Carbohydrate-deficient Glycoprotein Syndrome: Not an N-linked Oligosaccharide Processing Defect, But an Abnormality in Lipid-linked Oligosaccharide Biosynthesis?

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Abstract

The carbohydrate-deficient glycoprotein syndrome (CDGS) is a developmental disease associated with an abnormally high isoelectric point of serum transferrin. Carbohydrate analyses of this glycoprotein initially suggested a defect in N-linked oligosaccharide processing, although more recent studies indicate a defect in the attachment of these sugar chains to the protein. We studied both serum glycoproteins and fibroblast-derived [2-^{3}H]mannose-labeled oligosaccharides from CDGS patients and normal controls. While there was a decrease in the glycosylation of serum glycoproteins of affected individuals, differences were not seen in either monosaccharide composition or oligosaccharide structures. The lectin-binding profiles of glycopeptides from [2-^{3}H]-mannose-labeled fibroblasts were likewise indistinguishable. However, the incorporation of [2-^{3}H]mannose into both glycoproteins and the dolichol-linked oligosaccharide precursor was significantly reduced. Thus, at least in some patients, CDGS is not due to a defect in processing of N-linked oligosaccharides, but rather to defective synthesis and transfer of nascent dolichol-linked oligosaccharide precursors. This abnormality could result in both a failure to glycosylate some sites on some proteins, as well as secondary abnormalities in overall glycoprotein processing and/or function. (*J. Clin. Invest.* 1994. 94:1901–1909.) Key words: mental retardation • serum glycoproteins • transferrin • dolichol • lipid-linked oligosaccharide

Introduction

The carbohydrate-deficient glycoprotein syndrome (CDGS)[1] was initially described 10 years ago (1, 2) as an inherited disorder affecting multiple organ systems including the central and peripheral nervous systems, liver, bone, adipose tissue, and genital organs (1–9). Within the first few months of life, affected children present with neurological abnormalities, and their development is marked by variable but often severe psychomotor retardation, lower motor neuron dysfunction, abnormal facies, skeletal anomalies, variable hepatomegaly, and other clinical symptoms and signs. Since its initial description, the syndrome has been reported in populations from around the world, and the existence of distinct subtypes based on the severity of clinical symptoms has been suggested (10–14). A characteristic biochemical abnormality of this syndrome was discovered serendipitously in the isoelectric focusing of serum transferrin (1–3, 15), a test originally devised to screen for alcohol abuse in normal adults (16). A consistent cathodal migration of serum transferrin was noted, and attributed to a reduction in the number of sialic acid (Sia) residues from a normal of four to five, to predominantly two, giving rise to the initial name "disialotransferrin development deficiency syndrome" (3). Compositional studies indicated that, in addition to sialic acid, serum transferrin from affected individuals was deficient in other monosaccharides characteristic of N-linked glycans including galactose (Gal) and *N*-acetyl-glucosamine (GlcNAc) (3, 15). Similar deficiencies were also reported in other serum proteins, in addition to serum transferrin (2).

The typical side chains ("antennae") of complex-type N-linked oligosaccharides on most normal human serum glycoproteins have the following sequence:

Sia α2-(3 or 6)Galβ1-(3 or 4)GlcNAcβ1-(2,4, or 6)Manα1-

These antennae arise from the processing (remodeling) of mannose (Man)-containing structures, and are therefore the net product of multiple exoglycosidases and glycosyltransferases (17, 18). Thus, it was first suggested that the defect in CDGS arose from a deficiency of one or more of these processing enzymes, leading to truncated oligosaccharide structures on glycoproteins. Based on a partial decrease in total GlcNAc transferase activity in serum (2, 15), abnormalities were postulated of one or more of the specific GlcNAc transferases responsible for the initial extension of the antennae of N-linked oligosaccharides. However, at least one previously known human defect in GlcNAc transferase activity gives rise to a completely different clinical syndrome, that of Hereditary Erythrocytic Multinuclearity with Positive Acidified Serum test (HEMPAS) (19). Also, one report on transferrin isolated from a patient with CDGS indicated a decrease in Man content as well (10), making the situation even less clear. Finally, two recent reports indicate that the N-linked glycans on CDGS serum transferrin are completely normal in structure, but are simply reduced in number (20–23). These authors therefore suggest a completely different defect, one of

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1. *Abbreviations used in this paper:* CDGS, Carbohydrate-deficient Glycoprotein Syndrome; Con A, concanavalin A; Dol, dolichol; ER, endoplasmic reticulum; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; GlcNAc, *N*-acetyl-glucosamine; HPAE, HPLC anion exchange chromatography with pulsed amperometric detection; LLO, lipid-linked oligosaccharide; Man, mannose; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparigine amidase; Sia, sialic acid; TBA, thiobarbituric acid assay; TFA, trifluoroacetic acid.

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failure of attachment of one of the two N-linked oligosaccharides normally found on serum transferrin. While a specific biochemical defect was not identified, possible explanations discussed included an oligosaccharyltransferase deficiency, or a defect in the biosynthesis of the lipid-linked oligosaccharide (LLO) precursor of N-linked oligosaccharides (20, 21).

The compositional analyses reported in the original studies were actually composite data, obtained using a variety of different colorimetric and enzymatic methods for measurement of individual monosaccharides. While this approach can yield useful information, it may be prone to error. Regardless, the global abnormality reported in total serum proteins suggest that the patients do indeed have a basic defect involving the biosynthesis of N-linked oligosaccharides (2). Based on this assumption, we reexamined the matter using several different approaches. Focusing on N-linked oligosaccharides, an initial series of experiments were directed towards identifying a defect in the processing of N-linked oligosaccharides, presuming that the defect(s) occurred after the transfer of the high-mannose oligosaccharide from dolichol to the nascent protein chain in the endoplasmic reticulum (ER). Surprisingly, these experiments failed to show a specific processing defect. However, further experiments suggested a defect in the synthesis of the lipid-linked precursor itself, with reduced levels of [2-^{3}H]Man incorporation into both the LLO precursor and nascent glycoproteins. As protein synthesis itself is not affected, the net result is a relative underglycosylation of glycoproteins in the CDGS samples relative to controls. In some but not all experiments, the LLO of CDGS patients was of an abnormally small size, as well. Thus, in the CDGS cases examined here, the data indicate a biosynthetic defect in the assembly of the LLO precursor.

Methods

Materials. Most of the materials used were obtained from Sigma Chemical Co. (St. Louis, MO), except for: concanavalin A (Con A) Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ); E_4-PHA and L_4-PHA agarose (E.Y. Laboratories, Inc., San Mateo, CA); MicroPak AX-5 HPLC column (Varian Instrument Business, San Fernando, CA); Jack bean α-mannosidase (V-labs Inc., Covington, LA; bovine testicular β-galactosidase (Oxford GlycoSystems, Inc.); *Arthrobacter ureafaciens* sialidase (Calbiochem Corp., La Jolla, CA). Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparigine amidase (PNGase F) was purified as described (24).

Patients. Two sisters were diagnosed with CDGS on the basis of their clinical presentation including low birth weight, poor feeding, hypotonia, ataxia, strabismus, hepatomegaly, seizures, autistic-like behavior, characteristic fat pads, and a cathodal shift of serum transferrin in isoelectric focusing. Based upon the severity and extent of the symptoms, these patients fall into the Type I classification of Jaeken (9, 14).

Preparation of glycopeptides from serum proteins. Serum samples (480 μl), from the two CDGS patients and two age- and sex-matched controls, were adjusted to pH 8.3 with 60 μl of 3 M Tris-Cl, pH 8.3 followed by 15 μl of Proteinase K (20 mg/ml). After 16 h at 37°C, an equal aliquot of Proteinase K was added and then digested for 36 h. Analysis by SDS-PAGE demonstrated complete digestion of serum proteins. Samples were delipidated by mixing with 1.6 ml of methanol, 0.8 ml of chloroform, and 0.52 ml of 0.2 M KCl. After vortexing and centrifuging, the upper (aqueous) layer was recovered, and the lower layer extracted twice with prepared upper phase. The combined upper phases were dried, resuspended in water, and further delipidated by passage through a reverse phase cartridge (Sepak C18 cartridge, Millipore Corp., Marlborough, MA) and elution with water. Finally, the samples were desalted over a 15-ml BioRad P-2 column run in 10 mM ammonium formate and lyophilized.

Total protein and monosaccharide analysis. Protein concentrations were measured by the BCA protein assay (Bio-Rad Laboratories Richmond, CA) or by the Lowry method (25) using BSA as a standard. Hexose concentrations were determined by the phenol sulfuric assay (26), using Man as a standard. Monosaccharide compositional analyses were performed by hydrolyzing serum glycopeptides with 2 N trifluoroacetic acid (TFA) for 2 h at 121°C in a sealed vial under nitrogen. The samples were subsequently lyophilized and analyzed by HPLC anion exchange chromatography with pulsed amperometric detection (HPAE-PAD; Dionex Corp., Sunnyvale, CA) on a Carbopac PA-1 column Dionex Corp. (27). Molar amounts of each monosaccharide were calculated by comparison with a standardized mixture of each monosaccharide. Detector response by the PAD was demonstrated to be linear for each monosaccharide over the range examined. By this technique, approximately half of the hexose predicted by the phenol sulfuric assay could be accounted for. The discrepancy most likely reflects a combination of factors, including nonuniform color yield of different hexoses in the phenol sulfuric acid assay (e.g., Man and Gal differ by twofold), and incomplete hydrolysis of sugars and/or their partial destruction during TFA hydrolysis. However, each methodology is reproducible within 3–5%, and comparisons utilizing the same technique are valid.

Sialic acid content was determined by digestion with *Arthrobacter ureafaciens* sialidase in 50 mM sodium acetate, pH 5.4, overnight at 37°C, and quantitating released sialic acid by a modified thiobarbituric acid (TBA) assay (28). The amount of β-Gal exposed at the nonreducing termini of the glycoconjugates was determined by digestion with bovine testicular β-galactosidase. After digestion, the samples were mixed with 200 pmol of 2-deoxyglucose (as an internal standard), desalted on Amberlite mixed bed ion exchange resin (Sigma Chem. Co.) and analyzed by HPAE-PAD. In parallel, identical samples were treated with sialidase prior to β-galactosidase digestion, and the difference in Gal release was used to calculate the amount of Sia-capped β-Gal.

Examination of serum N- and O-linked oligosaccharides by HPAE-PAD. Serum samples were dialyzed extensively against 0.15 M NaCl and 2 mM EDTA, pH 7.0, before hydrazinolysis. Samples from controls and patients (containing 1 mg protein) were submitted to hydrazinolysis using a hydrazinolysis instrument (Glycoprep 1000; Oxford GlycoSystems, Inc., Rosedale, NY) Under the settings used (95°C for 4 h), the instrument gives good recovery of released N- and O-linked chains that have also been re-*N*-acetylated. Alternatively, N-linked oligosaccharides were released and purified from serum glycoproteins by digestion with PNGase F as described (26).

Released oligosaccharides were analyzed by HPAE-PAD using a GlycoStation equipped with a Carbo-Pac PA-1 column (Dionex Corp.). A linear gradient of sodium acetate (20–250 mM) with a constant concentration of sodium hydroxide (100 mM) capable of separating O- and N-linked oligosaccharides was used (27).

Cell lines. Primary fibroblast cultures, established from punch biopsies of skin, were grown in α-MEM supplemented with 10% FCS, and serially passaged by trypsinization. Control fibroblasts included normal pediatric samples, which were limited in availability, as well as pediatric samples from patients suffering from disorders known not to express the transferrin abnormality diagnostic of CDGS (including a seizure disorder and an unclassified movement disorder associated with ataxia and tremor). Additionally, normal adult fibroblasts (CRL 1826) obtained from American Type Culture Collection (Rockville, MD) were examined.

Metabolic labeling of fibroblasts with [2-^{3}H]Man and preparation of glycopeptides. Subconfluent cultures of the fibroblasts were labeled with [2-^{3}H]Man (100 μCi/5 ml) in complete α-MEM with 10% FCS and allowed to grow to confluency (3 d). Labeled cells from a single 15-cm dish were harvested by scraping, washed twice in ice-cold Tris-buffered saline (TBS, 20 mM Tris-Cl, pH 7.3, 140 mM NaCl), and then solubilized in TBS/0.1% NP-40 containing 1% (vol/vol) aprotinin on ice for 15 min. Nuclei were removed by centrifugation at 2,000 rpm $\times$ 10 min, and the supernatant, containing the solubilized glycoproteins, digested with Proteinase K (80 μg) for 24 h at 37°C. The reaction was boiled for 10 min, insoluble material removed by centrifugation, and

the soluble glycopeptides desalted on a Sephadex G-25 column eluted in water.

Lectin affinity chromatography. [2-^{3}H]Man-labeled glycopeptides were analyzed on a 2-ml column of Con A Sepharose as previously described (29). L_4-PHA Sepharose and E_4-PHA Sepharose affinity chromatography was carried out as described (29), except that a [^{14}C]-ManNAc internal marker was added to accurately mark the position of unretained molecules.

Determination of incorporation of [2-^{3}H]Man into cultured fibroblasts relative to total protein synthesis. Fibroblasts were labeled with [2-^{3}H]Man (20 μCi/ml) and [^{35}S]Methionine (2 μCi/ml) for 60 min in DMEM containing 0.1 mg/ml glucose. After removal of the radioactive medium, cells were quickly washed twice with ice-cold PBS, and harvested by trypsinization. Cell pellets were surface washed with 1 ml water, sonicated, and solubilized in 0.1% SDS. A portion of the material was used for protein determination, and the remainder was precipitated with 10% TCA, resolubilized, mixed with scintillation fluid, and counted. Incorporation was normalized to protein content.

Isolation and characterization of the LLO precursor from fibroblasts. Fibroblasts grown in glass petri dishes were labeled with [2-^{3}H]Man (100 μCi/ml) in DMEM, 0.1 mg/ml glucose, for 30 min. After removing the radioactive medium, cells were quickly washed twice with ice-cold PBS, and then 5 ml of $CHCl_3$/MeOH (2:1, vol/vol) was added at 0°C (30). Cells were scraped, extracted twice with the same solvent, and then dried in a stream of nitrogen. A small amount of water was added to the dried cells, which were then sonicated and centrifuged. The pellet was washed several times with water to remove the free label, and then LLO were extracted with $CHCl_3$/MeOH/H_2O (10:10:3 vol/vol/vol). Oligosaccharides were released from the lipid by mild acid hydrolysis (30). After extraction of the LLO, the residual pellet was solubilized in 0.1% SDS and subjected to PNGase F digestion to obtain the oligosaccharides from proteins as described (26). The released free oligosaccharides obtained were reduced with sodium borohydride in sodium borate buffer, pH 9.8 (31). After evaporation of methyl borate, the labeled products were desalted on Amberlite MB3 mixed bed resin and analyzed by HPLC on an AX5 column using a linear gradient of water in acetonitrile (31) at a flow rate of 1 ml/min using a 60-min program.

Results

Carbohydrate compositional analyses of total serum proteins. Prior work by others (2, 3, 15) has suggested that the biochemical abnormality of CDGS is a defect in the processing of N-linked oligosaccharides of serum glycoproteins, leading to decreased molar amounts of sialic acid, Gal, and GlcNAc, but not Man. These monosaccharides are found on the outer antennae of N-linked oligosaccharides. These analyses were performed with a combination of colorometric and enzymatic assays to quantitate individual monosaccharides, assays that are known to be susceptible to variable interference. Therefore, we sought to repeat these analyses utilizing HPAE-PAD, a technique that permits the direct quantitation of all possible monosaccharides (except sialic acid) in a single chromatographic run. Moreover, the sensitivity of this approach requires only ~ 10 μl serum per analysis. For these analyses, glycopeptides were first generated from serum glycoproteins from two children with CDGS and from two age- and sex-matched controls. By the method employed, the resulting samples would contain most of the serum N- and O-linked carbohydrate structures but would be substantially free of glycolipids. The total hexose content of these samples was determined by the phenol sulfuric acid assay (utilizing Man as a standard), and equivalent amounts of hexose subjected to total acid hydrolysis followed by quantitation of the released sugars by HPAE-PAD. No major differences are seen in the monosaccharide compositions other than a slight increase in GalNAc content (Table I). However, GalNAc is not a major constituent of serum protein N-linked oligosaccharides, being a more common constituent of O-linked chains and glycolipids. More relevant to N-linked oligosaccharide structure, the molar ratios of Gal/Man and GlcNAc/Man show no significant differences between patients and controls (Table I). Altered ratios of these sugars were reported in some of the earlier studies (3, 15). In contrast to these negative results, the total hexose content of the patient samples show a significant reduction relative to the amount of protein (Table I), although the albumin and total protein contents of the samples were within normal limits (Table I, and data not shown).

Table I. Neutral Monosaccharide Analysis of Serum-derived Glycopeptides

	Sample			
	CDGS	CDGS	Control	Control
Total serum protein (mg/ml)*	64	54	52	53
Total hexose (μmol/ml serum)‡	2.9	2.53	3.48	3.7
nmol total hexose/mg serum protein	45.3	46.8	66.9	69.8
Compositional analyses§ (nmol sugar/10 nmol hexose)				
Gal	2.6	2.4	2.3	2.3
GlcNAc‖	6.7	6.2	5.9	5.5
Man	2.5	2.8	2.3	2.5
GalNAc‖	0.55	0.46	0.27	0.23
Glc¶	0.29	0.47	0.24	0.35
Molar ratio of:				
Gal/Man	1.02	0.86	0.99	0.91
GlcNAc/Man	2.62	2.21	2.54	2.15

* BCA protein assay, using BSA as a standard. ‡ Phenol-sulfuric acid assay, using Man as a standard. § Total TFA hydrolysis and HPAE-PAS, as described in *Methods.* ‖ After TFA hydrolysis, GlcNAc and GalNAc are recovered and measured as glucosamine and galactosamine, respectively. ¶ Glucose is found variably in sample blanks as well, suggesting environmental contamination.

Amounts of sialic acids and β-Gal residues in serum glycopeptides. As hydrolysis in TFA destroys sialic acids, this sugar was quantitated separately by the TBA assay after enzymatic release. These results indicate no significant differences in the sialic acid content of the glycopeptides derived from patient or control samples (Table II). Given that the majority of sialic acid is found linked to underlying β-Gal residues, the amount of sialic acid-capped and non-capped β-Gal was determined as a further measure of terminal sialylation. This was accomplished by quantitating Gal, released by bovine testicular β-galactosidase, before and after sialidase treatment. Thus, while all terminal sialic acids can be removed by sialidase, only terminal (non-sialylated) Gal residues can be removed by β-galactosidase. No differences are seen in the ratios of sialic acids to Gal between patient and control samples, nor in the amount of terminal Gal accessible to β-galactosidase (Table II).

Analysis of total serum N-linked oligosaccharides. The compositional analyses reported above could still miss subtle changes in the overall structure of the N-linked oligosaccharides, especially if they selectively affected only certain subsets

Table II. Release of Sialic Acid or Galactose from Serum-derived Glycopeptides by Exoglycosidases

	Sample			
	CDGS	CDGS	Control	Control
Monosaccharide*				
sialic acid (Sia)	4.72	2.90	3.60	3.07
Terminal β-Gal	0.82	0.69	0.66	0.93
Sia-β-Gal	5.1	3.9	5.0	5.6
% Terminal β-Gal‡	16	18	13	16.6

Serum-derived glycopeptides were digested with bovine testicular β-galactosidase with or without prior *A. ureafaciens* sialidase treatment. The released sialic acid and Gal were quantitated as described in *Methods.* * Nmol of sialic acid (measured by TBA assay) or Gal (measured by HPAE-PAD) per 10 nmol total hexose (measured by phenol–sulfuric acid assay, using Man as a standard). ‡ Percent of total β-Gal residues not capped by sialic acid.

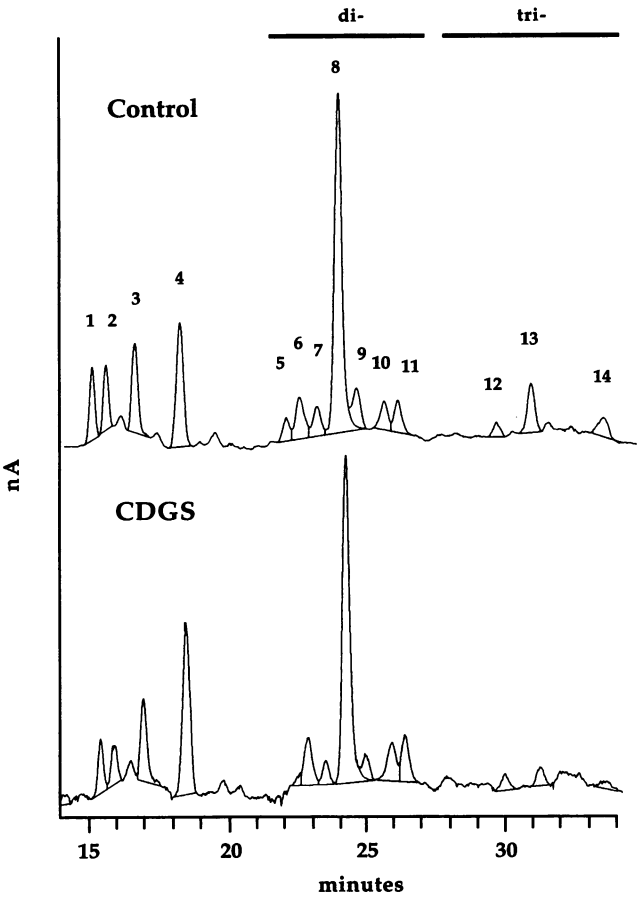

Figure 1. Examples of HPAE-PAD profiles of total serum oligosaccharides released by hydrazinolysis. Oligosaccharides were released from total serum glycoproteins by hydrazinolysis and subsequently analyzed by HPAE-PAD as described in *Methods.* Representative profiles are presented of oligosaccharides derived from a control sample and a CDGS sample. The general elution positions of bi- and triantennary oligosaccharides are indicated by the horizontal bars. The regions of elution of small O-linked oligosaccharides (before 15 min) and of tetra-antennary N-linked chains (after 35 min) are not shown because the peaks were insignificant and/or no significant differences could be discerned. The individual peaks are numbered by order of elution for purposes of comparison between samples and with Figs. 2 and 3.

of molecules. We therefore studied the total oligosaccharide population released by hydrazinolysis from unfractionated serum proteins. After controlled treatment with hydrazine under conditions which release both N- and O-linked structures, the oligosaccharides were profiled by HPAE-PAD. This method separates oligosaccharides on the basis of multiple parameters including relative size and charge. Furthermore, the detection technique used relies on the ionization of hydroxyl groups at high pH values (27), and thus is rather specific for sugars as opposed to other molecules. The data for one patient and one control sample are presented in Fig. 1, which demonstrate that the overall profile does not appear substantially different between these samples. Similar results are seen with the other patient and control samples as well (data not shown). The pattern of total serum N-linked oligosaccharides, derived from PNGase F digestion, likewise show no differences (Fig. 2, and data not shown).

When the individual peaks are considered on a percentage basis (arbitrarily assuming equivalent detector responses for each unique structure), qualitative and quantitative differences are noted between the patient and control samples. The data for two patient and two control samples, released by hydrazinolysis or by PNGase F digestion, are presented graphically in Fig. 3. In particular, there appear to be lower levels of the peaks corresponding to the tri- and tetra-antennary chains (peaks 12–14, as labeled in Fig. 1). However, these are minor components even in the normal serum samples, and the differences are not consistent between the samples. In the hydrazinolysis-released material, the patients' samples appear to contain slightly higher levels of the early eluting peak 4 (Figs. 1 and 3). This peak is not present in the oligosaccharides released by PNGase F (see Figs. 2 and 3), and likely represents O-linked oligosaccharides which are present on a few serum glycoproteins and are released by hydrazinolysis. The major peak, 8, coelutes with a bisialylated biantennary N-linked oligosaccharide standard, and most of the remaining peaks run in positions expected for other complex-type chains.

Incompletely processed N-linked chains could also be coeluting with these molecules and remain undetected. Such chains would be either of the high mannose type or the hybrid type and are expected to have terminal α-linked Man residues (17). However, treatment of the oligosaccharide mixtures with α-mannosidase caused no significant change in any of the profiles (see example in Fig. 2), indicating that all serum oligosaccharides are processed to complex type chains in both the patient and control samples. Treatment of the samples with sialidase caused all of the peaks to elute earlier than 13 min, indicating that all structures eluting after 13 min represent sialylated oligosaccharides (data not shown).

Metabolic labeling of fibroblasts with [2-^{3}H]Man. Prior studies have shown that even after prolonged labeling periods, this radioactive monosaccharide remains exclusively in [2-^{3}H]-Man and [2-^{3}H]fucose (32). As only a minority of the isotope is converted to [2-^{3}H]fucose and Man is found exclusively in N-linked oligosaccharides, labeling with [2-^{3}H]Man is a convenient approach to screen for abnormalities in the processing pathway of these sugar chains (32). Glycopeptides prepared from cells labeled for 3 d with [2-^{3}H]Man were passed over

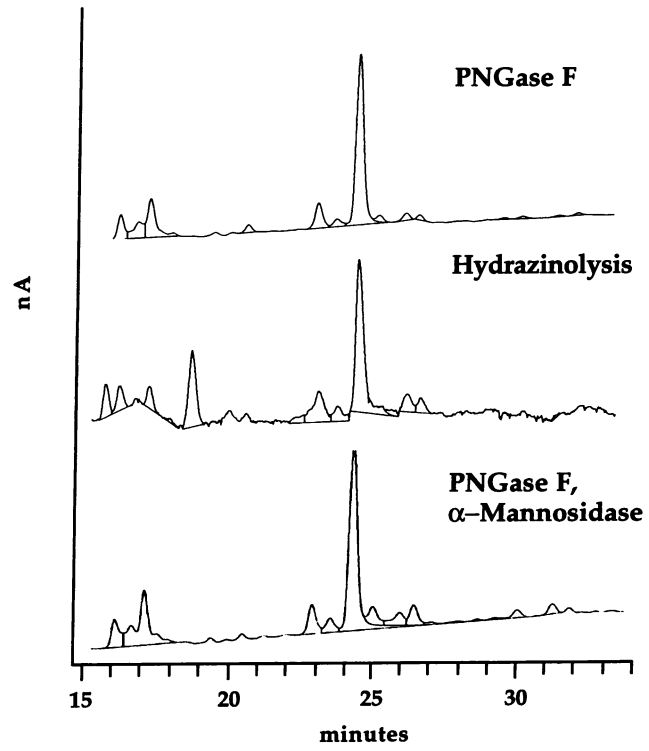

Figure 2. Examples of HPAE-PAD profiles of total serum oligosaccharides. Oligosaccharides were released from total serum glycoproteins of a CDGS case either chemically by hydrazinolysis or enzymatically with PNGase F and analyzed by HPAE-PAD as in Fig. 1. The effects of treatment with α-mannosidase are presented in the third panel.

Con A Sepharose, fractionating them into multiantennary or bisected types (Con A I, runs through the column), biantennary types (Con A II, elutes with 10 mM α-methyl glucoside), and high-mannose or hybrid types (Con A III, elutes with 100 mM

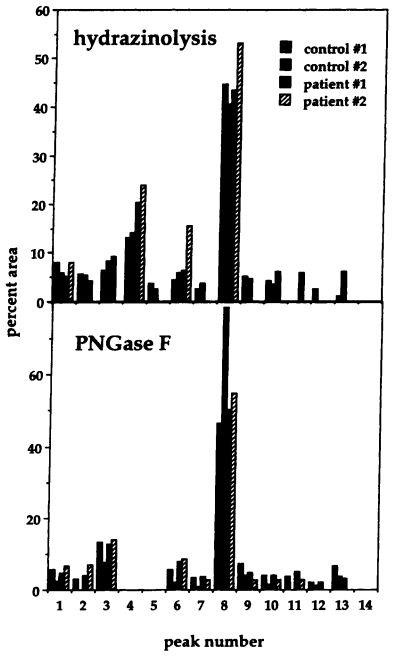

Figure 3. Relative ratios of oligosaccharide species released from total serum by hydrazinolysis or PNGase F. From the HPAE-PAD chromatographs, the areas corresponding to all of the peaks (as labeled in Fig. 1) were totalled together, and each peak is presented here as a percentage of this total. The data for oligosaccharides from the serum of two patient and control samples released by hydrazinolysis (*top panel*) or PNGase F (*bottom panel*) are presented. The data are derived from the chromatograms presented in Figs. 1 and 2, as well as from chromatograms not shown.

Table III. Analysis of [2-^{3}H]Man-labeled Glycopeptides from Primary Fibroblast Cultures by Lectin Affinity Chromatography

Lectin	CDGS	CDGS	Control
Con A I, % of total	30	25	25
Con A II, % of total	30	25	25
Con A III, % of total	40	49	49
L-PHA % bound	20	ND	13
E-PHA % bound	35	ND	42

Primary fibroblast cultures were labeled with [2-^{3}H]Man for 3 d, and glycopeptides were prepared and desalted as described in *Methods*. Aliquots were analyzed by affinity chromatography on Con A Sepharose, L_4-PHA Sepharose, and E_4-PHA Sepharose, as described in *Methods*. ND, not done.

α-methyl mannoside) (29). The last group contains biosynthetic precursors of the other two. Thus, any major defect in the processing of N-linked oligosaccharides from high-mannose type to complex type should have a gross effect on the Con A elution profiles; however, none are seen (Table III). In one sample, further analysis was done using L_4-PHA and E_4-PHA Sepharose chromatography, to uncover certain changes in the branching of N-linked oligosaccharides (29). Again, no significant differences were seen. To ensure that the analysis was confined to N-linked oligosaccharides alone, the labeled oligosaccharides from one pair of normal and CDGS fibroblasts were released and isolated from total cellular glycoproteins using PNGase F. Analysis of these structures by Con A affinity chromatography again showed no obvious differences (data not shown). In view of these predominantly negative results, complete studies by lectin affinity chromatography were not done. Taken together, the data indicate that, as in the case of serum glycoproteins (synthesized primarily by hepatocytes and hematopoietic cells), there are no major changes in the N-linked oligosaccharides synthesized by cultured human fibroblasts.

Abnormalities in the synthesis of LLO. The data above indicate that while minor differences are present, no substantial structural abnormality is seen in the N-linked oligosaccharides of the CDGS patients when compared with normal individuals. However, they do not address events that take place in the earliest stages of the N-linked oligosaccharide biosynthetic pathway (i.e., the formation of the LLO precursor and its en bloc transfer to newly synthesized proteins) (17, 18). To explore these steps, we labeled the LLO metabolically in cultured fibroblasts using a short pulse label (60 min) of [2-^{3}H]Man (to measure protein N-glycosylation) and [^{35}S]methionine (to measure protein biosynthesis). These experiments demonstrate no decrease in the rate of protein biosynthesis between CDGS and normal fibroblasts. However, the amount of [2-^{3}H]Man incorporated is markedly reduced when examined both in terms of [2-^{3}H]Man per mg protein and in terms of [2-^{3}H]Man/[^{35}S]-methionine incorporation. The results of one representative experiment are presented in Table IV.

As these results could reflect either a decrease of incorporation of [2-^{3}H]Man into the LLO or a decrease in the rate of transfer of [2-^{3}H]Man-LLO to protein, levels of [2-^{3}H]Man in LLO relative to protein were measured directly in fibroblasts. These experiments revealed similar decreases in [2-^{3}H]Man incorporation into both LLO and nascent proteins in the CDGS

Table IV. Comparison Between N-Linked Glycosylation and Protein Biosynthesis in Fibroblasts

Fibroblasts	[^{3}H]Man	[^{35}S]methionine	^{3}H/^{35}S ratio
	cpm/mg cell protein	*cpm/mg cell protein*	
Normal	882,510	3,575	246
	835,270	3,245	257
Non-CDGS*	307,730	1,500	204
	354,095	1,515	234
CDGS	410,570	4,024	102
	249,440	4,170	60
CDGS	113,685	4,955	23
	111,020	4,830	23

Incorporation of [^{3}H]Man or [^{35}S]methionine into macromolecules of fibroblasts during a 60-min labeling was determined by TCA precipitation as described in *Methods*. * From a child with a seizure disorder but normal transferrin isoelectric focusing pattern.

Table V. [^{3}H]Man Incorporation into LLO and Glycoproteins in Fibroblasts

Fibroblasts	LLO	Glycoproteins	LLO/protein ratio
	cpm/mg cell protein	*cpm/mg cell protein*	
Normal	718,000	507,500	0.70
CDGS	341,000	210,000	0.62
CDGS	189,000	94,900	0.50

Incorporation of [^{3}H]Man into LLO and glycoproteins was determined from the same labeled cell population, as described in *Methods*.

samples; representative results are presented in Table V. While the ratio of ^{3}H in LLO versus protein of the CDGS samples is reduced slightly (10–25%) compared to normal, this level of reduction is much less dramatic than the 3–10-fold reduction in total [2-^{3}H]Man incorporation (Table IV). Thus, the rate of transfer of oligosaccharide from the lipid precursor to protein is not likely to be the primary defect in CDGS; rather, some earlier step involved in the biosynthesis of the LLO is implicated.

An alternative explanation for the decreased levels of [2-^{3}H]Man incorporated into LLO and protein presented in Tables IV and V is that the size of the precursor is smaller, resulting in the same amount of LLO but with fewer Man residues and thus less ^{3}H label. To examine this possibility, cells were labeled with [2-^{3}H]Man as before, the LLO was extracted, and the oligosaccharides were released from the lipid by mild acid hydrolysis. In parallel, the N-linked oligosaccharides were released from proteins by digestion with PNGase F. These two populations of oligosaccharides were fractionated by size, by chromatography on an AX5 column (31). Normal cells make a heterogeneous array of oligosaccharides in the LLO and protein fraction. Their sizes correspond to those with five to nine Man residues, with the greatest portion having nine (data not shown). In contrast, the oligosaccharides from CDGS cells are much more variable in size. In approximately half of the experiments, the LLO and protein oligosaccharides contain a higher proportion of structures containing five or fewer Man residues, while in the remaining experiments, the structures are similar in size to those from normal fibroblasts. The cause of this variability could not be traced to cell density, labeling conditions, or passage number, and is currently under investigation. Smaller oligosaccharides would contain fewer [2-^{3}H]Man residues, and thus have a lower amount of radioactivity than normally sized structures. However, even when the decrease in incorporation is normalized for the difference in size of the LLO (three separate experiments), CDGS patients make only 30–35% of the amount of LLO found in normal cells. Thus, the decreased protein glycosylation in CDGS does not require an altered LLO structure; rather, it is a reflection of a reduced amount of LLO itself. Once transferred to proteins, these truncated oligosaccharides can be processed normally, and thus may not be apparent on the fully processed glycoproteins. No evidence for abnormally processed structures was found in our studies (Figs. 1–3) nor in those of others (21).

Discussion

The original biochemical description of CDGS focused on abnormal isoforms of serum transferrin. Five different charge isoforms can be identified by isoelectric focusing, which represent polypeptide chains containing from zero to four sialic acid residues (3, 15). Transferrin is known to contain two N-linked glycosylation sites, consisting of 80% biantennary structures and 20% tri- and tetraantennary structures (33, 34). In normal individuals, isoforms corresponding to three and four sialic acid residues predominate. In contrast, individuals with CDGS phenotype predominantly express a disialylated form of transferrin. While most researchers have focused exclusively upon transferrin, other serum glycoproteins can also show an abnormal cathodal migration in isoelectric focusing (7, 21) (D. M. Krasnewich and G. D. Holt, personal communication), and alterations in monosaccharide content of total serum glycoproteins have been reported (15). More recently, the finding of absent glycosylation at specific sites on serum transferrin has permitted speculations about possible defects in the oligosaccharyltransferase and/or earlier steps in LLO biosynthesis (20, 21). However, a subsequent study indicates that the defect is not the synthesis of dolichyl phosphate or *N*-acetylglucosaminyl-pyrophosphoryl-dolichol (35).

Utilizing several different approaches, we have explored the biochemical basis of CDGS in two patients. Contrary to prior reports, we find no differences in the composition of monosaccharides in total serum glycoproteins in this disease. The disparity may be because the prior studies utilized enzymatic and colorimetric assays, whereas the current work used direct chromatographic determination by HPAE-PAD analysis. Based upon the well known structure of the typical antennae of N-linked oligosaccharides and the invariant nature of the core structure ($Man_3GlcNAc_2$), the constant ratios of monosaccharide composition indicate that there is no major change in the overall extent of processing of the N-linked oligosaccharides found on serum glycoproteins. Profiling of the total N-linked oligosaccharides by HPAE-PAD confirms this conclusion. Although we did not identify the structure of each of the oligosaccharide peaks, the overall similarity in the profile indicates that only minor differences are present. In fact, the only obvious difference involves oligosaccharides released by hydrazinolysis but not by PNGase F, which represent O-linked or possibly PNGase F–

resistant structures. A difference in O-linked structures would also fit with the increase in content of GalNAc, a monosaccharide more commonly found in O-linked oligosaccharide structures. However, such abnormalities could still be secondary due to a primary failure in N-linked glycosylation.

Since the majority of serum glycoproteins are produced either by hepatocytes or hematopoietic cells, the molecules we first studied represent the final biosynthetic products of these cell types. For another perspective, we also examined skin fibroblasts metabolically labeled with [2-^{3}H]Man, which primarily enters into N-linked oligosaccharides. Using lectin affinity chromatography as a screening tool, we could find no major changes in the N-linked oligosaccharides synthesized by CDGS fibroblasts during a 3-d "equilibrium" label. However, examination of the earliest steps in the pathway revealed strikingly different results. There is two- to fourfold less labeled LLO precursor in CDGS fibroblasts compared to normal, and those from CDGS sometimes show a range of sizes smaller than normal. However, the rate of transfer of oligosaccharide from the precursor to proteins in intact cells does not appear to be markedly different. Taken together, the data suggest a primary defect in LLO biosynthesis. Of note, several eukaryotic cell lines have been identified that have mutations involving LLO biosynthesis (36–44), but in none of these lines do the biochemical defect(s) mirror those seen here. For instance, two different *Dictyostelium* lines have been identified which have apparent defects in the mannosyl transferase(s) involved in LLO biosynthesis (41, 43). The predominant structure made is $Man_6GlcNAc_2$-P-P-Dol, rather than the array of structures seen here. One of these mutant lines also shows a reduced rate of in vivo glycosylation, although both have reduced rates in an in vitro assay, demonstrating the complexity of protein glycosylation in intact cells (43). Several carbohydrate mutants have been identified with defects in dolichol biosynthesis. Some of these mutants have multiple abnormalities in the structures of their N-linked oligosaccharides including underglycosylation of some glycoproteins and reduced levels of [^{3}H]Man incorporation into total cellular glycoproteins (37–40). The enzymatic defects in these cell lines include reduced levels of polyprenol reductase activity (required for the biosynthesis of dolichol), and of mannosylphosphoryldolichol synthase (required for the biosynthesis of Man-P-Dol; see 37, 39, 45, 46). The abnormal LLO structure in the last case has been determined to be $Glc_3Man_5GlcNAc_2$-P-P-Dol (36, 37), again in contrast to the heterogeneous sizes of the LLO seen here. *Saccharomyces cerevisiae* sec59 cells, which are deficient in dolichol kinase activity (47), show reduced levels of Man incorporation into nascent proteins, as seen with the CDGS samples. However, in these mutants, the oligosaccharides are consistently smaller than in normal cells (48), again unlike the situation here. As a further complexity, recent studies have indicated that dolichol biosynthesis is tightly controlled by cAMP levels in some cell lines (49), suggesting that mutations of enzymes involved in this regulatory mechanism could also produce alterations in LLO biosynthesis. Likewise, other researchers have shown that *cis*-isoprenyltransferase, involved in Dol-P synthesis, is a key enzyme regulating LLO biosynthesis in mitogen-activated B cells (50); thus, mutations affecting this enzyme could likewise produce the results seen here.

Given our results, it is more likely that the defect in CDGS involves one or more early steps in LLO biosynthesis, such as the availability of monosaccharides, dolichol phosphate, and sugar nucleotides, which could secondarily affect the structure and/or amount of the LLO (see Fig. 4). The most consistent finding is the decreased incorporation of Man into both the LLO and newly synthesized proteins. An early defect in the pathway of LLO biosynthesis may thus explain the variable results seen in the size of the LLO precursor. Regardless of the nature of the primary defect, these findings can also explain many of the seemingly disparate abnormalities previously reported in this disease (Fig. 4). Reduced levels of LLO could result in a random failure of glycosylation of available Asn-X-Ser/Thr sequons. Abnormalities in the size of the LLO may also affect the rate of transfer, although this was not obvious in the data presented here. The consequences of under glycosylation may include altered protein folding and/or function, altered ER–Golgi–plasma membrane secretion rates, and/or altered processing of other oligosaccharide chains. A possible outcome of these multiple changes could be multisystem abnormalities as seen in CDGS. In some respects, this picture is similar to that seen with the inhibitor tunicamycin, which blocks the assembly of the LLO in the ER. Depending upon the protein under study, tunicamycin can result in reduced rates of ER–Golgi transport, partial glycosylation, altered functions, and/or altered half lives, or can be relatively without effect except to change electrophoretic mobility (51). Likewise, glucosamine feeding is also known to inhibit the formation of LLOs in some cell types (52). Thus, the defect in CDGS may result in a somewhat heterogeneous pattern of glycosylation abnormalities, some of which may be only apparent when specific glycoproteins are studied in detail (e.g., transferrin). However, specific glycosylation abnormalities of individual glycoproteins can be of significance. For example, underglycosylation of gonadotrophic hormones is known to convert them to potent antagonists of their normally

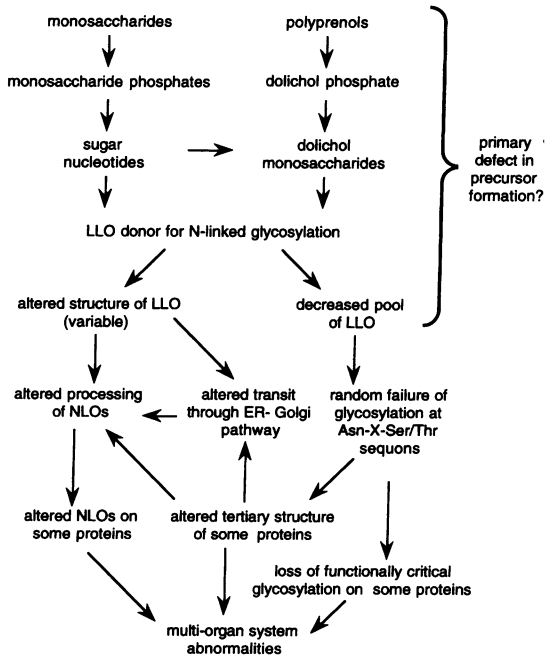

Figure 4. Possible explanations for the biochemical defects reported in CDGS. An outline of the major steps in LLO biosynthesis that could be defective is presented, along with potential consequences for subsequent oligosaccharide processing, as well as glycoprotein structure and function.

glycosylated counterparts (53), and suggests a plausible hypothesis for the hypogonadism reported in some cases of CDGS (7, 9, 54).

Based on variabilities in clinical phenotype of CDGS patients, three subtypes have recently been suggested, and the proposal that these may correspond to abnormalities at widely differing steps in the N-linked oligosaccharide biosynthetic pathway has been extended (12). While this may explain both the clinical and biochemical heterogeneity of this disorder, as well as some of the conflicting reports in the literature, sufficient data have yet to be presented to support this model. The assay of several of the processing GlcNAc transferases in serum and fibroblasts of the Type I CDGS patients studied here showed no obvious deficiency (Vij, R., L. Powell, and A. Varki, unpublished data). Also, fibroblasts from two other patients with Type I CDGS that we have very recently studied (Paneerselvam, K., and H. Freeze, unpublished data), as well as those studied by others (Krasnewich, D. M., and G. D. Holt, personal communication) demonstrated similar abnormalities in [2-^{3}H]Man incorporation. Thus, it is probable that for Type I CDGS, all clinical features will be explained by primary defects confined to early ER steps of the N-linked oligosaccharide pathway. At least one other human genetic disorder (olivopontocerebellar atrophy of neonatal onset) has been reported to show "disialotransferrin deficiency" (55). Although some similarities in the clinical phenotype have been pointed out, it remains to be seen if similar defects in LLO synthesis are seen in this syndrome. In the final analysis, it is clear that elucidation of the primary defect in CDGS will not be straightforward. However, this experiment of nature does demonstrate the importance of N-linked glycosylation to many aspects of normal human biology.

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Note Added in Proof. While this paper was in review we became aware of work by others showing deficiency of the Golgi enzyme *N*-acetylglucosaminyltransferase II in a patient with Type II CDGS (Jaeken et al. *Arch. Dis. Child.* In press). This is a markedly different defect from that found in the Type I patients studied here, supporting the notion that CDGS may encompass several different abnormalities in the synthesis and processing of N-linked oligosaccharides (12).

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