ELSEVIER

Altered glycan structures: the molecular basis of congenital disorders of glycosylation

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Congenital disorders of glycosylation (CDG) are a group of diseases that affect glycoprotein biogenesis. Eighteen different types of CDG have been defined genetically. They result from deficiencies in either the biosynthesis of oligosaccharide precursors or specific steps of *N*-glycan assembly, resulting in the absence or structural alteration of *N*-glycan chains. These diseases have a broad range of clinical phenotypes and affect nearly every organ system, with special emphasis on normal brain development and the multiple functions of the nervous, hepatic, gastrointestinal and immune systems. Although most of the deficiencies observed in CDG patients are only partial, the severity of the clinical manifestations signifies the relevance of protein *N*-glycosylation and shows the importance of defined glycan structures.

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Introduction

For 10 years, physicians and scientists collaborated in the discovery of 18 inherited human disorders that result from altered protein *N*-glycosylation [1•,2•]. These congenital disorders of glycosylation (CDG) are listed in Table 1. The rapid identification of the molecular cause of these deficiencies relied on the sequenced human genome, robust model systems and a simple blood test to indicate abnormal protein glycosylation. Oligosaccharide structural analysis provided critical clues to many of these discoveries. The clinical features span a broad spectrum and affect many organ systems, especially the development of certain regions of the brain, and the gastrointestinal, hepatic, visual and immune systems, showing that their functions depend heavily on normal glycosylation. The clinical features vary enormously, making it difficult for physicians to recognize CDG patients; abnormal protein glycosylation provides the key to identifying many of these patients [3]. CDG patients were first identified 25 years ago, based on clinical symptoms and deficiencies in multiple plasma glycoproteins. Patients had psychomotor retardation, low muscle tone, incomplete brain development, visual problems, coagulation disorders and endocrine abnormalities [4,5]. The common defect identified was the incomplete addition of sialic acid to a proportion of serum transferrin molecules (Tf). A simple blood test using isoelectric focusing (IEF) or ion-exchange separation gave physicians a litmus test to identify patients without knowing the molecular basis of the disease.

CDG group I patients were found to lack sialic acid residues on Tf because they had unoccupied Asn-X-Ser/Thr glycosylation sequons that are normally glycosylated (i.e. entire sugar chains were absent). However, the *N*-glycan structures present on serum proteins of CDG type I patients were normal or only slightly altered [6–8]. By contrast, patients in group II presented with Tf protein for which all glycosylation sequons were glycosylated, but the structures of the protein-bound oligosaccharides were altered.

The explanation for these two types of *N*-glycosylation deficiencies lies in the bipartite nature of the *N*-glycosylation pathway.

First, we will sketch the *N*-glycan biosynthetic pathway. Then, we provide examples showing how glycan structural analysis of patient-derived molecules has guided the search for the faulty genes and subsequent assessment of the impact of their mutations. We end with a glance toward the future of glycan analysis in CDG diagnostics and other areas of medicine.

The *N*-glycosylation pathway

N-linked protein glycosylation takes place in two distinct cellular compartments: the endoplasmic reticulum (ER) and the Golgi apparatus. In the ER, a core oligosaccharide ($Glc_3Man_9GlcNAc_2$) is assembled on a lipid carrier, dolichylpyrophosphate, and subsequently transferred to selected asparagine residues of nascent polypeptide chains (Figure 1) [9]. The acceptor sequence is characterized by the sequon Asn-X-Ser/Thr, where X can be any amino acid except proline [10]. After transfer, the protein-bound oligosaccharide serves specific functions in glycoprotein folding, quality control, secretion and degradation in the ER [11]. The second phase of the glycosylation process involves the ordered removal of selected monosaccharides from the protein-bound oligosaccharide, and the addition of others in the ER and Golgi.

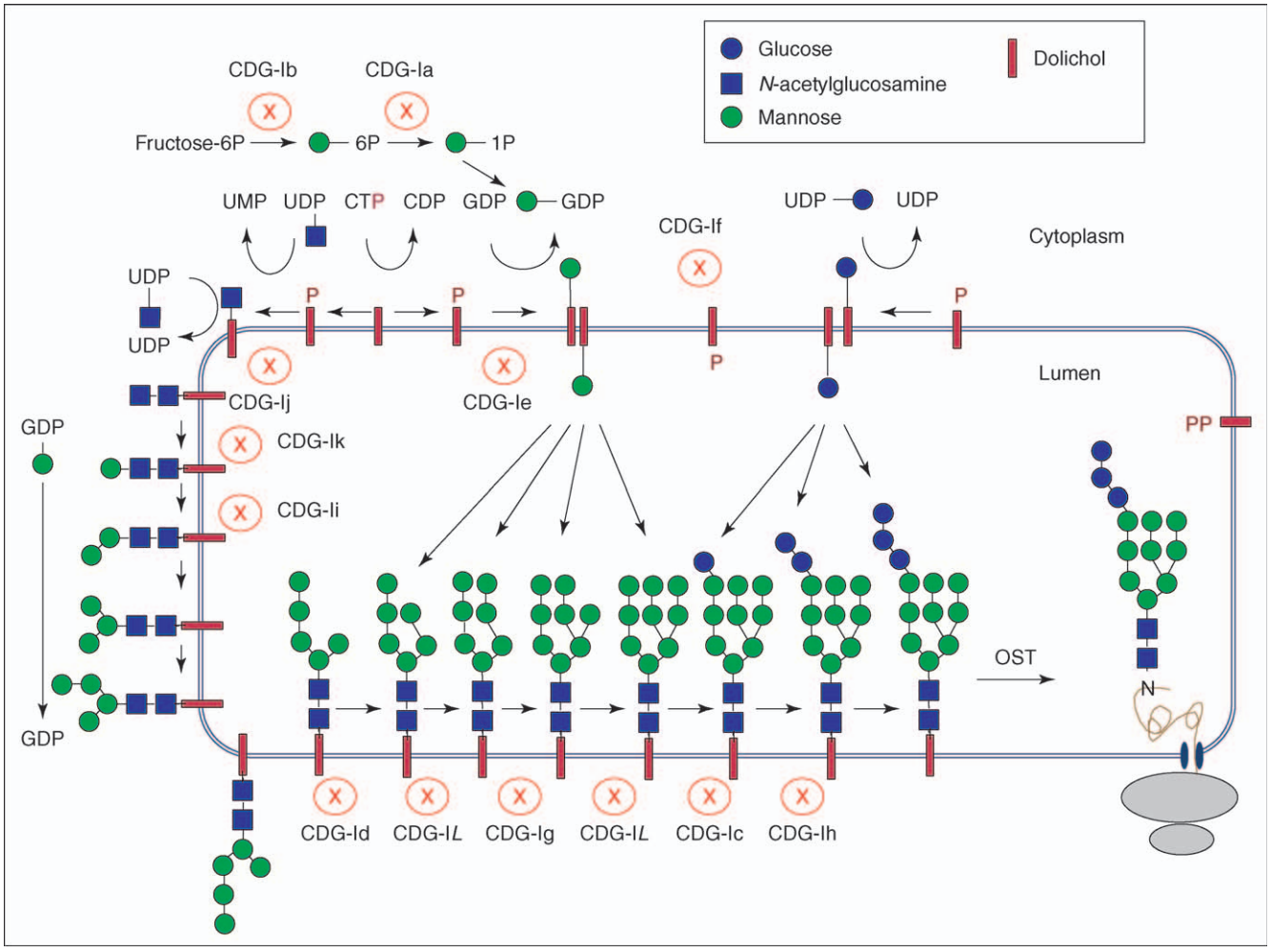
Table 1

Causes and symptoms of CDG.

CDG	Gene	Enzyme	Online Mendelian Inheritance in Man	Typical symptoms	References
CDG-Ia	*PMM2*	Phosphomannomutase II	212065	Mental retardation (MR), hypotonia, esotropia, lipodystrophy, cerebellar hypoplasia, seizures	[44]
CDG-Ib	*MPI*	Phosphomannose isomerase	602579	Hepatic fibrosis, protein-losing enteropathy (PLE), coagulopathy, hypoglycemia	[45]
CDG-Ic	*ALG6*	Dol-P-Glc: $Man_9GlcNAc_2$-PP-Dol glucosyltransferase	603147	MR, hypotonia, epilepsy	[46,47]
CDG-Id	*ALG3*	Dol-P-Man: $Man_5GlcNAc_2$-PP-Dol mannosyltransferase	601110	Severe MR, optic nerve atrophy	[48]
CDG-Ie	*DPM1*	Dol-P-Man synthase I GDP-Man: Dol-P-mannosyltransferase	608799	Severe MR, epilepsy, hypotonia, mildly dysmorphic, coagulopathy	[49,50]
CDG-If	*MPDU1*	Mannose-P-dolichol utilization defect 1/ Lec35	609180	Short stature, icthyosis, MR, retinopathy	[51,52]
CDG-Ig	*ALG12*	Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase	607143	Hypotonia, MR, facial dysmorphism, microcephaly, frequent infections	[53]
CDG-Ih	*ALG8*	Dol-P-Glc: $Glc_1Man_9GlcNAc_2$-PP-Dol glucosyltransferase	608104	Hepatomegaly, coagulopathy, PLE, renal failure	[54]
CDG-Ii	*ALG2*	GDP-Man: $Man_1GlcNAc_2$-PP-Dol mannosyltransferase	607906	Normal at birth, hepatomegaly, coagulopathy, MR, hypomyelination, intractable seizures	[55]
CDG-Ij	*DPAGT1*	UDP-GlcNAc: dolichol phosphate *N*-acetylglucosamine-1 phosphate transferase	608093	Severe MR, hypotonia, seizures, microcephaly	[56]
CDG-Ik	*ALG1*	GDP-Man: $GlcNAc_2$-PP-Dol mannosyltransferase	608540	Severe MR, hypotonia, acquired microcephaly, intractable seizures, fever, coagulopathy, nephrotic syndrome	[57]
CDG-I*L*	*ALG9*	Dol-P-Man: $Man_{6\ and\ 8}$ $GlcNAc_2$-PP-Dol mannosyltransferase	608776	Severe microcephaly, hepatomegaly, hypotonia, seizures	[58]
CDG-IIa	*MGAT2*	GlcNAcT-II	212066	MR, facial dysmorphism, seizures	[59,60]
CDG-IIb	*GLS1*	Glucosidase I	606056	Dysmorphism, hypotonia, seizures, hepatomegaly, hepatic fibrosis (death at 2.5 months), normal Tf	[61]
CDG-IIc	*SLC35C1/FUCT1*	GDP-fucose transporter	266265	Recurrent infections, neutrophilia, MR, microcephaly, hypotonia, normal Tf	[20,21]
CDG-IId	*B4GALT1*	β1,4-galactosyltransferase	607091	Hypotonia and myopathy, spontaneous hemorrhage	[19]
CDG-IIe	*COG7*	COG complex, subunit 7	608779	Fatal in infancy, dysmorphism, hypotonia, intractable seizures, hepatomegaly, progressive jaundice, recurrent infections, cardiac failure	[26••]
CDG-IIf	*SLC35A1*	CMP-sialic acid transporter	605634	Thrombocytopenia, abnormal platelet glycoproteins, but no neurologic symptoms and normal Tf	[24•]

The biosynthesis of the lipid-linked core oligosaccharide (LLO) itself is a bipartite process that begins on the cytoplasmic side and ends on the luminal side of the ER membrane (Figure 1). Monosaccharides are transferred to the lipid carrier (dolichylpyrophosphate) by highly specific monosaccharyltransferases [9]. For the covalent linkage of the first seven sugars, nucleotide-activated GlcNAc or mannose serves as the donor substrate for cytoplasm-oriented glycosyltransferases. The $Man_5GlcNAc_2$ intermediate is then flipped (translocated) into the lumen of the ER, where four additional mannose residues and three glucose residues are added; dolichylphosphomannose and dolichylphosphoglucose, respectively, serve as substrates for these reactions. Analysis of the folding and quality control processing of glycoproteins in the ER has revealed essential functions of individual residues of these oligosaccharides [11] and it is therefore of central importance that only completely assembled oligosaccharides, rather than biosynthetic intermediates, are transferred to the nascent protein. This is ensured by the defined order of LLO assembly and by the specificity of the oligosaccharyltransferase for the

Figure 1

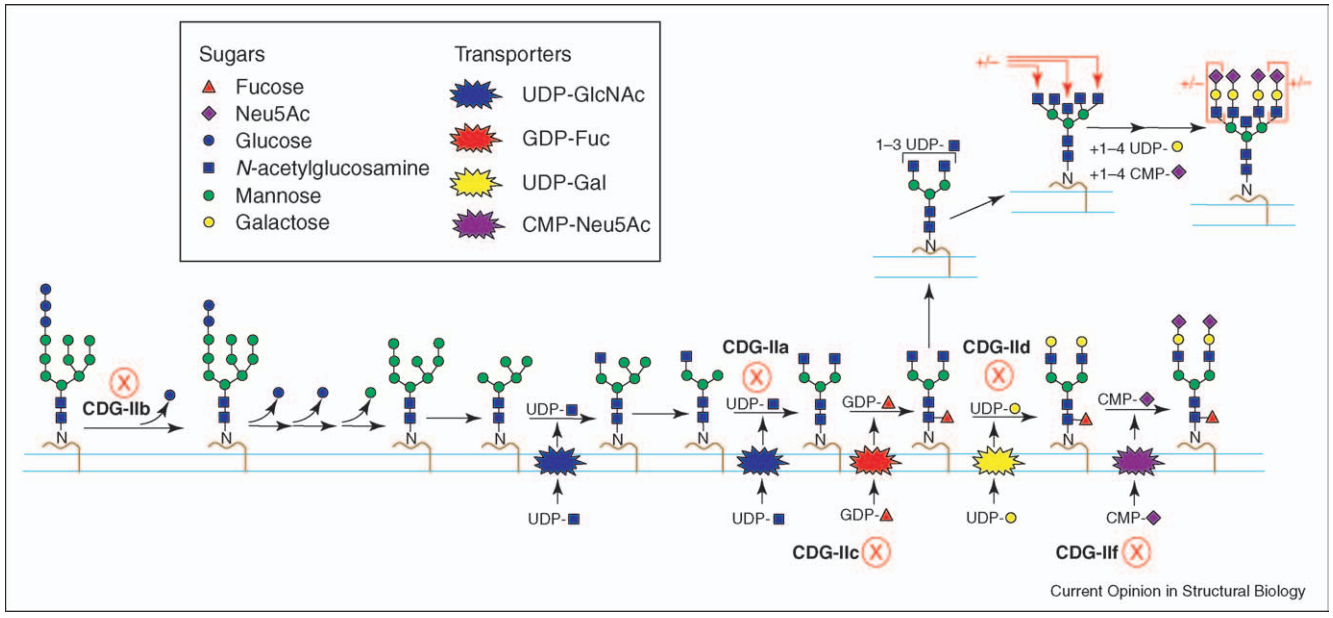


The pathway of *N*-linked protein glycosylation in the ER is affected in different forms of CDG type I. The assembly of the oligosaccharide and its transfer to nascent polypeptide chains are shown. This takes place at the membrane of the ER and the polyisoprenoid lipid dolichol serves as the lipid anchor for oligosaccharide assembly. The initial steps occur on the cytoplasmic side of the ER membrane. Nucleotide-activated sugars (UDP-GlcNAc and GDP-Man) serve as donors in these reactions. Completion of the $Glc_3Man_9GlcNAc_2$ oligosaccharide is achieved in the lumen of the ER. Dolichylphosphoglucose and dolichylphosphomannose are the monosaccharide substrates for the glycosyltransferases involved in this part of biosynthesis. Oligosaccharyltransferase (OST), an oligomeric membrane protein complex, catalyzes the transfer of the oligosaccharide to defined asparagine residues (N) of nascent polypeptide chains. CDG type I affects the assembly of the oligosaccharide substrate. The biosynthetic steps affected in the different CDG subtypes are indicated.

completely assembled oligosaccharide. Even though the oligosaccharide is a highly branched structure and could allow a whole series of biosynthetic routes, the specificity of the glycosyltransferases involved ensures a defined assembly pathway ending with the addition of the α1,2-linked glucose residue, an important determinant of substrate recognition by the oligosaccharyltransferase [12].

The second part of the pathway begins soon after glycan transfer and is initiated by the removal of the first two glucose residues, followed by the reversible removal/readdition of the third glucose. In the ER and the Golgi compartment, up to five mannose units are removed and several GlcNAc, galactose, sialic acid and fucose residues are added (Figure 2). For each addition, the activated donors must be transported into the Golgi compartment by nucleotide-sugar transporters. The order is 'prescribed', but it is not template driven. This generates a broad spectrum of *N*-glycan structures, because cells vary the expression and organization of this ensemble of biosynthetic enzymes. In addition, some enzymes compete for the same substrate at branch points in the pathway. Depending on abundance, affinity or location, the dominant glycosyltransferase can favor or prevent the synthesis of selected glycans as proteins move through the Golgi.

Figure 2



Type II CDG defects are caused by mutations that affect the processing of oligosaccharide chains after they are transferred to proteins in the ER. Initially, the three glucose residues are removed as part of the quality control of protein folding. Extensive removal of mannose residues is interspersed with the addition of multiple GlcNAc residues in a prescribed order, followed by capping of the multiple branches by galactose and sialic acid (Neu5Ac). In some cases, fucose is added near the protein linkage region or to the outer branches of the glycan. All the reactions, except the removal of the first mannose residue, occur in the Golgi and specific transporters are required to carry the nucleotide-sugars from the cytoplasm into the Golgi compartment. Defects that cause CDG-II are indicated.

The molecular basis of congenital disorders of glycosylation

Glycosylation is an essential process in eukaryotic cells. Many enzymatic activities contribute to the biosynthesis of *N*-linked glycans and deficiencies in this process lead to aberrant protein glycosylation. However, due to the bipartite pathway, the effect of different mutations is dramatically different. A deficiency in any of the steps required for the assembly of the LLO in the ER (e.g. the synthesis of nucleotide-activated sugars or individual glycosyltransferases) results in a structurally incomplete LLO. As the oligosaccharyltransferase has specificity for the complete LLO, this results in hypoglycosylation of glycoproteins. If incomplete oligosaccharides happen to be transferred to proteins, their subsequent trimming leads to *N*-glycans that are indistinguishable from those that were transferred as complete structures. Therefore, patients with a deficiency in the ER pathway generally display *N*-glycoproteins that lack whole oligosaccharides; however, any oligosaccharides present have normal structures. Such deficiencies are characterized by the accumulation of LLO biosynthetic intermediates. As LLO assembly is conserved from yeast to humans, identification of the accumulating intermediates and comparison with the phenotype of corresponding yeast mutant strains provided substantial clues to the defects. Most of the LLO assembly steps are not readily amenable to biochemical assays, but a functional assay could be done using mutants defective in LLO assembly, which had been identified primarily in *Saccharomyces cerevisiae* and in several mammalian cells (mostly Chinese hamster ovary cells) [13]. The close homology between yeast and human genes enabled the normal human ortholog to rescue defective glycosylation in yeast strains, whereas cDNA from patients with mutations did not. This approach was invaluable for tracking the human defects that cause CDG Ic, Id, Ig, Ih, Ii, Ik and IL. For the few types of CDG for which the yeast and mammalian pathways have diverged, mammalian cell lines with known glycosylation defects were available for complementation by a similar strategy. This was especially important for identifying the defects that cause CDG Ie and If. Established enzyme assays helped to identify the defects in CDG Ia, Ib, Ie and Ij. One critical clue to solving the defect in CDG-Ia, the most common type, was that patient cells synthesized a series of truncated LLO species when incubated in reduced glucose, whereas normal cells did not [14]. This pointed to a general limitation in early precursors that led to the identification of phosphomannomutase as the defective enzyme in CDG-Ia, because it reduced GDP-mannose. More recent studies using a full glucose medium led to the synthesis of normal-sized LLO in CDG-Ia cells [15•]. However, these studies suggest that mannose-6-phosphate, the

biosynthetic intermediate, might accumulate in CDG-Ia cells and affect the availability of completely assembled $Glc_3Man_9GlcNAc_2$ oligosaccharide directly, thereby exacerbating the hypoglycosylation phenotype [15•]. Whether this happens in CDG-Ia patients is unknown.

Detailed analysis of the mutations in different CDG type I cases has shown that there is always some residual enzymatic activity. It is most likely that complete loss of any of the biosynthetic steps required for the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide results in embryonic lethality, a conclusion supported by the analysis of mice carrying deletions in corresponding loci [16,17••].

Analysis of complex-type glycans solves some group II defects

The structural analysis of glycans on serum glycoproteins was essential to solving several group II defects. For instance, lack of complex-type chains and their replacement with monosialylated hybrid chains occurred concomitantly with loss of *N*-acetylglucosaminyltransferase II (GlcNAcT-II) enzymatic activity and mutations in the *MGAT2* gene [6]. Structural analysis of glycans from a mouse line ablated in *Mgat2* further confirmed the importance of this enzyme in the synthesis of complex-type sugar chains [18]. In another disorder, CDG-IId, structural analysis showed the loss of both galactose and sialic acid from Tf [19]. This pattern was consistent with the loss of nearly all β1,4-galactosyltransferase enzymatic activity and pathological mutations in β1,4-galactosyltransferase. Solving the defect in CDG-IIc, which is also called leukocyte adhesion deficiency type II (LAD-II), was more complex [20,21]. Tf sialylation was normal, so CDG-IIc was not detected by the usual test. However, *N*-glycosylation of IgM and *O*-linked glycans on leukocyte surface proteins were deficient in fucose. The latter include sialyl-Lewis^x glycans, which are required for selectin-mediated leukocyte rolling before extravasation [17••,22]. This defect results in greatly increased numbers of circulating leukocytes and leads to frequent infections. In patient fibroblasts, however, decreased fucosylation was found to be largely confined to the core region of biantennary *N*-glycans [23]. This finding emphasizes that the easily available fibroblasts do not always reflect the full impact of the defect, as seen in other cells, where the output of glycosylated proteins or cellular turnover rates are higher. One key discovery was that CDG-IIc patient cells did not bind the α1,3- and α1,6-fucose-specific lectin from *Aleuria aurantia* (AAL), whereas normal cells showed strong binding. This difference provided an avenue to identifying the defective gene by complementation cloning based on restoration of AAL binding [20,21]. The defect was identified as a deficiency in the GDP-fucose transporter, which provides fucose for both *N*- and *O*-linked glycans. Before this, all CDG defects were thought to affect only the *N*-linked pathway. This discovery stresses the importance of analyzing multiple classes of glycans in patients.

The well-established paradigm of 'determine glycan structure, find the genetic defect' encountered further complications. For example, the only reported case of CDG-IIf is caused by a defect in the CMP-sialic acid transporter [24•]. This would be predicted to affect the sialylation of all glycans, but, surprisingly, *N*-linked glycans on serum glycoproteins, including Tf, were sialylated normally. On the other hand, the patient completely lacked sialyl-Lewis^x on leukocytes due to a deficiency in sialic acid, which led to severe neutropenia, formation of giant platelets and abnormal platelet glycoproteins [25]. The abnormalities in this patient were confined to hematopoietic cells, perhaps due to the cell-type specificity of the patient's splicing site mutation, or to the rapid turnover or high output of glycoproteins by these cells. In any event, for the mutation seen in this patient, structural analysis of Tf glycans was not useful to identify the defect, but analyses of *O*-linked glycans were critical.

Expanding the spectrum of glycosylation disorders

All of the CDG types described above are caused by defects in glycan biosynthetic pathways. In most cases, *N*- or *O*-glycan structural analysis provided the major clues to solving the defect. Going forward, the road is likely to become more complicated. This is illustrated by the case of two sibs with an abnormal Tf pattern typical of type II disorders [26••]. They also had undersialylated *O*-glycans on apolipoprotein CIII (apo-CIII), one of the few serum proteins with only *O*-linked glycans [27]. Their fibroblasts showed enhanced binding of peanut agglutinin, implying defective addition of sialic acid to *O*-glycans. Also, there was a 2–3-fold reduction in the activity of CMP-sialic acid and UDP-galactose transporters, and a similar decrease in the activity of several glycosyltransferases. The relevant genes did not have any mutations. Clearly, the defect affected many steps of multiple glycosylation pathways. The defect was identified as a splice site mutation in *COG7*, which encodes one of the proteins of the eight-subunit COG (conserved oligomeric Golgi) complex (Cogs1–8). This complex associates with the cytoplasmic face of the Golgi apparatus and is required for normal intracellular trafficking of components of the glycosylation machinery [28,29,30•,31]. Loss of this subunit destabilized the complex, and led to the degradation and/or mislocalization of the other subunits. Expression of normal Cog7 corrected COG complex formation, and trafficking and glycosylation deficiencies.

Like much of the trafficking machinery, the COG complex is well conserved. In yeast, deletion of the individual subunits can be lethal or impair glycosylation, depending on which subunit is deleted [29]. CHO cell mutants ldlB and ldlC, which are deficient in Cog1 and Cog2,

respectively, have major deficiencies in multiple glycosylation pathways, again stressing the importance of these cellular model systems for understanding defects [28,32].

Moving forward with structural analysis

Knowing the defect in Cog7-deficient patients shows that, even though their *N*-glycan structures are altered, their detailed analysis would not have solved this defect, although they provided the critical focus on glycosylation deficiencies. It is unlikely that the molecular basis of these deficiencies would have been identified by any other approach. As the number of genes involved in intracellular protein trafficking is large, this expands the range of potential causes of glycosylation abnormalities. A substantial number of diverse CDG type II patients are now being described with unknown defects in the *N*- and sometimes also in the *O*-linked biosynthetic pathways [6,33,34•,35]. Some of them will probably be defective in either cytoplasmic ushers, such as Cogs, or escorts that reside within the ER/Golgi lumen to organize an efficient glycosylation apparatus in the functional Golgi [36,37].

It can be argued that defects in trafficking should not be considered CDG, because the glycosylation abnormalities are actually secondary [37]. It is unknown whether all of the clinical manifestations result from altered glycosylation alone. Determining its impact will be challenging, as extracellular matrix (ECM) components such as proteoglycans and collagen are glycosylated, and their assembly requires proper glycosylation. Some mentally retarded dysmorphic patients with *cutis laxa*, a condition characterized by sagging skin, have abnormal *N*- and *O*-linked glycosylation, suggesting that ECM components, perhaps even collagen itself, display altered glycosylation [34•].

Increased awareness of CDG means that more patients will be tested. Clearly, the single Tf test does not identify all patients, but it remains the gold standard for detecting group I patients. Additional serum markers may be useful. Traditional IEF is an inexpensive, low-resolution method that monitors only charge differences. More powerful and specific methods, such as ESI-MS (electrospray ionization mass spectrometry), are preferred because they can distinguish type I and type II cases [38]. Tf ESI-MS should be combined with MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) and/or HPLC (high-pressure liquid chromatography) profiling of PNGaseF-released *N*-glycans from total serum to identify abnormal *N*-glycan branching and altered fucosylation. This approach is not yet refined, but it offers significant promise for finding patients with primary or secondary glycosylation disorders [6,7,33,39]. *N*-glycan profiling can already identify patients with liver cirrhosis and gauge its severity [40•]. Broader testing by glycan profiling will reveal more patients with non-traditional clinical CDG symptoms. The challenge then will be to distinguish whether they are acquired or inherited disorders.

Identifying the defective genes in type II CDG cases will be challenging. Mutations in proteins that alter the localization or trafficking of the glycosylation machinery, such as the COG complex, will not be revealed through glycan analysis. Sequencing all the COG genes in patients is uninspiring, but possible. A general cloning approach based on complementation of the glycosylation deficiencies and monitoring glycosylation using a battery of lectins would be ideal to identify the corresponding genes. This approach was successful for isolating null CHO cell mutants [41] and for identifying the defect in CDG-IIc [20,21]. Unfortunately, patients usually have hypomorphic alleles and, in contrast to the dramatic glycosylation changes in CDG serum glycoproteins, the changes are sometimes much more subtle in the patients' fibroblasts [26••]. Traditional lectin probes are unlikely to solve this unmet challenge.

Little is known about the impact of glycosylation on the initiation or development of acquired diseases. These may become evident during periods of environmental stress and infection [42], analogous to low glucose generating truncated LLO in CDG-Ia cells. Ongoing efforts to establish a database of normal glycan structures in plasma using MS, in conjunction with lectin binding assays, will help identify abnormal glycosylation profiles in both genetic and acquired diseases [39,40•,43]. Glycan structural analysis may provide pathological markers for both inherited metabolic disorders and more complex diseases.

Conclusions

Structural analysis of plasma and cellular glycans was the key to defining many types of human glycosylation disorders. Analysis of mutant yeast and mammalian cell lines with altered glycans provided the biochemical and genetic underpinning. Continued glycan structural analysis will help identify more patients with novel inherited disorders and will probably provide novel biomarkers for acquired disorders.

Update

Rare patients with uncontrolled galactosemia (galactose-1-phosphate uridyltransferase deficiency) show small changes in their serum *N*-glycans, such as branching, fucosylation and sialylation, but surprisingly they also have dramatically underglycosylated Tf that lacks one or both *N*-glycans. Removing galactose from the diet reverses these abnormalities [62•]. This important observation suggests that underglycosylation may be a general basis for pathology in galactosemia. Whether this is true would require analysis of other abundant serum glycoproteins besides Tf and this was not done.

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