Congenital Disorders of Glycosylation Type Ig Is Defined by a Deficiency in Dolichyl-P-mannose:Man₅GlcNAc₂-PP-dolichyl Mannosyltransferase*

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Type I congenital disorders of glycosylation (CDG I) are diseases presenting multisystemic lesions including central and peripheral nervous system deficits. The disease is characterized by under-glycosylated serum glycoproteins and is caused by mutations in genes encoding proteins involved in the stepwise assembly of dolichol-oligosaccharide used for protein N-glycosylation. We report that fibroblasts from a type I CDG patient, born of consanguineous parents, are deficient in their capacity to add the eighth mannose residue onto the lipid-linked oligosaccharide precursor. We have characterized cDNA corresponding to the human ortholog of the yeast gene ALG12 that encodes the dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl α-L-fucose-mannosyltransferase that is thought to accomplish this reaction, and we show that the patient is homozygous for a point mutation (T571G) that causes an amino acid substitution (F142V) in a conserved region of the protein. As the pathological phenotype of the fibroblasts of the patient was largely normalized upon transduction with the wild type gene, we demonstrate that the F142V substitution is the underlying cause of this new CDG, which we suggest be called CDG Ig. Finally, we show that the fibroblasts of the patient are capable of the direct transfer of Man₅GlcNAc₂ from dolichol onto protein and that this N-linked structure can be glucosylated by UDP-glucose:glycoprotein glucosyltransferase in the endoplasmic reticulum.

Oligosaccharides N-linked to glycoproteins are initially synthesized on a lipid carrier in the endoplasmic reticulum (ER)1

* This work was supported by INSERM, by an INSERM-AFM Research network grant “Réseau de Recherche sur les CDG,” and by a grant from the Association Vaïncre Les Maladies Lysosomales. A preliminary account of this study was presented at the International Symposium on Protein Traffic, Glycosylation, and Human Health, May 15. Although the molecular events leading to this complicated clinical picture are multifactorial and not well understood, the disease is characterized at the biochemical level by under-glycosylated serum glycoproteins of hepatic origin (16). Disruption of any one of the enzymatic steps involved in the biosynthesis of lid-linked oligosaccharides (LLO) and the transfer of the mature oligosaccharide onto protein could potentially lead to the glycoprotein hypoglycosylation that is considered to be the hallmark of CDG I. Work on the underlying genetic bases of CDG I is still at an early stage because genes encoding the proteins involved in glycoprotein biosynthesis are numerous and, at least in humans, have for the most part yet to be identified (9). Notwithstanding these difficulties, mutations in six of the genes encoding proteins of the glycosylation pathway have been shown to underlie CDG I, and these disease types have been classified as CDG I subtypes a–f as follows: (Ia, phosphomannomutase 2 (17, 18); Ib, phosphomannose isomerase (19, 20); Ic, dolichyl-P-Glc:Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man₅GlcNAc₂-PP-dolichyl α3-glucosyltransferase: (21–23);
EBV-transformed lymphoblasts were generated from peripheral blood using the pAS plasmid encoding the simian virus 40 large T antigen and were transformed by Dr. Thierry Levade (INSERM U466). For some experiments, immortalized fibroblasts were grown in modified Eagle’s medium containing 2 g/liter glucose, 10% fetal calf serum, and 1% penicillin/streptomycin. For other experiments, immortalized fibroblasts were grown in RPMI 1640 medium supplemented as described above.

CDG I patient have a reduced capacity to add the eighth mannose residue onto the dolichol-PP-oligosaccharide precursor. This disease is known as type I CDG or the "deficiency of dolichol-P-Man:Man7GlcNAc2-PP-dolichyl 4-phosphate 3-mannosyltransferase (24); Ie, dolichol-P-Man synthase I (25, 26); and If, the MPDU1 gene product of unknown function (27, 28). The CDG I cases for which the genetic origins remain unknown are defined as CDG subtype If. Identification of the molecular bases of type I CDG is crucial from the clinical standpoint in that CDG Ib is treatable by administering oral mannose to the patient (29). In addition, identification of the underlying genetic defects will ultimately facilitate the design of antenatal diagnostic tests for different disease subtypes.

Here we report on the genetic deficit underlying a new subtype of CDG I. We show that skin biopsy fibroblasts from a CDG I patient have a reduced capacity to add the eighth mannose residue onto the dolichol-PP-oligosaccharide precursor required for protein glycosylation. Sequencing of cDNA derived from the patient and control cells indicated that the patient was homozygous for a point (F142V) mutation in the human homolog of the yeast ALG12 gene that encodes dolichyl-P-Man:Man,GlcNAc2-PP-dolichyl α3-mannosyltransferase.

**EXPERIMENTAL PROCEDURES**

**EST Clone and Primers**—The human EST accession number AI923828, I.M.A.G.E. Clone ID 2451352 was obtained from the I.M.A.G.E. Consortium, Livermore, CA (30). The different primers used for PCR, sequencing, hybridization, or subcloning are listed in Table I.

**Western Blotting**—Cells were rapidly disrupted in 7 ml of 4 M guanidine thiocyanate, and total RNA was isolated (31). RNA (20 μg) was denatured, fractionated by electrophoresis, transferred, and hybridized as described previously (32). 18 S rRNA was monitored using a specific antisense primer (9 AS, see Table I) or reverse transcriptase-PCR, PCR, and sequencing for restriction analysis.

**Isolation and Analysis of Lipid-linked and N-Linked Oligosaccharides**—Cells were extracted by a modification of the method of Folch (see Refs. 36 and 37). The lower (chloroform) and upper (methanolic) phases were removed and kept, and the interphase proteins were extracted with 2 × 2 ml CHCl₃/MeOH/H₂O, 10:10:3. The lower and 10:3 phases were dried and hydrolyzed with 0.02 N HCl (36) in order to release oligosaccharides from LLO. After desalting on AG-1/AG-50 columns, oligosaccharide components were resolved by TLC on silica-coated plastic sheets (Merck) in n-propyl alcohol/acetic acid/H₂O, 3:3:2 for 36 h (38) and detected by fluorography after spraying the dried TLC plates with En³hance® (PerkinElmer Life Sciences).

**Structural Analysis of Oligosaccharides**—Oligosaccharides released from LLO or from Pronase-digested glycoproteins by endo H were analyzed by concanavalin A-Sepharose (ConA-Sepharose, Amersham Biosciences AB) chromatography as described previously (39) except that the elution buffer containing 0.5 mM methyl α-D-mannopyranoside (Toronto Research Chemicals Inc., ON, Canada) was heated to 60 °C prior to use (40). The standard N-linked Man₅GlcNAc₂ structure was generated by endo H digestion of glycopeptides obtained from Pronase digestion of cellular glycoproteins isolated from HepG2 cells that had been metabolically radiolabeled with 2[^3H]mannose in the presence of CST and kifunensin (39).

Reduced oligosaccharides were subjected to acetylation (41), and the resulting fragments were analyzed as described previously (42) by TLC on cellulose-coated plastic sheets (Merck). The N-linked Man₅GlcNAc₂ and Man₆GlcNAc₂ structures used to generate standard acetylation fragments were obtained as described above except that the latter structure was derived from normal human skin biopsy fibroblasts.

**Mutation Analysis**— cDNA was generated from 5 μg of total RNA employing the Invitrogen Superscript™ Preamplification System using either a specific antisense primer (9 AS, see Table I) or nonspecific hexamers. For each primer couple, PCR conditions were optimized using the EST and then applied to the cDNAs. PCR products were purified with the Qiaquick PCR purification kit (Qiagen SA, Northwich, UK) and kifunensin (Toronto Research Chemicals Inc.) or the protein synthesis and N-glycosylation inhibitors, cycloheximide and tunicamycin, respectively, were added to the cells 30 min prior to the onset of the radiolabeling period.

**Table I**

| Name* | Sequence | Use |
|-------|----------|-----|
| 1S    | TTCGGGCTCGTGTCTGTTTCGG | PCR and sequencing |
| 1AS   | AGGATGATGCTCTGCTTGGTTTG | PCR and sequencing |
| 2S    | CACTGTCCACTGTTGGTGTCTGT | PCR and sequencing |
| 2AS   | CAAGGAAAGACGATACAGCCGGCG | PCR and sequencing |
| 3S    | GGCGAATGTTGTACGTACGAGG | PCR and sequencing |
| 3AS   | GACAGATGGGCGCCCTCTCATC | PCR and sequencing |
| 4S    | CCTCTGTCATCAGAGCAGCCAGG | PCR and sequencing |
| 4AS   | GTACTTCTACTGACGTCGCCGC | PCR and sequencing |
| 5S    | CTTGCTGTCACGAGCCACCTG | PCR and sequencing |
| 5AS   | GTCTGCTGTCACGAGCCACCTG | PCR and sequencing |
| 6S    | GAATTCCGCTACTGAGGCCACAG | PCR and sequencing |
| 6AS   | GGCGCAAGTGGCTACGTTCCGA | PCR and sequencing |
| 7S    | CAGGGACACAACACCGGGTCCT | Reverse transcriptase-PCR, PCR, and sequencing |
| 7AS   | ACCCTGGCCCTGTCGTCTTGG | PCR and sequencing |
| 8S    | CCAAGACAGTCCAGGCGCTTC | PCR and sequencing |
| 8AS   | GTGGCGGCGGGGAGCCACTTG | PCR and sequencing |
| 9S    | CCTGGCCCGAGGGCTCTTTCG | PCR and sequencing |
| 9AS   | GAGGGTCACTAACATTTAGG | PCR and sequencing |
| MALG12S | GCGATGTTGCACACATTGTCG | PCR and sequencing |
| MALG12AS | AGGGCAGACTGTCGCTTGGG | PCR and sequencing |
| HALG12NheIS | CTAGCTAGACGTGTAAGCTGAAAG | PCR and sequencing |
| HALG12XhoI | CGCCTGGAGCCCTGGCTTCAGT | PCR and subcloning of ORF |
| 18 S rRNA | AGGGTCACTAACATTTAGG | Hybridization, Northern blot |

* S, sense; AS, antisense.

Dulbecco’s modified Eagle’s or RPMI 1640 containing 0.5 mm glucose and 5% dialyzed fetal calf serum. Where appropriate the glycosidase inhibitors castanospermine (CST, Cambridge Research Biochemicals, Northwich, UK) and kifunensin (Toronto Research Chemicals Inc.) or the protein synthesis and N-glycosylation inhibitors, cycloheximide and tunicamycin, respectively, were added to the cells 30 min prior to the onset of the radiolabeling period.
**HIV-1-derived Lentiviral Vectors—**The pSIN.PW.eGFP HIV-1-derived transfer vector (43) contains a 400-bp deletion in the promoter/enhancer U3 region of the long terminal repeat (Fig. 5A). This so-called self-inactivating (SIN) integrated proviral vector is thus able to drive transcription from its long terminal repeat and therefore prevents premature interference and oncoregene activation. The eGFP reporter transgene is under the control of the internal human phosphoglycerate kinase promoter (hPGK). The post-transcriptional cis-acting regulatory element of the woodchuck hepatitis virus was inserted in sense orientation in order to increase transgene expression (44). The central polyurine tract associated with the central termination site was inserted into the transfer vector because it has been shown to enhance the nuclear translocation of lentiviral pre-integration complexes (43, 45).

CDNA encoding the human α6-mannosyltransferase, hALG12p ORF, amplified by PCR from the EST, was inserted downstream of the hPGK promoter into a pSIN.PW.IRES2.eGFP transfer vector. The resulting bicistronic pSIN.PW.hALG12.IRES2.eGFP vector contained a modified internal ribosome entry site (IRES2, CLONTECH) of the encephalomyocarditis virus to allow better expression of the downstream gene.

**Lentiviral Vector Production and Titration—**Lentiviral SIN.PW.hPGK/eGFP and SIN.PW.hALG12.IRES2.eGFP transfer vector particles were separately produced by cotransfection of their respective plasmids into human kidney 293T cells along with the packaging and envelope constructs. The packaging plasmid (pCMVΔR8.91) provided gag, pol, rev, and neo helper genes under the control of the human cytomegalovirus promoter. The envelope plasmid (pMD.G) encoded the heterologous vesicular stomatitis virus glycoprotein. Forty eight and seventy two hours post-transfection, supernatants were collected and filtered. High titer stocks were obtained by ultracentrifugation.

**Lentiviral Vector Transduction in Patient Cells—**Three immortalized fibroblast cell lines originating from patient ME were transduced with SIN.PW.hPGK/eGFP or SIN.PW.hALG12.IRES2.eGFP transfer vectors at 10 or 100 multiples of infection. 2-[3H]Manose labeling was performed on the transduced cells 48 h after transduction.

**RESULTS**

**Patient ME, Clinical Picture and Biochemical Diagnosis of CDG I—**The patient (ME), a girl of healthy consanguineous Tunisian parents, was admitted to the neonatal unit for weak sucking. Clinical examination revealed generalized hypotonia and facial dysmorphism, and routine laboratory investigations showed normal blood chemistry except for hypocalcemia. Evolution of the condition of the patient was marked by persistent feeding difficulties, failure to thrive, severe psychomotor involvement, major hypotonia, and progressive microcephaly. Moreover, the patient developed frequent ear, nose, throat, and respiratory infections. Later examinations, performed at 6 months, showed normal blood chemistry, although immunologic screening showed low IgG levels (2.38 g/liter) with normal IgA and IgM. At 18 months, failure to thrive required gastrostomy, but neuroimaging of the brain remained normal.

Plasma proteins from ME were subjected to Western blot and isoelectric focusing as shown in Fig. 1A, and examination of serum transferrin revealed hypoglycosylation profiles characteristic to those observed in type I CDG (11). Phosphomannomutase and phosphomannose isomerase activities, known to be characteristic to those observed in type I CDG (11). Phosphomannose and phosphomannose isomerase activities, normal in ME fibroblasts (results not shown).

**Accumulation of Manα-GlcNAc2-PP-dolichol in ME Fibroblasts—**In order to understand the cause of glycoprotein hypoglycosylation in patient ME, skin biopsy fibroblasts were metabolically pulse-radioiodinated with 2-[3H]mannose and then extracted with organic solvents to yield CHCl3 and CHCl3/MEOH/H2O (10:10:3) lipid fractions. Oligosaccharides were released from LLO present in both these fractions and resolved by TLC as shown in Fig. 1B. Control fibroblasts yielded predominantly mature triglucosylated LLO in the 10:10:3 lipid fraction, whereas less mature species such as Manα-GlcNAc2-PP-dolichol and Manα6-GlcNAc2-PP-dolichol were also apparent in the CHCl3 fraction. By contrast, ME fibroblasts elaborated much less Glc3Manα-GlcNAc2-PP-dolichol in both the 10:10:3 and CHCl3 fractions; however, the latter fraction contained substantial amounts of an LLO whose oligosaccharide moiety migrated between Manα6-GlcNAc2 and Man6-GlcNAc2 labeled “a” in Fig. 1B. After treatment with endo H, as shown in Fig. 1C, component a yielded a product that migrated slightly slower than a Manα-GlcNAc2 structure that was generated by endo H treatment of 2-[3H]mannose-labeled glycoprotein material derived from HepG2 cells. However, ConA-Sepharose chromatography of oligosaccharide a and HepG2 glycoprotein-derived Manα-GlcNAc2 demonstrated these two structures to be quite different (see Ref. 46 and Fig. 2A). The endo H sensitivity of oligosaccharide a on the one hand and its weak binding to the lectin column on the other hand suggested that the α6-linked mannos of the Manα-GlcNAc2 core is substituted with an α3-linked mannos residue. Acetylation of a reaction that specifically cleaves α6-linkages of oligosaccharide a (shown in Fig. 2B), confirmed this and demonstrated that its mannose substituents possess an isomeric configuration identical to that which is known to occur in Manα-GlcNAc2-PP-dolichol under normal physiological conditions (oligosaccharide structure shown to the right in Fig. 2B, 46).
rose chromatography; similarly, an aliquot of Man 7GlcNAc (structure a suggested oligosaccharide) was also analyzed.

glycoproteins (closed symbols) were also reduced with NaBH4 (aH2) prior to being subjected to acetylation. The acetylation fragments were resolved by TLC on cellulose-coated plates. The migration positions of standard oligosaccharides, generated from the acetylation of Man$_8$GlcNAc$_2$ and Man$_6$GlcNAc$_2$H$_2$, are shown to the left of the chromatogram (structures are represented above the lanes, and dotted lines indicate the α-linkages preferentially cleaved during the acetylation reaction: closed squares; mannose, open squares; GlcNAc$_2$H$_2$). The isomeric configuration of the mannose residues deduced to occur in aH$_2$ is shown to the right of the chromatogram. The abbreviations used are: M$_5$GNH$_2$, Man$_5$GlcNAc$_2$H$_2$, M$_6$GNH$_2$, Man$_6$GlcNAc$_2$; M, mannose; M$_o$, mannotriose; M$_a$, mannotetraose; M$_g$, Man$_4$GlcNAc$_2$H$_2$; M$_u$, Man$_5$GlcNAc$_2$H$_2$; M$_b$, Man$_6$GlcNAc$_2$H$_2$.

Cloning of the Human Homolog of S. cerevisiae ALG12—The above results suggested that the block in biosynthesis of LLO observed in ME fibroblasts is due to inefficient addition of the eighth mannose residue. The gene encoding dolichyl-P-mannose:Man$_6$GlcNAc$_2$-PP-dolichyl mannosyltransferase has been cloned in yeast and corresponds to the ALG12 locus. Examination of the literature (47, 48) indicated that ALG12 belongs to a family of genes encoding several putative mannosyltransferases containing the sequence HKE$_1$RF flanked by two hydrophobic regions (motif 2, Fig. 3). We found several new members of this family in the data banks and constructed a phylogenetic tree that allowed us to delineate four subfamilies. The subfamily containing Saccharomyces cerevisiae ALG12 revealed a second peptide motif (TKVEESF, motif 1, Fig. 3) found to be absent from the other subfamilies. By using this second motif we looked for human expressed sequence tags (ESTs, I.M.A.G.E Consortium, Washington, D. C.) corresponding to hALG12. One such EST was found to be large enough to contain the entire open reading frame (ORF) and was therefore completely sequenced. We obtained an identical sequence when control fibroblast cDNA was used as template. This sequence, which we deposited in the data banks (GenBank accession number AJ303120), encoded a highly hydrophobic protein containing 488 amino acids (see Fig. 3).

Analysis of the hALG12 Derived from Patient ME and the Parents—Northern blot analysis (Fig. 4A) revealed only a single hALG12 transcript of 2.5 kb even using low stringency washes. No differences in either the size or level of expression of hALG12 mRNA between normal and ME fibroblasts could be observed. We noted that three different fibroblast populations showed similar levels of mRNA expression to those observed in HepG2 cells, whatever the growth state of these human hepatocellular carcinoma cells.

The similarity of expression of the hALG12 mRNA in the fibroblasts from the patient and normal fibroblasts prompted us to sequence the hALG12 cDNA from the patient. Our sequence data indicated that the patient was homozygous for a point mutation (T571G) that caused an amino acid substitution (F142V) in a conserved region of the peptide sequence (see Fig. 3). The mutation was observed in eight independent sequences derived from two independent RNA preparations. This base substitution generates a restriction site for AccI that allowed us to confirm that the patient ME is homozygous and that the parents are both heterozygous for this mutation (see Fig. 4B). The heterozygous status of both parents was confirmed by sequencing (results not shown). The primer couple designed for PCR amplification of the region around the mutation amplifies a DNA fragment that has the same size irrespective of whether cDNA or genomic DNA was used as template, indicating that AccI digestion of the restriction fragment amplified from genomic DNA can be used for a prenatal diagnostic test.

Transduction of ME Fibroblasts with Wild Type hALG12 cDNA—Next we evaluated the effects of introducing normal hALG12 into the fibroblasts from the patient. This was undertaken by constructing two HIV-1-derived lentiviral transfer vectors as shown in Fig. 5A and described under “Experimental Procedures.” One vector contained DNA encoding enhanced GFP (eGFP vector), and a second contained both wild type hALG12 cDNA, and the eGFP DNA in a bicistronic based expression system (eGFP/hALG12 vector). To facilitate these retroviral gene transfer experiments, we chose to transduce a line of the patient’s fibroblasts that had been immortalized by transfection with the SV 40 large T antigen. Under the experimental conditions used, it was determined that greater than 90% of the immortalized fibroblasts became eGFP-positive irrespective of whether the eGFP or eGFP/hALG12 vector was used to transduce the cells (results not shown). Fig. 5B shows that whereas immortalized ME fibroblasts transduced with the eGFP vector generated an LLO pattern close to that observed in their non-transduced counterparts, transduction with the eGFP/hALG12 vector caused a dramatic increase in the proportion of mature LLO (Glc$_2$Man$_6$GlcNAc$_2$-PP-dolichol) in immortalized ME fibroblasts. Indeed, when ME fibroblasts were treated with the higher viral dose the distribution of LLO was similar to that observed in normal non-transduced cells.

Transfer of Non-glucosylated Man$_6$GlcNAc onto Glycoproteins in ME Fibroblasts—Finally, the nature of the oligosaccharides transferred onto nascent glycoproteins in ME fibroblasts was examined. Cellular glycoproteins were digested with

\* Oriol, R., Martinez-Duncker, I., Chantret, I., Mellicone, R., and Codogno, P. (2002) Mol. Biol. Evol., in press.
Pronase, and the resulting glycopeptides were treated with endo H in order to release N-linked oligomannose-type oligosaccharides. Resolution of these structures by TLC (Fig. 6A) shows that control fibroblasts yield predominantly Man9GlcNAc and its monoglucosylated counterpart. These two N-linked structures are also prominent on ME fibroblast glycoproteins, but in these cells two additional structures are present. ConA-Sepharose chromatography and acetolysis indicated that these two components correspond to Glc1Man7GlcNAc and Man7GlcNAc and that the isomeric configuration of their mannose residues was the same as that observed in oligosaccharide a and different to that of the N-linked Man7GlcNAc intermediate commonly found associated with glycoproteins. Triglucosylated oligosaccharides are more efficiently transferred from dolichol onto polypeptide.

**Human ALG12p Deficiency**

![Fig. 3. The human (H) ALG12 peptide sequence corresponding to the ORF of the nucleotide sequence reported here (GenBank™ accession number AJ303120) is shown aligned with its Mus musculus (Mm, SWALL accession number CAD22101), Drosophila melanogaster (Dm, SPTREMBL accession number Q23361), S. pombe (Sp, SP accession number O74753), and Saccharomyces cerevisiae (Sc, SP accession number P53730) homologs. The alignments were performed using the ClustalW program (55). An asterisk indicates conserved amino acids. Sequencing of hALG12 from patient ME (GenBank™ accession number AJ290427) revealed a single base mutation (T571G) that caused an F142V substitution in the peptide sequence (**vertical arrow**). The horizontal line indicates a conserved (except in yeast) glycosylation consensus site.](http://www.jbc.org/)

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tides; however, the direct transfer of Man7GlcNAc2 from dolichol onto protein has been observed in mammalian cell lines (46). In order to test whether or not this phenomenon can occur in ME fibroblasts, we treated these cells with CST, the ER glucosidase I and II inhibitor. In the presence of this drug, oligosaccharides transferred from dolichol onto protein cannot be deglucosylated. Accordingly, when control fibroblasts are treated with CST, all N-linked oligosaccharides were found to be triglucosylated, indicating that in normal cells there is no transfer of non-glucosylated oligosaccharides from lipid onto nascent glycoproteins. By contrast, although treatment of ME fibroblasts with the glucosidase inhibitor caused the appearance of triglucosylated Man9GlcNAc species and reduced the amount of Man7GlcNAc, it did not lead to the disappearance of N-linked Glc3Man9GlcNAc. When similar 30-min pulse radiolabeling experiments were conducted in the presence of CST and the Golgi mannosiase I inhibitor, kifunensin, there was little change to the patterns of N-linked structures occurring in either cell population (results not shown). The inability of CST to inhibit the appearance of Glc1Man7GlcNAc observed in 2-[^3H]mannose-labeled normal fibroblasts, comigrated with the Glc1Man7GlcNAc observed in 2-[^3H]mannose-labeled ME fibroblasts (Fig. 6B). Although ME fibroblasts also generated the same Glc1Man9GlcNAc species, two additional reglucosylated N-linked structures were found. A Glc3Man9GlcNAc structure was observed that, in contrast to its counterpart observed in [3H]galactose-labeled normal fibroblasts, comigrated with the Glc3Man9GlcNAc observed in 2-[3H]mannose-labeled ME fibroblasts. In addition, a species that migrated as Glc1Man9GlcNAc was observed in [3H]galactose-labeled ME fibroblasts, irrespective of the presence or absence of the two inhibitors. These results are consistent with the transient reglucosylation of ER-situated unfolded glycoproteins by UDP-glucose:glycoprotein glucosyltransferase. In normal fibroblasts we observed three such reglucosylated N-linked oligosaccharides migrating as Glc1Man9GlcNAc, Glc3Man7GlcNAc, and Glc3Man7GlcNAc (Fig. 6B). Although ME fibroblasts also generated the same Glc3Man9GlcNAc species, two additional reglucosylated N-linked structures were found. A Glc3Man9GlcNAc structure was observed that, in contrast to its counterpart observed in [3H]galactose-labeled normal fibroblasts, comigrated with the Glc3Man9GlcNAc observed in 2-[3H]mannose-labeled ME fibroblasts. In addition, a species that migrated as Glc3Man9GlcNAc was observed in [3H]galactose-labeled ME fibroblasts (Fig. 6B).

In summary, the ensemble of these results demonstrates that in ME fibroblasts three oligosaccharide structures are transferred in approximately equal amounts from dolichol onto glycoproteins; Glc3Man9GlcNAc, Glc3Man7GlcNAc, and Man7GlcNAc. Furthermore, the N-linked Man7GlcNAc structure that is observed in the cells from patient ME can be glucosylated by UDP-glucose:glycoprotein glucosyltransferase.
ConA-Sepharose chromatography and upon examining their susceptibility to the action of Benner's enzyme (54), we found that the human ALG12p cDNA was responsible for the human ALG12p deficiency in the patient described here.

We found the hALG12 CDNA of the patient to be homozygous for a single point mutation that leads to replacement of a Phe residue by Val in the encoded protein. Several lines of argument suggest that this mutation is the underlying cause of the Man6GlcNAc2-PP-dolichyl accumulation observed in fibroblasts obtained from patient ME. First, examination of the size of the hALG12 mRNA transcript from the fibroblasts of the patient revealed it to be identical to that observed for the transcript present in other cell lines, indicating normal processing and the absence of an important deletion. Second, this mRNA transcript appeared to be as abundant as that observed in control fibroblasts, signifying on the one hand that both alleles are expressed and on the other hand an absence of mRNA instability. Third, analysis of the human EST banks allowed us to identify 28 separate human ESTs containing the region of the mutation, but we were unable to detect the T571G base change in any of these sequences, suggesting that the mutation is not simply a common polymorphism. Fourth, the F142V replacement occurs in one of the small highly conserved patches toward the NH2 terminus of ALG12p. In fact, in all species for which sequence data are available, we noted that the position of the replaced Phe residue is invariably occupied by the aromatic amino acids Phe or Tyr (Schizosaccharomyces pombe) and that this position is next to a pair of highly conserved aromatic amino acids in ALG12p function is not understood, but our observations suggest that they are situated in, or near, a transmembrane domain. Indeed, several point mutations in glycosyltransferases that lead to CDG type I have been noted to occur in the transmembrane regions of these proteins. To conclude, the above arguments strongly favor our assertion that the point mutation identified in the hALG12 CDNA of patient ME is responsible for the accumulation of Man6GlcNAc2-PP-dolichyl observed in skin biopsy fibroblasts obtained from this subject.

As observed for other types of CDG I, the observed mutation is leaky (18, 22, 24) because small amounts of fully mannosylated LLO can be detected in cells from the patient. This leakiness allows a substantial transfer of GlcMan2GlcNAc2 from dolichol onto nascent polypeptides in the fibroblasts of the patient. We also observed that N-linked GlcMan2GlcNAc2 and Man4GlcNAc2 account for the remaining two-thirds of the total oligosaccharide transferred onto protein from oligosaccharide lipid. The relative abundance of these latter two species linked on the one hand to lipid and on the other hand N-linked to

DISCUSSION

Here we report on a CDG I patient with a deficiency in the ability to add the eighth mannose onto LLO. Structural analysis of the lipid-linked Man7GlcNAc2 that was found to accumulate in fibroblasts from this patient revealed the same iso-meric configuration of mannose residues to that which has been shown to occur in the normal LLO intermediate generated during glycoprotein biosynthesis (2, 46). This result allowed us to eliminate the hypothesis that the block was due to a rare “change of function” mutation potentially causing the seventh mannose residue to be added to an inappropriate position of the LLO acceptor. Additionally, it is noteworthy that in a yeast strain deficient in ALG11p, the enzyme that adds the fifth mannose residue to be added to an inappropriate position of the LLO acceptor. Furthermore, it is noteworthy that in a yeast strain deficient in ALG11p, the enzyme that adds the fifth mannose to the growing oligosaccharide-lipid (50), an abnormal Man7GlcNAc2 structure linked to dolichol has been observed (50) further emphasizing the necessity to carry out detailed structural analyses on the oligosaccharide species that occur in these unusual circumstances. The most likely hypothesis to explain the block in LLO biosynthesis was a deficiency in dolichyl-P-Man-Man,GlcNAc2-PP-dolichyl 6-mannosyltransferase. In yeast, this enzyme is thought to be encoded by the ALG12 gene (48, 51). The proteins encoded by the ALG12 genes of different species are all hydrophobic, and like some membrane transporters may possess up to 10-12 transmembrane domains. Nevertheless, the peptide sequences of the ALG12 gene products have features in common to those of the ALG3p which has been shown to have dolichyl-P-mannose:Man5

GlcNAc2-PP-dolichyl mannosyltransferase activity (52), indicating that ALG12p is also likely to have mannosyltransferase activity. In the work presented here we characterized the human homolog of this gene, and the accuracy of the sequence we deposited in the data banks was attested to by the subsequent appearance of an identical “anonymous” cDNA sequence (GenBankTM accession number BC001729). Interestingly, we noted the presence of a consensus glycosylation site (NK/R/S, see Fig. 3) which was conserved in all species except yeast. Theoretical predictions of hALG12p transmembrane regions indicate that this glycosylation site is situated in an extramembrane loop sufficiently large to support glycosylation. The question of whether or not hALG12p is glycosylated will have to await experimental evidence.

When the human wild type ALG12 gene was introduced into the fibroblasts of the patient, we noted a remarkable normalization of LLO biosynthesis (Fig. 5B), and a concomitant increase in the fraction of fully mannosylated oligosaccharides was transferred from dolichol onto polypeptide (results not shown). These observations strongly suggest that the ALG12 gene is defective in the patient described here.

We found the hALG12 CDNA of the patient to be homozygous for a single point mutation that leads to replacement of a Phe residue by Val in the encoded protein. Several lines of argument suggest that this mutation is the underlying cause of the Man6GlcNAc2-PP-dolichyl accumulation observed in fibroblasts obtained from patient ME. First, examination of the size of the hALG12 mRNA transcript from the fibroblasts of the patient revealed it to be identical to that observed for the transcript present in other cell lines, indicating normal processing and the absence of an important deletion. Second, this mRNA transcript appeared to be as abundant as that observed in control fibroblasts, signifying on the one hand that both alleles are expressed and on the other hand an absence of mRNA instability. Third, analysis of the human EST banks allowed us to identify 28 separate human ESTs containing the region of the mutation, but we were unable to detect the T571G base change in any of these sequences, suggesting that the mutation is not simply a common polymorphism. Fourth, the F142V replacement occurs in one of the small highly conserved patches toward the NH2 terminus of ALG12p. In fact, in all species for which sequence data are available, we noted that the position of the replaced Phe residue is invariably occupied by the aromatic amino acids Phe or Tyr (Schizosaccharomyces pombe) and that this position is next to a pair of highly conserved aromatic amino acids in ALG12p function is not understood, but our observations suggest that they are situated in, or near, a transmembrane domain. Indeed, several point mutations in glycosyltransferases that lead to CDG type I have been noted to occur in the transmembrane regions of these proteins. To conclude, the above arguments strongly favor our assertion that the point mutation identified in the hALG12 CDNA of patient ME is responsible for the accumulation of Man6GlcNAc2-PP-dolichyl observed in skin biopsy fibroblasts obtained from this subject.

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polypeptide suggests the following: first, the lipid-linked Man$_{n}$GlcNA$_{c}$ is poorly glycosylated; second, the small amount of lipid-linked Glc$_{3}$Man$_{n}$GlcNA$_{c}$ that is formed can be more efficiently transferred to protein than its non-glycosylated counterpart. The observation that substantial amounts of non-glycosylated Man$_{n}$GlcNA$_{c}$ can be transferred from lipid onto protein was surprising considering that human skin biopsy fibroblasts deficient in dolichol-P-Glc-Man$_{n}$GlcNA$_{c}$-PP-dolichol transferase do not appear to be able to transfer Man$_{n}$GlcNA$_{c}$ directly onto protein (22). However, this phenomenon has been observed in mouse F9 teratocarcinoma cells (46). It would be interesting to look for mutations in the mouse ALG12 gene in the F9 cell line. To summarize, it appears that in addition to leading to only a partial block in hALG12p function, the truncated Man$_{7}$GlcNA$_{c}$ intermediate that accumulates can be transferred directly onto protein. Despite these relieving factors, it is apparent the clinical picture for patient ME is severe, comprising both central nervous system and peripheral deficits. At present, it is unclear whether or not a deficiency in hALG12p will invariably lead to a severe type of CDG I; only characterization of other patients with the same enzymic deficit will answer this question. However, one compounding factor may contribute to the severity of the symptoms observed in this case. In fact, observations made with a yeast strain deficient in ALG12 led investigators to suspect that misfolded glycoproteins bearing N-linked Man$_{n}$GlcNA$_{c}$ are poor substrates for ER-associated glycoprotein degradation (48). Although these observations have yet to be extended to mammalian cells, it is possible that in fibroblasts from ME such a phenomenon could exacerbate the ER accumulation of glycoproteins that may result from the misfolding of hypoglycosylated polypeptides (53). Under these conditions, the resulting stress generated in the ER could have profound effects on the cellular homeostasis of such cells. Furthermore, it has been shown in vitro that the “processing” isoforms of Man$_{n}$GlcNA$_{c}$ (generated by ER mannosidases) that are substrates for UDP-glucose:glycoprotein glucosyltransferase have only 15% of the acceptor activity of Man$_{n}$GlcNA$_{c}$ (54). However, we show here that the “biosynthetic” Man$_{n}$GlcNA$_{c}$ structure that is transferred directly from dolichol onto protein in cells from patient ME can be glycosylated by UDP-glucose:glycoprotein glucosyltransferase, presumably as part of the glycoprotein “quality control” system.

To conclude, our results indicate that a F142V replacement in hALG12p is the cause of inefficient addition of the eighth mannose residue onto Man$_{n}$GlcNA$_{c}$-PP-dolichol during glycoprotein biosynthesis in a patient with type I CDG. The insufficiency in this step of the pathway for lipid-linked oligosaccharide biosynthesis defines a new subtype of the disease that we suggest should be called type Ig.

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Note Added in Proof—The GenBank™ AL671710 genomic sequence located in chromosome 22g13.3 contains the ALG12 gene.
Congenital Disorders of Glycosylation Type Ig Is Defined by a Deficiency in Dolichyl-P-mannose:Man 7GlcNAc2-PP-dolichyl Mannosyltransferase
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