A Deficiency in Dolichyl-P-glucose:Glc₃Man₉GlcNAc₂-PP-dolichyl α₃-Glucosyltransferase Defines a New Subtype of Congenital Disorders of Glycosylation*

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The underlying causes of type I congenital disorders of glycosylation (CDG I) have been shown to be mutations in genes encoding proteins involved in the biosynthesis of the dolichyl-linked oligosaccharide (Glc₃Man₉GlcNAc₂-PP-dolichyl) that is required for protein glycosylation. Here we describe a CDG I patient displaying gastrointestinal problems but no central nervous system deficits. Fibroblasts from this patient accumulate mainly Man₉GlcNAc₂-PP-dolichyl, but in the presence of castanospermine, an endoplasmic reticulum glucosidase inhibitor Glc₃Man₉GlcNAc₂-PP-dolichyl predominates, suggesting inefficient addition of the second glucose residue onto lipid-linked oligosaccharide. Northern blot analysis revealed the cells from the patient to possess only 10–20% normal amounts of mRNA encoding the enzyme, dolichyl-P-glucose:Glc₃Man₉GlcNAc₂-PP-dolichyl α₃-glucosyltransferase (hALG8p), which catalyzes this reaction. Sequencing of hALG8 genomic DNA revealed exon 4 to contain a base deletion in one allele and a base insertion in the other. Both mutations give rise to premature stop codons predicted to generate severely truncated proteins, but because the translation inhibitor emetine was shown to stabilize the hALG8 mRNA from the patient to normal levels, it is likely that both transcripts undergo nonsense-mediated mRNA decay. As the cells from the patient were successfully complemented with wild type hALG8 cDNA, we conclude that these mutations are the underlying cause of this new CDG I subtype that we propose be called CDG Ih.

Type I congenital disorders of glycosylation (CDG I)² are often severe multisystemic diseases characterized by the presence of hypoglycosylated glycoproteins in the serum of affected individuals (1–5). Although glycoproteins play vital roles in many aspects of human cellular physiology (6), the precise relationship between glycoprotein hypoglycosylation and the clinical symptoms of these diseases that include hypotonia, seizures, failure to thrive, psychomotor retardation, and various dysmorphias is not understood (2).

Hypoglycosylation of glycoproteins bearing N-glycans is caused by either an insufficiency in the biosynthesis of the lipid-linked oligosaccharide (LLO) precursor, Glc₃Man₉GlcNAc₂-PP-dolichyl, that is required for protein glycosylation or inefficient transfer of the sugar moiety of this LLO onto nascent glycoproteins in the lumen of the endoplasmic reticulum (ER). Theoretically, mutations in any of the genes encoding for the 30 or so proteins involved in this biosynthetic pathway could lead to glycoprotein hypoglycosylation in CDG I patients. However, mutations in only 7 of the genes encoding proteins of the glycosylation pathway have so far been shown to underlie CDG I, and these 7 cases have been classified as CDG I subtypes a–g (Ia, phosphomannomutase 2 (7, 8); Ib, phosphomannose isomerase (9, 10); Ic, dolichyl-P-Glc₃Man₉GlcNAc₂-PP-dolichyl α₃-glucosyltransferase (11–13); Id, dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl α₃-mannosyltransferase (14); Ie, dolichyl-P-Man synthase I (15, 16); If, the MPDU1 gene product known to facilitate dolichyl-P-Glc₃Man₉GlcNAc₂-PP-dolichyl utilization (17, 18); and Ig, dolichyl-P-Man:Man₉GlcNAc₂-PP-dolichyl α₆-mannosyltransferase (19–21)). Although there are too few patients representing each subtype of the disease to draw precise genotype/phenotype relationships, CDG Ib (PMI deficiency) generally presents as a disease in which central nervous system defects are absent (9, 10). Often, PMI deficiency leads to a less severe form of the disease because as well as the PMI-catalyzed conversion of fructose 6-phosphate to mannose 6-phosphate the cell possesses an alternative route for the generation of the latter intermediate. In fact, serum mannose can be taken up by cells (22, 23) and phosphorylated by hexokinase to yield mannose 6-phosphate. In PMI deficiency, the flux through this alternative metabolic route can be augmented by giving patients oral mannose, a treatment that has been shown to reverse serum glycoprotein hypoglycosylation and alleviate the symptoms of this form of the disease (24, 25).

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The abbreviations used are: CDG, congenital disorders of glycosylation; LLO, lipid-linked oligosaccharide; ER, endoplasmic reticulum; CDG Ib, disorder of N-glycosylation; hALG8, human ALG8; LH, lipid-linked oligosaccharide; ER, endoplasmic reticulum; CDG Ia, phosphomannomutase 2 (7, 8); CDG Ib, phosphomannose isomerase (9, 10); CDG Ic, dolichol-P-Glc₃Man₉GlcNAc₂-PP-dolichyl α₃-glucosyltransferase (11–13); CDG Id, dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl α₃-mannosyltransferase (14); CDG Ie, dolichyl-P-Man synthase I (15, 16); CDG If, the MPDU1 gene product known to facilitate dolichyl-P-Glc₃Man₉GlcNAc₂-PP-dolichyl utilization (17, 18); CDG Ig, dolichyl-P-Man:Man₉GlcNAc₂-PP-dolichyl α₆-mannosyltransferase (19–21).
Here we report on a patient presenting clinical symptoms similar to those observed in CDG Ib patients but whose PMI levels were found to be normal. We demonstrate that cells derived from this patient accumulate Glc$_2$Man$_{5}$GlcNAc$_2$-PP-dolichyl and display dramatically reduced levels of mRNA encoding dolichyl-P-glucose;Glc$_2$Man$_{5}$GlcNAc$_2$-PP-dolichyl α3-glucosyltransferase (hALG8p). Sequencing of the hALG8 genomic DNA from the patient revealed each allele to possess mutations that generate premature stop codons.

**EXPERIMENTAL PROCEDURES**

**Western Blot**—Western blotting of serum transferrin was performed as described previously (26) using a rabbit polyclonal anti-transferrin antibody. Phosphomannomutase (27, 28) and phosphomannose isomerase (29) activities were assayed as described previously. Cells, Cell Culture, and Metabolic Labeling of Cells—Skin biopsy fibroblasts, obtained from patient M. P. and a patient diagnosed with CDG Ib (35), were grown in Dulbecco’s modified Eagle’s medium containing 2 g/liter glucose, 10% fetal calf serum, and 1% penicillin/streptomycin. Primary skin fibroblasts were immortalized by Dr. Thierry Levade (INSERM U466), as reported previously (30), and cultivated as described above. EBV-transformed lymphoblasts were generated from peripheral blood mononuclear cells isolated using a Ficoll-Paque Plus gradient and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Cytokine-stimulated fibroblasts in 24-well tissue culture flasks or EBV-transformed lymphoblasts were pulse-radioabeled with 100 μCi of [2-3H]mannose (23.9 Ci/mmol, PerkinElmer Life Sciences) in 1 ml of glucose-free Dulbecco’s modified Eagle’s medium or RPMI 1640 supplemented with 0.5 mM glucose and 5% dialyzed fetal calf serum, where appropriate. 1 mg of dolichyl phosphate, in a final volume of 50 μl. Finally, 0.5 μCi of UDP-[3H]glucosamine (11.5 Ci/mmol, PerkinElmer Life Sciences) was added to the cells 30 min prior to the onset of the radiolabeling period, at concentrations of 2 mM and 100 μM, respectively. Analysis of LLO Glucosylation in Streptolysin-O-permeabilized Lymphoblasts—EBV-transformed lymphoblasts (2 × 10⁶ cells) were pulse-radioabeled with 200 μCi [2-3H]mannose as described above. The cells were then permeabilized with streptolysin-O (SLO, Bacto-Streptolysin O, product reference 0482, BD Biosciences) as described previously (31). Briefly, cells were washed into cell permeabilization buffer (PB; 130 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 5 mM HEPES/KOH, pH 7.3, containing 2 mg/ml bovine serum albumin and 1 mM dithiothreitol) and incubated with 5 units of SLO per 10⁶ cells for 20 min at 40°C. After washing twice with ice-cold PB, the cells were warmed to 37°C for 5 min and then placed on ice for 30 min. The cells were again washed in PB prior to being resuspended in the same buffer, dispersed into 1.5-ml centrifuge tubes, and incubated for different times at 37°C in the absence or presence of 2 mM UDP-glucose and/or 4 mM CST. Isolation and Analysis of Lipid-linked and N-Linked Oligosaccharides—Radiolabeled cells and SLO-permeabilized cells were extracted with organic solvents as described previously (19). Briefly, cells were rinsed with ice-cold phosphate-buffered saline and then resuspended in MeOH, 100 mM Tris/HCl, pH 7.4, containing 4 mM MgCl₂, and 2 M NaCl. After washing twice with ice-cold PB, the cells were warmed to 37°C for 5 min and then placed on ice for 30 min. The cells were again washed in PB prior to being resuspended in the same buffer, dispersed into 1.5-ml centrifuge tubes, and incubated for different times at 37°C in the absence or presence of 2 mM UDP-glucose and/or 4 mM CST. Northern Blot—LLO was isolated and analyzed as described previously (32). The LLO homogenate was centrifuged at 1,500 g for 30 min at 4°C, and the resulting supernatant was subjected to ultracentrifugation at 84,000 g for 20 min at 4°C. The LLO was resuspended in buffer A, which, UK) and kifunensin (KIF, Toronto Research Chemicals Inc.) were added to the cells 30 min prior to the onset of the radiolabeling period, at concentrations of 2 mM and 50 μM, respectively. The LLO was then permeabilized with streptolysin-O (SLO, Bacto-Streptolysin O, product reference 0482, BD Biosciences) as described previously (31). Briefly, cells were washed into cell permeabilization buffer (PB; 130 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 5 mM HEPES/KOH, pH 7.3, containing 2 mg/ml bovine serum albumin and 1 mM dithiothreitol) and incubated with 5 units of SLO per 10⁶ cells for 20 min at 40°C. After washing twice with ice-cold PB, the cells were warmed to 37°C for 5 min and then placed on ice for 30 min. The cells were again washed in PB prior to being resuspended in the same buffer, dispersed into 1.5-ml centrifuge tubes, and incubated for different times at 37°C in the absence or presence of 2 mM UDP-glucose and/or 4 mM CST. RNAs were extracted from the different samples, digested with DNase I, and separated on 1% agarose gels containing ethidium bromide. Isolated RNAs were then transferred to nylon membranes and hybridized with specific probes (see Table I) as described previously (36). In some experiments cells were treated with the translation inhibitor emetine (Sigma, product reference E-2375, dissolved in H₂O) for 8 h before RNA extraction. 18 S rRNA was monitored using a complementary oligonucleotide (37) or, where indicated, methylene blue staining (38). Mutation Analysis—The different primers used for PCR, sequencing, subcloning, and hybridization are listed in Table I. All sequencing was performed on both strands and on two independent PCRs. PCR products were purified with the QIAquick PCR purification kit (Qiagen, S. Franchise) prior to automated sequencing. The genomic DNA from the patient and parents was extracted with Trizol from peripheral blood mononuclear cells isolated using a Ficol-Paque Plus gradient. Trizol and Ficol-Paque were used according to the manufacturer’s instructions. DNA extracted exon 4 from the ALG8 gene from the patient was subcloned from genomic DNA after PCR amplification using the primers described in Table I. The PCR products obtained in this way were subcloned into the pCR3.1 plasmid employing the Eukaryotic Taq Expression Bi-directional kit (Invitrogen). HIV-1-derived Lentiviral Vectors and Transduction of Fibroblasts—The hALG8 DNA sequence was amplified from the human expressed sequence tag (accession number AJ224875, I.M.A.G.E. Clone ID 265381, obtained from the I.M.A.G.E. Consortium, Livermore, CA (39), using the primers indicated in Table I, and subcloned into the pSIN.PW.EFFP HIV-1-derived transfer vector as described previously (19). Transduction and labeling of cells were performed as reported before (19).

**RESULTS**

A Patient with Gastrointestinal Problems but Without Central Nervous System Deficits Presents with Hypoglycosylated Serum Glycoproteins—A girl, M. P., the first child of unrelated healthy parents, was referred at 4 months of age for edematous ascitic syndrome related to severe hypoalbuminemia resulting from protein losing enteropathy. Upon admission, she had no dysmorphic symptoms and normal psychomotor development, but had severe diarrhea and moderate hepatomegaly. Routine blood tests showed severe hypoalbuminemia (9 g/liter), normal aminotransferase activities, increased cephalin kaolin time (3 s), and low factor XI (12%), protein C (21%), and antithrombin III (17%) levels. Abdominal ultrasonography, echocardiography, and cerebral magnetic resonance imaging were normal, but electroretinography showed slight anomalies. The combined presence of coagulation factor anomalies and protein losing enteropathy was suggestive of CDG. This diagnosis was confirmed upon investigation of the glycosylation status of the serum glycoproteins from the patient by Western blot as shown in Fig. 1A. Although the electrophoretic profile of transferrin derived from serum of a normal subject displays a single band,
indicate the migration positions of transferrin species bearing 0, 1, and 2 radiolabeled with [2-3H]mannose and extracted with organic solvents. Ic M. P. (normal) and a patient diagnosed with CDG Ic (MP) were pulse-labeled for dolichyl phosphate as described under "Experimental Procedures." Radioactive components were visualized by fluorography, and the migration positions of standard oligosaccharides were indicated: M₉, Man₉GlcNAc₂; G₉₆, Man₉GlcNAc₂; M₉0, Man₉GlcNAc₂; G₉₆, Glc₂Man₉GlcNAc₂; G₉₆, Glc₂Man₉GlcNAc₂; C, lymphoblasts from a normal subject (N) and patient M. P. (MP) were assayed for dolichyl-P-glucose synthase activity, in both the absence (−Dol-P) and presence (+Dol-P) of dolichyl phosphate as described under "Experimental Procedures."

that from the patient reveals three distinct components whose migration positions coincide with transferrin species observed to occur in patients previously diagnosed with type I CDG (Fig. 1A). Initially, the girl required total parenteral nutrition and albumin infusions because of severe digestive complications. Oral mannose treatment was ineffective, but digestive indications improved with a low fat diet in association with essential fatty acid supplementation. After 18 months of dietary treatment, the diarrhea and protein-losing enteropathy were resolved, but despite normal liver function tests there was mild hypercholesterolemia without portal hypertension, and coagulation anomalies persisted. Psychomotor development continued normally, and electroretinographic observations remained unchanged.

Accumulation of Hypoglycosylated Dolichyl-linked Oligosaccharides in Skin Biopsy Fibroblasts Obtained from Patient M. P.—Further diagnosis was performed by assaying phosphomannomutase and phosphomannose isomerase (PMI) enzyme activities that are known to be deficient in CDG Ia and Ib, respectively. However, both these activities were found to be normal (phosphomannomutase, 4.1 units/g total protein (normal >3.4 units/g total protein), and PMI, 8.9 units/g total protein (normal >5.5 units/g total protein)). Next, skin biopsy fibroblasts from the patient were subjected to metabolic radiolabeling with [2-3H]mannose in order to examine LLO biosynthesis in these cells. After mild acid hydrolysis the oligosaccharide moieties of LLO from the patient and normal cells were resolved by TLC. A preliminary experiment revealed that although the control cells yielded predominantly glycosylated LLO, accumulations of LLO whose oligosaccharide structures comigrated with Man₉GlcNAc₂ and to a lesser extent Glc₃Man₉GlcNAc₂, were apparent in the cells from the patient (Fig. 1B). Similar results were observed when cells from a CDG Ic patient (deficiency in dolichyl-P-glucose:Man₉GlcNAc₂-PP-dolichyl α3-glucosyltransferase) were examined, but in these fibroblasts the monoglycosylated structure was less apparent (Fig. 1B). These observations suggested that in patient M. P. there is inefficient addition of glucose residues onto the growing LLO in the lumen of the ER. However, enzymic assay of dolichyl-P-glucose synthase (hALG5p) revealed that this enzyme that is responsible for the synthesis of the glucose donor molecules required for LLO glycosylation was not impaired in the fibroblasts from the patient (Fig. 1C). Finally, the dolichyl-P-glucose:Man₉GlcNAc₂-PP-dolichyl glucosyltransferase (hALG6) gene from the patient was sequenced, but no mutations were found.

Glc₃Man₉GlcNAc₂-PP-dolichyl Accumulates upon Treatment of Fibroblasts from the Patient with the Glucosidase Inhibitor Castanospermine—When normal and M. P. fibroblasts were radiolabeled in the presence of the ER glucosidase I and II inhibitor CST, we noted that LLO profiles were different from those generated in the absence of this agent. Thus, as demonstrated in Fig. 2A, whereas the CHCl₃ phase derived from organic solvent extraction of normal fibroblasts yields LLO containing mainly fully mannosylated oligosaccharides bearing between zero and three glucose residues (Glc₀–Glc₃Man₉GlcNAc₂), the chloroform/methanol/H₂O (10:10:3) phase was observed to comprise mainly an LLO possessing the fully glycosylated structure (Glc₃Man₉GlcNAc₂) that is known to be efficiently transferred onto glycoprotein in the lumen of the ER. Similar examination of the oligosaccharide species derived from LLO recovered from CST-treated normal cells revealed the almost exclusive appearance of fully glycosylated Glc₃Man₉GlcNAc₂ in both organic phases. By contrast, CST treatment of the cells from the patient reduced the accumulation of Man₉GlcNAc₂ but brought about substantial increases of Glc₂Man₉GlcNAc₂ as well as Glc₃Man₉GlcNAc₂ (Fig. 2B). When a similar experiment was conducted on fibroblasts derived from a patient diagnosed as having CDG Ic (13, 14), the glucosidase inhibitor had no effect on the accumulation of Man₉GlcNAc₂, and furthermore, no Glc₂Man₉GlcNAc₂ was apparent under these conditions (results not shown). These results can be explained by the glucosyltransferase-glucosidase shuttle proposed by Spiro and co-workers (40–42). According to this mechanism, represented in Fig. 2B, there are two ways in which Man₉GlcNAc₂-PP-dolichyl can be formed in mammalian cells. These authors showed that in addition to nascent glucose-containing glycoproteins in the lumen of the ER, glycosylated LLO are also susceptible to trimming by ER glucosidases, and it was hypothesized that the ability to both add and remove glucose residues
from both the CHCl$_3$ (ternary) or methanol/H$_2$O (binary) phases. Subsequent to mild acid hydrolysis of the lipid-linked species, the released oligosaccharides were resolved by TLC. The migration positions of standard oligosaccharides are indicated, and the abbreviations used are as defined in the legend to Fig. 1. The degradative steps have been proposed to be catalyzed by ER glucosidases I and II (GLS1, and -42 gene products, respectively).

Examination of LLO Glucosylation in Intact and SLO-permeabilized Lymphoblasts Derived from Patient M. P. A. Analysis of LLO biosynthesis in EBV-transformed lymphoblasts from patient M. P. revealed similar results to those obtained from fibroblasts, except that the addition of CST to these cells caused a less dramatic redistribution of glucosylated LLO species when compared with that observed for fibroblasts (results not shown). Therefore, EBV-transformed lymphoblasts from this subject were permeabilized with the plasma membrane pore-forming reagent SLO in order to perform in vitro analyses of LLO glucosylation. Permeabilized cells were incubated in either the absence or presence of UDP-Glc as shown in Fig. 3A.

Under these conditions there is no transfer of oligosaccharide from LLO onto protein (43), and after these brief 10-min incubations less than 10% of the LLO fraction is lost (probably as free oligosaccharides (43)). After permeabilization, LLO is glucosylated such that the majority of LLO glucosylation is at the level of Glc$_1$Man$_9$GlcNAc$_2$-PP-dolichyl. Crucially, these experiments revealed that the initial quantity of Man$_9$GlcNAc$_2$-PP-dolichyl declines at the same rate in both cell types, indicating that addition of the first glucose residue is not

![Image](http://www.jbc.org/)

**Fig. 2.** LLO glucosylation in M. P. cells and the glucosyltransferase/glucosidase shuttle. A, normal (N) and M. P. (MP) fibroblasts were pulse-radiolabeled with [2-3H]mannose in either the absence (-) or presence (+) of CST. Cells were extracted with organic solvents as described under "Experimental Procedures," and LLO were recovered from both the CHCl$_3$ (chloroform), and chloroform/methanol/H$_2$O, 10:10:3 (10:10:3), phases. Subsequent to mild acid hydrolysis of the lipid-linked species, the released oligosaccharides were resolved by TLC. The migration positions of standard oligosaccharides are indicated, and the abbreviations used are as defined in the legend to Fig. 1. B, the glucosyltransferase/glucosidase shuttle which has been proposed by Spiro and coworkers (40–42) to operate in mammalian cells. In human cells the biosynthetic steps are thought to be carried out by the three glucosyltransferases which are now known to be encoded by the human orthologs of the yeast ALG6, ALG8, and ALG10 loci. The degradative steps have been proposed to be catalyzed by ER glucosidases I and II (GLS1, and -42 gene products, respectively).

**Fig. 3.** Analysis of LLO glucosylation in SLO-permeabilized lymphoblasts derived from patient M. P. A. EBV-transformed lymphoblasts from a normal person (N) and the patient (MP) were pulse-radiolabeled with [2-3H]mannose prior to being permeabilized with SLO as described under "Experimental Procedures." The permeabilized cells were then incubated in either the absence or presence of UDP-Glc for the indicated times at 37°C. Subsequently, the cells were extracted with organic solvents, and LLO recovered from the CHCl$_3$, and 10:10:3 phases were pooled. 40,000 cpm of the oligosaccharides liberated from LLO by mild acid hydrolysis were resolved by thin layer chromatography as described in the legend to Fig. 1. The abbreviations used are as described for Fig. 1. B, a similar experiment was performed in either the presence of UDP-Glc alone (left panel) or UDP-Glc and 4 mM CST (right panel). After resolution of oligosaccharides released from LLO, the indicated radioactive species were eluted from the thin layer chromatography plate and quantitated by scintillation counting. The recovery of each oligosaccharide is expressed as the % of the total (M$_3$ + G$_2$M$_9$ + G$_3$M$_9$ + G$_2$M$_9$). Open circles, cells from control subject; closed circles, cells from patient M. P.
limiting in M. P. cells. Furthermore, when the same experiments were performed in the presence of CST, we were still able to detect the Glc₃Man₃GlcNAc₂-PP-dolichyl intermediate, demonstrating that this component is not generated by ER glucosidase I/II action on fully glucosylated LLO (Fig. 3B). Finally, in both cell populations LLO glucosylation also occurs to a lesser extent in the absence of UDP-Glc and is probably driven by an endogenous pool of dol-P-Glc.

**Reduced hALG8 mRNA Expression in Cells from Patient M. P.—**In yeast, the ALG8 gene thought to encode the dolichyl-P-glucose:Glc₃Man₃GlcNAc₂-PP-dolichyl α₃-glucosyltransferase has been cloned (44), and the complete sequence of the putative human ortholog of this gene (EMBL: BC001133 and AJ224875) is available (45). This information allowed us to create a probe in order to examine hALG8 mRNA in the cells from the patient by Northern blot. As demonstrated in Fig. 4A, there was a dramatic reduction in the quantity of the hALG8 message in the cells from the patient when compared with that observed in normal fibroblasts. By contrast, the level of the **hALG6** message was similar in the two cell populations. As the quantity of the **hALG8** message in the cells from the patient was less than 10% of that observed in control cells, it is apparent that both of the transcripts of the alleles from the patient at this locus are affected (Fig. 4A).

**Identification of Two Mutations Leading to Premature Stop Codons in the ALG8 Genomic DNA from the Patient—**The availability of the hALG8 cDNA sequence allowed us to find five partial genomic sequences from chromosome 11q14. By using these data, we were able to define the structure of the gene that comprised 13 exons (Fig. 4B). All the exon/intron boundaries follow the AG/GT rule, with the exception of intron 6 which starts with GC instead of GT. This observation was confirmed in the four available genomic sequences comprising exon 6 and the entire 276 bp of intron 6. By using the hALG8 gene structure, we designed intronic primers (see Table I) in order to amplify the different exons. The **ALG8** alleles from the patient were sequenced and compared with those obtained from the genomic DNA from the parents. As shown in Fig. 5A, two mutations were found in exon 4 of the ALG8 sequence from the patient: the allele originating from the father of the patient contained a deletion (413 del C) and that originating from the mother contained an insertion (396 ins A). In order to read the patient’s sequence between these two mutations, we subcloned this region from genomic DNA. Twenty independent clones corresponding to either of the two alleles were sequenced and found to occur in equal proportions, and additional mutations in this region of the gene were not found. The 396 ins A and 413 del C mutations generated premature stop codons (underlined bases in Fig. 5A) whose translation is predicted to generate severely truncated polypeptides (Fig. 5B). We also found a G665A variation in the sequence of exon 6; the father was found to be heterozygous at this position, but taking into account that both the mother and the patient possess only A at this position, and that this variation occurs after the two premature stop codons, we believe that this variation does not lead to the hALG8p deficiency and may correspond to a polymorphism.

**Effect of Translation Inhibition on the Quantity of ALG8 mRNA Recovered from Normal and M. P. Lymphoblasts—**It is known that the presence of premature stop codons can lead to a type of mRNA degradation that is accomplished by a translation-dependent process known as nonsense-mediated mRNA decay (NMD) (46, 47). In order to examine the possibility that the low expression of ALG8 mRNA in cells from patient M. P. is the result of such a process, we treated the cells from the patient and the control with different concentrations of the translation inhibitor emetine, as has been described previously (48). As shown in Fig. 6, Northern blot analysis reveals that emetine provokes a concentration-dependent increase of ALG8 mRNA in both control and M. P. lymphoblasts suggesting that the message is stabilized in both cell lines. However, whereas the message is only stabilized 3.5-fold in the control cells treated with 10 μg/ml emetine, a 20-fold increase is observed in the cells from the patient.

**Transduction of M. P. Fibroblasts with hALG8 cDNA—**In order to demonstrate unambiguously that a deficiency in hALG8p is the cause of the accumulation of underglucosylated LLO in cells from patient M. P., we have transduced immortalized M. P. fibroblasts with hALG8 cDNA using HIV-1-de-
rived lentiviral transfer vectors. Results presented in Fig. 7 show that whereas a vector harboring GFP cDNA alone had little effect on the distribution of LLO in CST-treated M. P. fibroblasts, a vector containing both hALG8 and GFP cDNA markedly reduced the appearance of underglucosylated LLO in the cells from the patient and restored the distribution of LLO to a pattern similar to that observed in normal, GFP-transduced, fibroblasts treated with CST. In this experiment it was noted that the distribution of LLO between the CHCl$_3$ and 10:10:3 organic phases was different from that usually observed. Although incubation of cells with CST favors the recovery of triglucosylated LLO in the 10:10:3 phase (see Fig. 2 S), sense; AS, antisense.

### Table I

**Primer sequences used in this study**

| Name  | Sequence                          | Use                                      |
|-------|-----------------------------------|------------------------------------------|
| ALG8  |                                   |                                          |
| 1S    | GGCACGGCTTCAGAGGGCT               | Genomic PCR and sequencing               |
| 1AS   | TCTCTCCGGCCTTGACCGGCA             | Idem                                     |
| 2S    | AAGCATAACTGGGGAATCTAGA            | Idem                                     |
| 2AS   | GCACCCAGCCAGAAACATT               | Idem                                     |
| 2ASbis| AAAATGAAAAATTTAAAAAC              | Sequencing                              |
| 3S    | CACCATCTTTGGTTTATCTAGCT           | Genomic PCR and sequencing               |
| 3AS   | CATATACTACCTTTCTCTCTCTCTC         | Idem                                     |
| 4S    | CATGATAGTTGTCAGAGGATTGA           | Idem                                     |
| 4AS   | CTAACCACCACCACTACAT               | Idem                                     |
| 5S    | ATTTGTTGCTTCTGTTGCTCT            | Idem                                     |
| 5AS   | TTACCTATCACCACTACTAC             | Idem                                     |
| 6S    | TAATGTCTCTTGGTCTGTTCC            | Idem                                     |
| 7AS   | CTAACCAACAAACAAAGAACAT           | Idem                                     |
| 7ASbis| AAAAAAAAAAAAGGCCT                  | Sequencing                              |
| 8S    | CCCATACTTTCATACACAT              | Genomic PCR and sequencing               |
| 8AS   | CAGCAGACTCCAACAAACAAA             | Idem                                     |
| 9S    | ATGAAAATCTCTGGCTTTTAAAGCA         | Idem                                     |
| 9AS   | TACAGTTGGAAGACCTTGGAA            | Idem                                     |
| 10S   | GAACCTGTGTTTGCATGAAGTCT           | Idem                                     |
| 10AS  | ACAAGAGGAGGAGAGAGCAGAAAT          | Idem                                     |
| 11S   | CTAATGCTCAGGCACTTCTAA            | Idem                                     |
| 12AS  | AACATTCCACAGAGATGAT             | Idem                                     |
| 12S   | ACTCTGGCTTGGCTTCTATAA            | Idem                                     |
| 13AS  | CTAATGGAGAAATGCTGCTT             | Idem                                     |
| 13S   | CTATGCTCCAAGGATGCC               | Idem                                     |
| 4BS   | CTTCTTCTGGCAATGTTGAGTAT          | Idem                                     |
| 4BSAS | ATGAAACGAAACAAACAAGACAT          | Idem                                     |
| 1BS   | ATGTGGATCAAGTGTGGGGGA            | Idem                                     |
| 1BSAS | CATACCTAAGCCAGTCTCTTATCC         | Idem                                     |
| 7TeraAS| CACATCAACTGGCTCTCTC              | Idem                                     |
| PetIS | AACTGAGACATATGGGCGGCGTCACTGCCC   | PCR of ALG8 probe                       |
| PetISNsmIAS| TCCCCGGCGCGCTGACGTTCTTCTCTCTCTCTCCTCATAAT | PCR of ALG8 probe |
| ALG6  |                                   |                                          |
| 6S    | TTTAAAGCTCTCGGACTGTTG            | PCR of ALG8 probe                       |
| 6AS   | TTTCAGGGACTGTTCTCAGAT             | PCR of ALG8 probe                       |

\* S, sense; AS, antisense.

**Transfer of Underglucosylated Oligosaccharides from LLO onto Glycoprotein in M. P. Fibroblasts**—Finally, we examined the nature of oligosaccharides that are transferred from LLO onto polypeptides in the lumen of the ER. Cells were treated with CST and KIF, an inhibitor of ER mannosidase I and Golgi mannosidase. When fibroblasts were pulse-radiolabeled with [2-3H]mannose under these conditions, N-linked oligosaccharides remain untrimmed and may reflect the nature of oligosaccharides that are transferred from LLO onto polypeptides. Accordingly, whereas in uninhibited control fibroblasts the predominant N-linked oligosaccharides are Glc$_0$–Man$_9$GlcNAc and Man$_8$GlcNAc, in inhibited cells Glc$_0$–Man$_9$GlcNAc is the predominant species detected (Fig. 8, A and B). Similar observations were made for the cells from the patient, but, in inhibited cells, we noted a modest increase in the proportion of an oligosaccharide migrating as Glc$_2$Man$_9$GlcNAc, when compared with that occurring in CST + KIF-treated control cells, indicating that either this structure is transferred directly from LLO onto polypeptide or that Man$_9$GlcNAc is transferred onto polypeptide prior to being post-translationally monoglucosylated by UDP-glucose:glycoprotein glucosyl transferase (UGGT).

**DISCUSSION**

In the present work we demonstrate that cells from a child displaying serum glycoprotein hypoglycosylation, and some clinical symptoms suggestive of type I CDG, reveal an inefficiency in their ability to add the second glucose residue onto LLO. Under normal radiolabeling conditions the predominant LLO is Man$_9$GlcNAc$_2$–PP-dolichyl. However, we were unable to detect any changes in the expression, or mutations, in the gene that encodes dolichyl-P-Glc:Man$_9$GlcNAc$_2$–PP-dolichyl glucosyltransferase, nor were we able to detect a reduction in dolichyl-P-glucose synthase activity in the cells from this patient. In fact, striking accumulations of monoglucosylated LLO displaying serum glycoprotein hypoglycosylation, and some clinical symptoms suggestive of type I CDG, reveal an inefficiency in their ability to add the second glucose residue onto LLO. Under normal radiolabeling conditions the predominant LLO is Man$_9$GlcNAc$_2$–PP-dolichyl. However, we were unable to detect any changes in the expression, or mutations, in the gene that encodes dolichyl-P-Glc:Man$_9$GlcNAc$_2$–PP-dolichyl glucosyltransferase, nor were we able to detect a reduction in dolichyl-P-glucose synthase activity in the cells from this patient. In fact, striking accumulations of monoglucosylated LLO only became apparent when M. P. fibroblasts were treated with CST, the ER glucosidase I and II inhibitor. Although the LLO species recovered from the 10:10:3 organic phase are generally more heavily glucosylated than those species recovered from the CHCl$_3$ fraction, our experience with primary human fibroblasts has shown that the proportion of total LLO that is glucosylated is quite variable. At present the factors that lead to this variability are not understood. However, where control pulse-radiolabeling experiments do yield large quantities of Man$_9$GlcNAc$_2$–PP-dolichyl, parallel incubations conducted in the presence of CST lead to a block in the appearance of this structure and a concomitant appearance of triglucosylated...
LLO, suggesting that the glucosyltransferase/glucosidase shuttle functions under certain cellular growth/stress conditions. Indeed it has been shown that the deglucosylating reactions occur when tissue slices or cells are deprived of oxygen (41), and it has been suggested that this stress leads to inefficient glucosylation of LLO due to reduced availability of dolichyl-P-glucose (41). More evidence for an ALG8p deficiency in M. P. cells was obtained by examining LLO glucosylation in SLO-permeabilized lymphoblasts. In this in vitro system we were able to demonstrate that whereas M. P. cells could efficiently add the first glucose to LLO, the addition of the second glucose residue was slow when compared with that observed in control cells. It was noted that in the absence of exogenously added UDP-Glc, the cells from the patient accumulated predominantly Man9GlcNAc2-PP-dolichyl, whereas in the presence of the sugar nucleotide Glc1Man9GlcNAc2-PP-dolichyl was more abundant than Man9GlcNAc2-PP-dolichyl. Thus, successful triglucosylation of LLO in cells from the patient may be particularly sensitive to cellular UDP-Glc levels that are known to fluctuate during hypoxia (49) and glucose insufficiency (50).

Interestingly, examination of LLO biosynthesis in a yeast strain deficient in ALG8p reveals the accumulation of Glc1Man9GlcNAc2-PP-dolichyl and not Man9GlcNAc2-PP-dolichyl as has been observed here in M. P. fibroblasts (51). It is not clear why yeast and mammalian cells deficient in ALG8p should behave differently in this respect, but although mammalian glucosidases have been shown to be active toward glucosylated LLO (52, 53), we have been unable to find any data in the literature indicating that the yeast glucosidases are active toward this substrate. In fact, in a yeast strain deficient in both ALG8p and ER glucosidase I, the structure Glc2Man9GlcNAc2 was detected N-linked to protein (51). In that study it was proposed that the elevated levels of Glc1Man9GlcNAc2-PP-dolichyl, caused on the one hand by the absence of ALG8p and on the other by the slow transfer of Glc1Man9GlcNAc2 onto protein, allowed ALG10p to "cap" the monoglucosylated LLO with an α2-linked glucose residue. Apparently then, in yeast and mammalian cells accumulated Glc1Man9GlcNAc2-PP-dolichyl may have different fates, and in the latter cell type, under certain conditions, this structure can be deglucosylated, whereas in yeast it can be glucosylated by ALG10p.

Whatever the significance of the glucosyltransferase/glucosidase shuttle, it is apparent that treatment of cells from the patient with CST gave us an early clue as to the underlying defect in this patient. Indeed, incubations performed in the

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**FIG. 5.** The genomic DNA from the patient contains two mutations that lead to premature stop codons in the hALG8 gene. A, the region of hALG8 exon 4 in which two mutations were found (numbering of the cDNA sequence starts with ATG). The mutated allele from the father contained a deletion (413 del C), and the mutant allele from the mother contained an insertion (396 ins A). Both mutations induced frame shifts that led to premature stop codons (underlined). B, amino acid sequences were generated from normal hALG8, and the two mutant alleles and Kyte-Doolittle (62) hydropathy plots were derived. A hydropathy index greater than 2 often indicates the presence of a series of amino acids capable of forming a transmembrane region. New peptide sequences between the frameshift and the premature stop codon are underlined.

**FIG. 6.** The effect of protein synthesis inhibition on ALG8 transcript levels in M. P. lymphoblasts. A, EBV-transformed lymphoblasts obtained from the patient (MP) or a normal subject (N) were treated with the indicated concentrations of emetine for 8 h. Total RNA was then extracted from the cells and subjected to Northern blot analysis as described for Fig. 4. B, ALG8 mRNA was quantitated by densitometric scanning, and the results were normalized with respect to the quantity of 18 S ribosomal RNA detected in the same gel lanes.
oligosaccharides were eluted from the chromatography plates and the abbreviations are as described in the legend to Fig. 1. The indicated CDG Ix cases where Man9GlcNAc2-PP-dolichyl is seen to accumulate in this case, and it is likely that, in other places if the PSC is dication of mRNA containing PSCs, but this process only takes place if the mRNA transcript is translated to stabilize certain mRNA transcripts containing PSCs. Indeed, our results show that this reagent stabilizes the patient’s ALG8 transcript such that its level is the same as that of its normal counterpart found in control cells treated with the same concentration of the drug. We noted that emetine also increases the expression of ALG8 mRNA in normal cells. In fact, in yeast many of the ALG gene mRNAs behave like the transcripts of “early growth-response” genes that are known to be stabilized in the presence of protein synthesis inhibitors (54–56). To date, this is the first time that NMD has been shown to be operative in cells from patients with CDG. In other diseases it has been shown that when NMD of PSC-containing mRNA is operational, the symptoms are less severe than in those patients for which the mutant mRNA is stable (46, 47). This is probably due to the fact that NMD can clear the cell of PSC-containing open reading frames, which, if translated would lead to the accumulation of potentially deleterious, dominant negative, truncated proteins (46, 47). If any mRNA is translated in patient M. P., it is apparent that only the first ~20% of the protein, containing only two potential transmembrane regions, is produced rendering it highly unlikely that these two alleles could give rise to active proteins. The dolichyl-P-monomosaccharide requiring mannosyltransferases and glucosyltransferases of the LLO pathway are extremely hydrophobic enzymes comprising 10–14 transmembrane regions (45). The importance of the transmembrane regions for enzyme function is attested to by the observation that several of the disease causing mutations in CDG Ii are found in or near the membrane spanning regions that occur along the entire length of the defective glycosyltransferases. By
taking into account the paucity of hALG8 mRNA, the above observations strongly suggest that patient M. P. has little or no ALG8p activity.

The paradox raised by our work is that despite the probable low leakiness of the hALG8 mutations presented here, the patient’s disease presents as a CDG with a less severe clinical picture than that generally associated with CDG Ic in which a deficit in LLO glucosylation is also the underlying cause. It is clear that cells from this patient manage to synthesize a deficit in LLO glucosylation is also the underlying cause. It is clear that cells from this patient manage to synthesize

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51. Runge, K. W., and Robbins, P. W. (1986) *J. Biol. Chem.* **261**, 15582–15590
52. Elting, J. J., Chen, W. W., and Lennarz, W. J. (1980) *J. Biol. Chem.* **255**, 2325–2331
53. Grinna, L. S., and Robbins, P. W. (1980) *J. Biol. Chem.* **255**, 2255–2258
54. Kukuruzinska, M. A., and Lennon, K. (1994) *Glycobiology* **4**, 437–443
55. Kukuruzinska, M. A., and Lennon-Hopkins, K. (1999) *Biochim. Biophys. Acta* **1426**, 359–372
56. Lennon, K., Pretel, R., Kesselheim, J., te Heesen, S., and Kukuruzinska, M. A. (1995) *Glycobiology* **5**, 633–642
57. Inon de Iannino, N., and Dankert, M. A. (1988) *Arch. Biochem. Biophys.* **260**, 139–145
58. D’Souza-Schorey, C., and Elbein, A. D. (1993) *J. Biol. Chem.* **268**, 4720–4727
59. Reiss, G., te Heesen, S., Zimmerman, J., Robbins, P. W., and Aebi, M. (1996) *Glycobiology* **6**, 493–498
60. Burda, P., and Aebi, M. (1998) *Glycobiology* **8**, 455–462
61. Chao, K. M., Pearson, W. R., and Miller, W. (1992) *Comput. Appl. Biosci.* **8**, 481–487
62. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
A Deficiency in Dolichyl-P-glucose:Glc1Man9GlcNAc2-PP-dolichyl α 3-Glucosyltransferase Defines a New Subtype of Congenital Disorders of Glycosylation

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