow$ 3)-linked NeuAc on the β-(1 $\rightarrow$ 6)-linked branch [14–16]. A small proportion of these structures is normally α-(1 $\rightarrow$ 6) fucosylated at the core [17].

There was an increase in total fucosylation of the N-linked glycans of transferrin in CDG-Ia, -Ib and -Ic, and in two out of three of the CDG-Ix variants, compared with the controls (Figures 1 and 2, Table 1). The CDG-Ix sample [CDG-Ix(iii)] did

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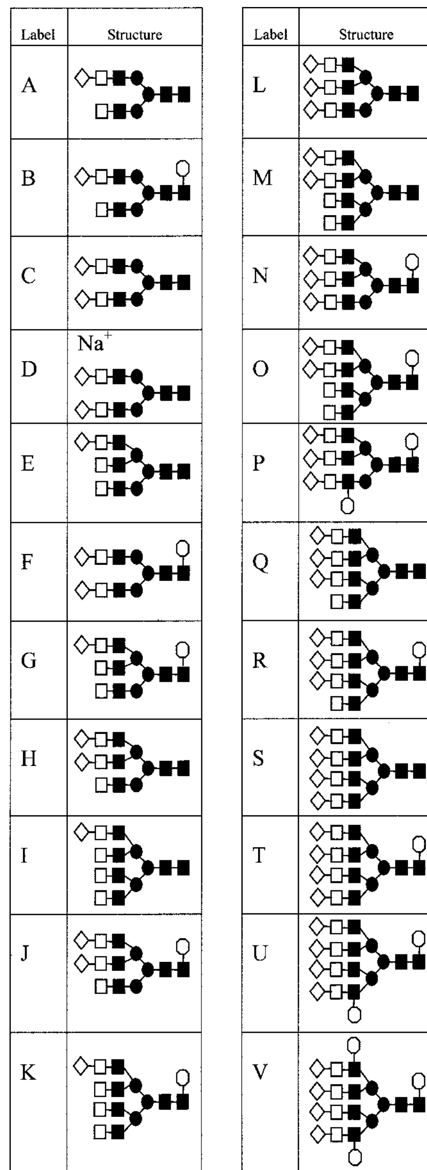

Figure 2 Proposed structures of glycans released from rivanol-purified transferrin and α_1-antitrypsin then identified by MALDI–TOF-MS (see Figure 1)

(■) *N*-Acetylglucosamine; (●) mannose; (□) galactose; (◇) sialic acid; (○) fucose.

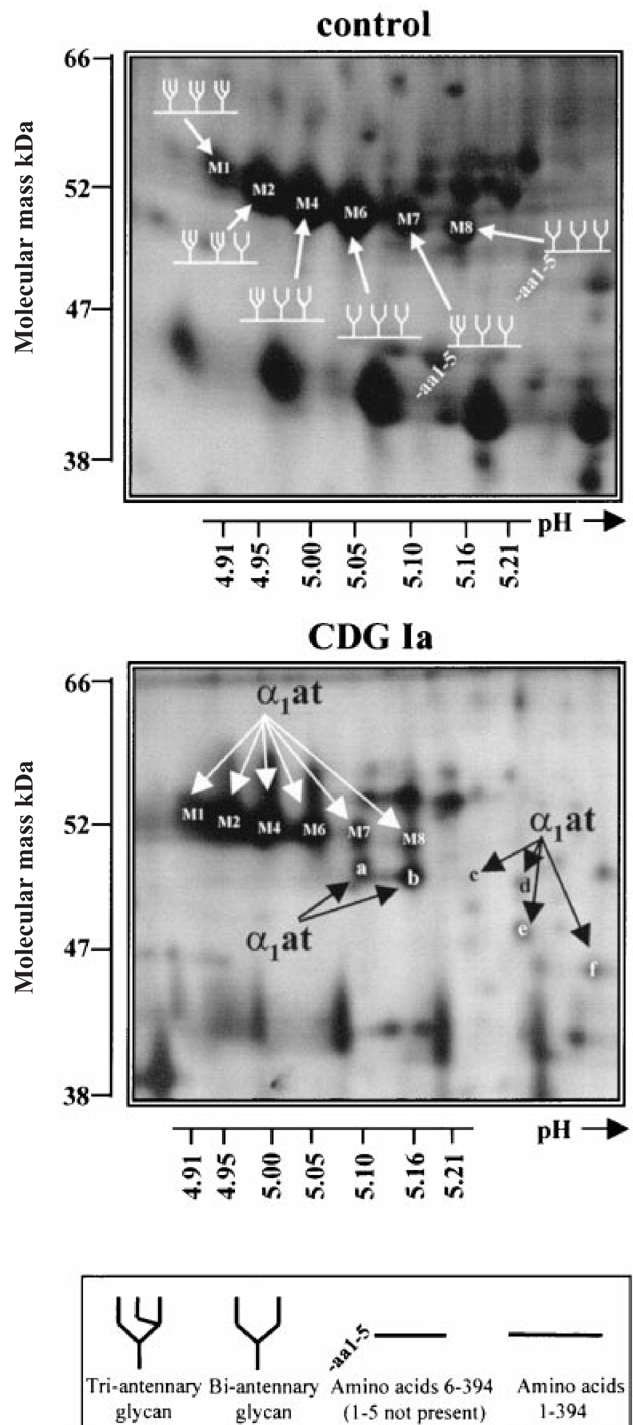

Figure 3 Enlarged section of two-dimensional PAGE of normal and CDG-Ia plasma α_1-antitrypsin

The isoforms of α_1-antitrypsin ($\alpha_1$at) were separated by using a narrow range of pH 4.5–5.5 for focusing in the first dimension. Six additional α_1-antitrypsin isoforms (a–f) were identified in the two-dimensional PAGE of serum from a patient with CDG-Ia.

not have an increased level of fucosylation and also lacked elevated lysosomal enzyme activities, suggesting that it might not be a typical case of CDG-I. The level of fucosylation of transferrin was not increased in the CDG-II patients.

The fucosylation of both bi- and triantennary glycans was increased in all cases of CDG-I (Table 1). There was also an increase in the proportion of tri- and tetra-antennary glycans, both fucosylated and unfucosylated, in the CDG-I samples, especially in CDG-Ic, with 31 % tetra-antennary glycans (Figure 1).

An ion corresponding to a bifucosylated triantennary glycan (Figure 2, structure P) was detected in CDG-Ia and -Ib. Ions corresponding to a bifucosylated tetra-antennary (Figure 2, structure U) glycan were found in CDG-Ib and -Ic, and a trifucosylated tetra-antennary (Figure 2, structure V) glycan was detected in CDG-Ic (Figure 1), but not in the control samples. The presence of the bi- and trifucosylated glycans indicated that peripheral fucosylation of the antennae, as well as core α-(1 $\rightarrow$ 6) fucosylation, must occur in CDG-I.

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Characterization of N-linked glycans of α_1-antitrypsin

As both the fully glycosylated and underglycosylated isoforms of the glycoproteins occur in the serum of CDG-I patients (K. Mills, P. B. Mills, P. T. Clayton, A. W. Johnson and B. G. Winchester, unpublished work), it is possible that the observed altered processing was only on the underglycosylated isoforms, i.e. was due to the underglycosylation itself. To test this hypothesis, both the fully glycosylated and underglycosylated individual isoforms of α_1-antitrypsin were separated by two-dimensional PAGE (Figure 3), and their glycans released by in-gel digestion with PNGase F for analysis by MALDI–TOF-MS.

α_1-Antitrypsin has three N-glycosylation sites at asparagine residues 46, 83 and 247, which are occupied by mixtures of complex bi- and triantennary glycans. This gives rise to multiple isoforms with different pIs and mass, the structural basis of which has been established previously [18,19] and recently confirmed ([6]; K. Mills, P. B. Mills, P. T. Clayton, A. W. Johnson, D. B. Whitehouse and B. G. Winchester, unpublished work). The structures are shown in Figure 3. Six major isoforms of α_1-antitrypsin (designated M1, M2, M4 and M6–M8 in Figure 3, upper panel) were identified by peptide mass fingerprinting in control plasma. The glycan compositions of the three major isoforms, M2, M4 and M6 of control α_1-antitrypsin, are given in Table 2. The M2 and M4 isoforms are composed of a mixture of biantennary and triantennary N-linked glycans and their monofucosylated derivatives. The M6 isoform is composed of biantennary and fucosylated biantennary glycans exclusively. Less than 5 % of the biantennary glycans in M2 and M4 were fucosylated, but the proportions of fucosylated triantennary glycans were much higher, 48 ± 4 % and 41 ± 6 % for the M2 and M4 isoforms respectively (Table 2). The M6 isoform, which only contains biantennary glycans, had a very low level of fucosylation (< 2 %).

Four prominent additional isoforms of α_1-antitrypsin (a–d), with masses approx. 2 kDa less than the M series, were detected in plasma from a CDG-Ia patient (Figure 3, lower panel). The decrease in mass and the shifts in pI are consistent with the loss of one complex N-linked glycan compared with the M series, i.e. they are the underglycosylated analogues containing only two glycans. Other components with masses approx. 4 kDa and 6 kDa lower than the M series are also present, and we have shown that these are the monoglycosylated and non-glycosylated forms of α_1-antitrypsin respectively (K. Mills, P. B. Mills, P. T. Clayton, A. W. Johnson, D. B. Whitehouse and B. G. Winchester, unpublished work). Similar patterns of α_1-antitrypsin isoforms were obtained for all the CDG-I patients. Analysis of glycans of the fully glycosylated M series of isoforms of α_1-antitrypsin from CDG-I patients revealed a 3–7-fold increase in fucosylation of the biantennary glycans in all isoforms compared to controls (Table 2). Similarly, the fucosylation of the biantennary glycans in the underglycosylated analogue of M6 (Figure 3, lower panel; spot b) was greater than that of biantennary glycans in normal isoforms for all CDG-I patients (Table 2). Interestingly, a small amount of triantennary glycan was found in the M6 isoform of CDG-Ia. This could arise from an underglycosylated form with two triantennary glycans, which would have a similar pI.

Comparative analysis of the triantennary glycans in the M2 isoforms from CDG-I and controls showed that there was no increase in monofucosylated glycans in CDG-I, but there was an increase in bifucosylated glycans and the appearance of both fucosylated and non-fucosylated tetra-antennary glycans (Table 2). Approx. 10 % of the glycans in the M2 isoforms from CDG-I were tetra-antennary. In the M4 isoform, there was a marked increase in mono- and bifucosylated glycans, but tetra-antennary glycans were not detected. These results clearly show that the increased fucosylation and branching occur on both the fully glycosylated and underglycosylated isoforms of α_1-antitrypsin in CDG-I.

It was possible to confirm that the altered processing was not due to underglycosylation by analysing the glycans on α_1-antitrypsin from a patient who was heterozygous for a mutation in the N-glycosylation sequon, Asn-Leu-Thr (residues 83–85), in α_1-antitrypsin [4]. The two-dimensional PAGE pattern of α_1-antitrypsin isoforms from this patient contained both the M series and diglycosylated forms comparable with spots a–d in Figure 3. The levels of fucosylation and branching in both the fully glycosylated isoforms and the major underglycosylated isoform were similar to those in the fully glycosylated control isoforms.

DISCUSSION

This study provides evidence for a marked increase in the fucosylation and branching of N-linked glycans of serum trans-

Table 1 Percentage composition of glycans and their fucosylated derivatives on transferrin from CDG patients

Number of patients analysed is indicated in parentheses. Where $n > 1$, results are represented as mean ± S.D. Abbreviation: n.d., no fucosylated structures or tetra-antennary glycans detected.

	Glycan composition (% of fraction fucosylated)			
CDG type	All	Bi-antennary	Tri-antennary	Tetra-antennary
Controls ($n = 10$)	100 (11.9 ± 3.3)	85.2 ± 2.2 (8.1 ± 2.8)	14.8 ± 2.2 (34.3 ± 11.9)	n.d
Ia ($n = 3$)	100 (37.3 ± 19.4)	79.1 ± 8.2 (27.7 ± 18.8)	20.9 ± 8.2 (64.0 ± 31.8)	n.d.
Ib ($n = 1$)	100 (23.5)	71.0 (16.0)	29.0 (40.5)	n.d.
Ic ($n = 2$)*	100 (31.1 ± 2.1)	52.0 ± 1.8 (11.3 ± 1.8)	17.5 ± 1.4 (49.9 ± 2.2)	30.5 ± 3.2 (54.1 ± 2.8)
Ix(i)†	100 (30.4)	92.1 (26.4)	7.9 (76.8)	n.d.
Ix(ii)†	100 (35.0)	80.4 (25.1)	19.6 (76.0)	n.d.
Ix(iii)†	100 (8.7)	91.8 (9.4)	8.2 (n.d.)	n.d.
IIx(i)‡	100 (8.0)	84.1 (6.4)	15.9 (16.3)	n.d.
IIx(ii)‡	100 (10.6)	82.3 (9.6)	17.7 (26.8)	n.d.
IIx(iii)‡	100 (14.2)	82.9 (10.3)	16.0 (33.9)	1.1 (n.d.)

* Mean of two determinations.
† Patients diagnosed as CDG-I by isoelectric focusing, but of unknown genetic defect.
‡ Patients diagnosed as CDG-II by isoelectric focusing, but of unknown genetic defect.

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Table 2 Percentages of N-linked glycan structures of individual isoforms of α_1-antitrypsin from control and CDG-I patients

Isoforms of α_1-antitrypsin were separated by narrow range two-dimensional PAGE, and their glycans released by in-gel PNGase-F digestion prior to analysis of glycans by MALDI–TOF-MS as described in the Materials and methods section. Symbols identifying sugar residues are as detailed in Figure 2. The values for the M6 underglycosylated isoform are from Figure 3 (spot b). Abbreviations: Bi, biantennary; FBi, fucosylated biantennary; Tri, triantennary; FTri, fucosylated triantennary; FFTri, difucosylated triantennary; Tetra, tetra-antennary; FTetra, fucosylated tetra-antennary; FFTetra, difucosylated tetra-antennary; n.d., not detected.

	Total N-linked glycan structures (%)							
Isoform	Bi	FBi	Tri	FTri	FFTri	Tetra	FTetra	FFTetra
M2 isoform								
Control ($n = 4$)	78±5	3±1	10±1	9±trance	n.d.	n.d.	n.d.	n.d.
CDG-Ia ($n = 1$)	60	16	7	5	4	n.d.	3	5
CDG-Ic ($n = 1$)	69	9	5	5	1	4	6	1
M4 isoform								
Control ($n = 5$)	83±6	3±1	9±2	5±1	n.d.	n.d.	n.d.	n.d.
CDG-Ia ($n = 1$)	65	11	8	12	4	n.d.	n.d.	n.d.
CDG-Ic ($n = 1$)	67	9	9	12	3	n.d.	n.d.	n.d.
M6 isoform								
Control ($n = 2$)	98±2	2±2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CDG-Ia ($n = 1$)	80	14	2	4	n.d.	n.d.	n.d.	n.d.
CDG-Ic ($n = 1$)	89	11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M6 underglycosylated isoform								
CDG-Ia ($n = 1$)	84	16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CDG-Ic ($n = 1$)	89	11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

ferrin and α_1-antitrypsin from patients with CDG-I. As well as an increase in monofucosylated glycans, bi- and trifucosylated tri- and tetra-antennary glycans were detected, indicating that peripheral fucosylation of glycans must occur in CDG-I. Existing knowledge of the specificities of liver α-2,3- and α-2,6-sialyl-transferases, and α-1,3-fucosyltransferase [20,21], suggests that α-(1 → 3)-linked fucose can only be transferred to branches terminating in α-(2 → 3) sialic acid, creating the sialyl Lewis^x structure. The existence of an α-(1 → 3)-linked fucose on a branch of a biantennary glycan terminating in an α-(2 → 6)-linked NeuAc residue has not been reported in serum glycoproteins. Therefore we speculate that increased fucosylation of the biantennary glycans in transferrin and α_1-antitrypsin is predominantly due to core α-(1 → 6) fucosylation. Furthermore we postulate that there is increased α-(1 → 3) fucosylation of the β-(1 → 6)-linked antennae in tri- and tetra-antennary glycans to create the sialyl Lewis^x blood-group determinant in CDG-I serum glycoproteins. The expression of the sialyl Lewis^x determinant on a biantennary structure has been reported in human amniotic fluid transferrin, in which, however, 40% of biantennary N-linked glycans have terminal α-(2 → 3)-linked NeuAc residues [22].

Recently, increased expression of the sialyl Lewis^x determinant on the branches of tri- and tetrasialylated glycans of the acute-phase protein, α_1-acid glycoprotein, has been reported in CDG-Ia [23]. However, unlike normal serum transferrin and α_1-antitrypsin, α_1-acid glycoprotein normally contains α-(1 → 3)-linked fucose.

There are several possible explanations for the observed increases in the activity of the fucosylation and branching enzymic steps of glycosylation in CDG-I. Firstly, there could be an increase in the expression of the processing enzymes, as this has been observed under various abnormal physiological conditions. Elevated levels of the sialyl Lewis^x antigen on α_1-acid glycoprotein in inflammatory responses have been attributed to increases in the expression of α-1,3-fucosyltransferase [21,24]. Likewise, an increase in the relative amounts of tri- and tetra-antennary glycans compared with biantennary glycans has been described for a number of chronic inflammatory states, and similar changes have been seen in hyperoestrogenism [25]. Enhanced expression of both α-1,3- and α-1,6-fucosyltransferases has also been reported in cancer cells [26]. Increased core α-(1 → 6) fucosylation of the biantennary glycans of α_1-antitrypsin has been reported in various acute-phase proteins from patients with hepatocellular carcinoma, consistent with increased levels of expression of α-1,6-fucosyltransferase activity [27].

The increase in fucosylation and branching could also result from a decrease in the flux of the glycoproteins through the Golgi, leading to greater processing of individual glycoprotein molecules. This may account for the mixed pattern of fucosylation in CDG-I patients, with tri- and tetra-antennary glycans being better substrates for the α-1,3-fucosyltransferase than biantennary glycans. Alternatively non-glycosylation at one sequon could alter the conformation of the glycan/peptide at another sequon, thereby affecting its suitability as a substrate for a processing enzyme. However, both the fully glycosylated and partially glycosylated glycoforms of CDG-I α_1-antitrypsin were processed abnormally, whereas the underglycosylated α_1-antitrypsin variant ($PIZ_{bristol}$) was processed normally. Finally, it is possible that the processing enzymic steps are stimulated because of higher concentrations of the nucleoside phosphate sugar glycosyl donors in the Golgi, resulting from diversion of glycosylation precursors from the defective assembly of the dolichol-pyrophosphate-linked oligosaccharide precursor.

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The level of branching was much greater in the single case of CDG-Ic analysed than in the other forms of CDG-I. However, it is too early to judge whether the different enzymic defects in CDG-I result in significantly different patterns of glycosylation or whether the genotype affects the level of processing. The significance of the contribution of the altered processing of glycoproteins to the pathophysiology of CDG-I remains to be investigated.

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