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Congenital Disorders of Glycosylation Type Ig Is Defined by a Deficiency in Dolichyl-P-mannose:$Man_7GlcNAc_2$-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:$Man_7GlcNAc_2$-PP-dolichyl α6-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 $Man_7GlcNAc_2$ 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] membrane by the stepwise addition of monosaccharides onto dolichol pyrophosphate to form $Glc_3Man_9GlcNAc_2$-PP-dolichol (1, 2). The oligosaccharide moiety is then transferred from lipid onto nascent proteins in the lumen of the ER. The resulting oligomannose-type oligosaccharides *N*-linked to glycoproteins are involved in the folding (3, 4), degradation (5, 6), and efficient transport (7) of glycoproteins in the secretory pathway. During their passage through the Golgi apparatus, the oligomannose-type sugar units attached to glycoproteins are often partially demannosylated and subsequently decorated with GlcNAc, galactose, sialic acid, and fucose residues (2). The resulting glycoproteins, bearing complex-type oligosaccharide, are subsequently secreted into the extracellular space or exported to the cell surface where they are involved in diverse regulatory processes (8). The importance of this widespread cotranslational modification is attested to by the identification of a rapidly growing number of diseases whose molecular origins have been linked to abnormalities in glycoprotein biosynthesis (9–14).

Type I congenital disorders of glycosylation (CDG I) are newly described rare metabolic diseases presenting a severe clinical picture in which multisystemic lesions including central and peripheral nervous system deficits are apparent (9, 11, 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 enzymic steps involved in the biosynthesis of lipid-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:$Man_9GlcNAc_2$-PP-dolichyl α3-glucosyltransferase: (21–23); Id, dolichyl-P-Man:$Man_5GlcNAc_2$-

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[1] The abbreviations used are: ER, endoplasmic reticulum; CDG, congenital disorders of glycosylation; LLO, lipid-linked oligosaccharide; EST, expressed sequence tag; ORF, open reading frame; endo H, endo-β-D-*N*-acetylglucosaminidase H; HIV-1, human immunodeficiency virus, type 1; eGFP, enhanced green fluorescent protein; ConA, concanavalin A; CST, castanospermine; EBV, Epstein-Barr virus; SIN, self-inactivating; hPGK, human phosphoglycerate kinase promoter; IRES, internal ribosome entry site.

TABLE I
Primers used in this study

Name[a]	Sequence	Use
1S	TTCGGGCTCTGTTCTGTTTCCG	PCR and sequencing
1AS	ATGTGTGGCCTGCAGGTTGAAG	PCR and sequencing
2S	CACTGTTCCACCTGGTCATCTGT	PCR and sequencing
2AS	CAACGAAAAGCACGTAAACCGCG	PCR and sequencing
3S	GGGCCAGTGGTGATTCGCAGTG	PCR and sequencing
3AS	CAAGGCCAGCAGCAGCAGGAGG	PCR and sequencing
4S	GCACGAGTGGGCCCGCTTCATC	PCR and sequencing
4AS	CCTTCTGTCTACCAAGCCCAGG	PCR and sequencing
5S	GTACTTCTACTCAGCCCTGCCC	PCR and sequencing
5AS	CTCTGCATTGCGACGCCACCTG	PCR and sequencing
6S	GAATGCCGCCTACTCAGCCAC	PCR and sequencing
6AS	GGGCAAGTTGGGTCAGGTTCAG	PCR and sequencing
7S	CAGGGACACACACCGGGTCCT	PCR and sequencing
7AS	ACCTGGGCCCCTCGTTCTTTGG	PCR and sequencing
8S	CCAGACCAGGTCCAGGGCCTC	PCR and sequencing
8AS	GTGCGGCCGGGGCACCATGG	PCR and sequencing
9S	CTGCCCGAGGGCCTCTTGGG	PCR and sequencing
9AS	GAGGTTCCAATCAACATTTATTGCC	Reverse transcriptase-PCR, PCR, and sequencing
MALG12S	GCCATGGTGGCCACCATGTTCTGC	PCR for restriction analysis
MALG12AS	AGGGCCAGCACATTGGGCAGTGTC	PCR for restriction analysis
HALG12*Nhe*IS	CTAGCTAGCAGGTACGTGAAGCTGAAAG	PCR and subcloning of ORF
HALG12*Xho*IAS	CCGCTCGAGAGAAACTGGTAGTGATAAC	PCR and subcloning of ORF
18 S rRNA	ACGGTATCTGATCGTCTTCGAA	Hybridization, Northern blot

[a] S, sense; AS, antisense.

PP-dolichyl α3-mannosyltransferase (24); Ie, dolichol-P-Man synthase I (25, 26); and If, the MPDU1 gene product of unknown function, (27, 28). CDG I cases for which the genetic origins remain unknown are defined as CDG subtype Ix. 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:$Man_7GlcNAc_2$-PP-dolichyl α6-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.

Northern 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 complementary oligonucleotide (33).

Western Blot and Isoelectric Focusing—Western blotting and isoelectric focusing of human serum proteins were performed as described previously using a rabbit polyclonal anti-transferrin antibody (34).

Cells, Cell Culture, and Metabolic Radiolabeling of Cells—Skin biopsy fibroblasts were grown in Dulbecco's modified Eagle's medium containing 2 g/liter glucose, 10% fetal calf serum, and 1% penicillin/streptomycin. For some experiments, immortalized fibroblasts were used and were transformed by Dr. Thierry Levade (INSERM U466), using the pAS plasmid encoding the simian virus 40 large T antigen (35). 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 as described above. Fibroblasts in confluent 25-cm^2 tissue culture flasks or EBV-transformed lymphoblasts were pulse-radiolabeled for 30 min with 100 μCi of 2-[^{3}H]mannose (23.9 Ci/mmol, PerkinElmer Life Sciences) or 100 μCi of 6-[^{3}H]galactose (29.5 Ci/mmol, PerkinElmer Life Sciences) in 1 ml of 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.

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 $CHCl_3$/MeOH/H_2O, 10:10:3. The lower and 10:10:3 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/H_2O, 3:3:2 for 36 h (38) and detected by fluorography after spraying the dried TLC plates with En^{3}hance® (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 M methyl α-D-mannopyranoside (Toronto Research Chemicals Inc., ON, Canada) was heated to 60 °C prior to use (40). The standard *N*-linked $Man_7GlcNAc$ 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-[^{3}H]mannose in the presence of CST and kifunensin (39).

Reduced oligosaccharides were subjected to acetolysis (41), and the resulting fragments were analyzed as described previously (42) by TLC on cellulose-coated plastic sheets (Merck). The *N*-linked $Man_8GlcNAc$ and $Man_9GlcNAc$ structures used to generate standard acetolysis fragments were obtained as described above except that the latter structure was derived from normal human skin biopsy fibroblasts.

Mutation Analysis—First strand 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, France) prior to automated sequencing. The PCR products obtained with primer couple MALG12S/MALG12AS (see Table I) were submitted to *Acc*I (Ozyme, Saint Quentin en Yvelines, France) restriction digestion followed by electrophoresis in a 6% NuSieve agarose gel (FMC BioProducts, Rockland, ME). Parental mRNA was extracted by Trizol from peripheral blood mononuclear cells isolated using a Ficoll-Paque Plus gradient. Trizol and Ficoll-Paque were used according to the manufacturer's instructions.

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. 5*A*). This so-called self-inactivating (SIN) integrated proviral vector is thus able to drive transcription from its long terminal repeat and therefore prevents promoter interference and oncogene 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 polypurine 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 Titrations—Lentiviral SIN.PW.hPGK/eGFP and SIN.PW.hALG12.IRES2.eGFP transfer vector particles were separately produced by cotransfection of their respective transfer plasmids into human kidney 293T cells along with the packaging and envelope constructs. The packaging plasmid (pCMVΔR8.74) provided *gag*, *pol*, *tat*, and *rev* helper viral genes under the control of the human cytomegalovirus promoter. The envelop 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—3×10^6 immortalized fibroblast cells originating from patient ME were transduced with SIN.PW.hPGK/eGFP or SIN.PW.hALG12.IRES2.eGFP transfer vectors at 10 or 100 multiplicities of infection. 2-[^{3}H]Mannose 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 suckling. 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. 1*A*, 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 reduced in CDG types Ia and Ib, respectively, were normal in ME fibroblasts (results not shown).

Accumulation of $Man_7GlcNAc_2$-PP-dolichol in ME Fibroblasts—In order to understand the cause of glycoprotein hypoglycosylation in patient ME, skin biopsy fibroblasts were metabolically pulse-radiolabeled with 2-[^{3}H]mannose and then extracted with organic solvents to yield $CHCl_3$ and $CHCl_3$/MeOH/H_2O (10:10:3) lipid fractions. Oligosaccharides were released from LLO present in both these fractions and resolved by TLC as shown in Fig. 1*B*. Control fibroblasts yielded predominantly mature triglucosylated LLO in the 10:10:3 lipid fraction, whereas less mature species such as $Man_9GlcNAc_2$-PP-dolichol and $Man_5GlcNAc_2$-PP-dolichol were also apparent in the $CHCl_3$ fraction. By contrast, ME fibroblasts elaborated much less $Glc_3Man_9GlcNAc_2$-PP-dolichol in both the 10:10:3 and $CHCl_3$ fractions; however, the latter fraction contained substantial amounts of an LLO whose oligosaccharide moiety migrated between $Man_9GlcNAc_2$ and $Man_5GlcNAc_2$ (labeled "*a*" in Fig. 1*B*). After treatment with endo H, as shown in Fig. 1*C*, component *a* yielded a product that migrated slightly slower than a $Man_7GlcNAc$ structure that was generated by endo H treatment of 2-[^{3}H]mannose-labeled glycoprotein material derived from HepG2 cells. However, ConA-Sepharose chromatography of oligosaccharide *a* and HepG2 glycoprotein-derived $Man_7GlcNAc$ demonstrated these two structures to be quite different (see Ref. 46 and Fig. 2*A*). 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 mannose of the $Man_3GlcNAc$ core is substituted with an α3-linked mannose residue. Acetolysis, a reaction that specifically cleaves α6-linkages of oligosaccharide *a* (shown in Fig. 2*B*), confirmed this and demonstrated that its mannose substituents possess an isomeric configuration identical to that which is known to occur in $Man_7GlcNAc_2$-PP-dolichol under normal physiological conditions (oligosaccharide structure shown to the *right* in Fig. 2*B* (2, 46)).

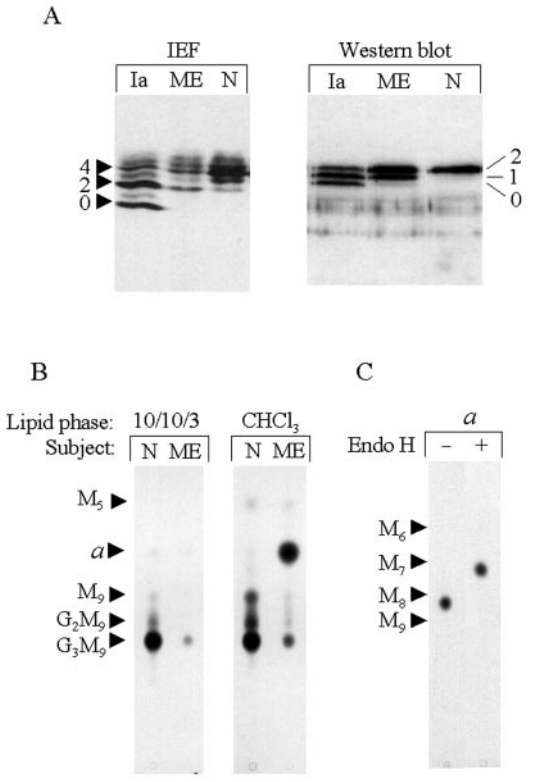

FIG. 1. **Analysis of serum glycoproteins and LLO biosynthesis in patient ME.** *A*, plasma samples from a normal subject (*N*), a CDG Ia patient (*Ia*), and patient ME (*ME*) were subjected to the following: *left panel*, isoelectric focusing (*IEF*), anode is at the *top* and the *numbers* to the *left* refer to the number of negative charges; *right panel*, SDS-PAGE followed by Western blot analysis, the number of carbohydrate chains are indicated to the *right* of the gel. *B*, after pulse radiolabeling with 2-[^{3}H]mannose, fibroblasts were extracted with organic solvents, and LLO from two solvent phases ($CHCl_3$ and 10:10:3) were subjected to mild acid hydrolysis. The liberated oligosaccharide components were resolved by TLC. The migration positions of standard oligosaccharides are shown to the *left* of the chromatogram. The radioactive material labeled *a* was eluted from the plate and further characterized. Radioactive oligosaccharides were detected by fluorography as described under "Experimental Procedures." The abbreviations used are: M_5, $Man_5GlcNAc_2$; M_9, $Man_9GlcNAc_2$; G_2M_9, $Glc_2Man_9GlcNAc_2$; G_3M_9, $Glc_3Man_9GlcNAc_2$. *C*, oligosaccharide *a* was subjected to endo H digestion and then rechromatographed. The migration positions of endo H-released oligosaccharides derived from metabolically radiolabeled HepG2 cell glycoproteins are shown to the *left* of the chromatogram. The abbreviation used is: M_{9-6}, $Man_{9-6}GlcNAc$.

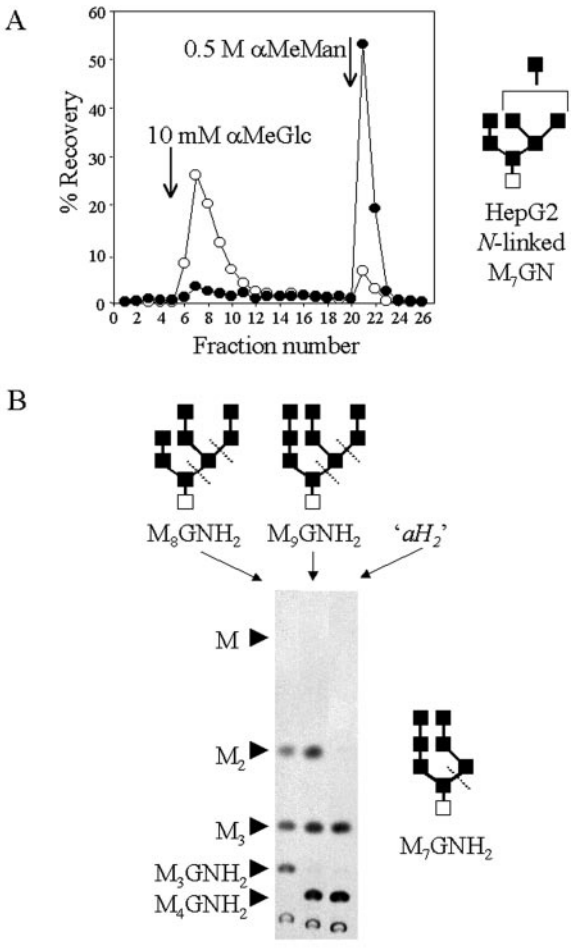

FIG. 2. **Characterization of oligosaccharide *a*.** *A*, endo H-digested oligosaccharide *a* (*open symbols*) was subjected to ConA-Sepharose chromatography; similarly, an aliquot of $Man_7GlcNAc$ (structure indicated to the *right* of the panel) released by endo H from HepG2 glycoproteins (*closed symbols*) was also analyzed. *B*, the endo H-digested oligosaccharide *a* was reduced with $NaBH_4$ (aH_2) prior to being subjected to acetolysis. The acetolysis fragments were resolved by TLC on cellulose-coated plates. The migration positions of standard oligosaccharides, generated from the acetolysis of $Man_9GlcNAcH_2$ and $Man_8GlcNAcH_2$, are shown to the *left* of the chromatogram (structures are represented *above* the lanes, and *dotted lines* indicate the α6-linkages preferentially cleaved during the acetolysis reaction: *closed squares*; mannose, *open squares*; $GlcNAcH_2$). 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_8GNH_2, $Man_8GlcNAcH_2$; M_9GNH_2, $Man_9GlcNAcH_2$; *M*, mannose; M_2, mannobiose; M_3, mannotriose; M_3GNH_2, $Man_3GlcNAcH_2$; M_4GNH_2, $Man_4GlcNAcH_2$.

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:$Man_7GlcNAc_2$-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*X*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.[2] 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. 4*A*) revealed only a single *ALG12* 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 *Acc*I that allowed us to confirm that the patient ME is homozygous and that the parents are both heterozygous for this mutation (see Fig. 4*B*). 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 *Acc*I 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. 5*A* 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. 5*B* 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 ($Glc_3Man_9GlcNAc_2$-PP-dolichyl) 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 $Man_7GlcNAc$ 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

[2] Oriol, R., Martinez-Duncker, I., Chantret, I., Mollicone, R., and Codogno, P. (2002) *Mol. Biol. Evol.*, in press.

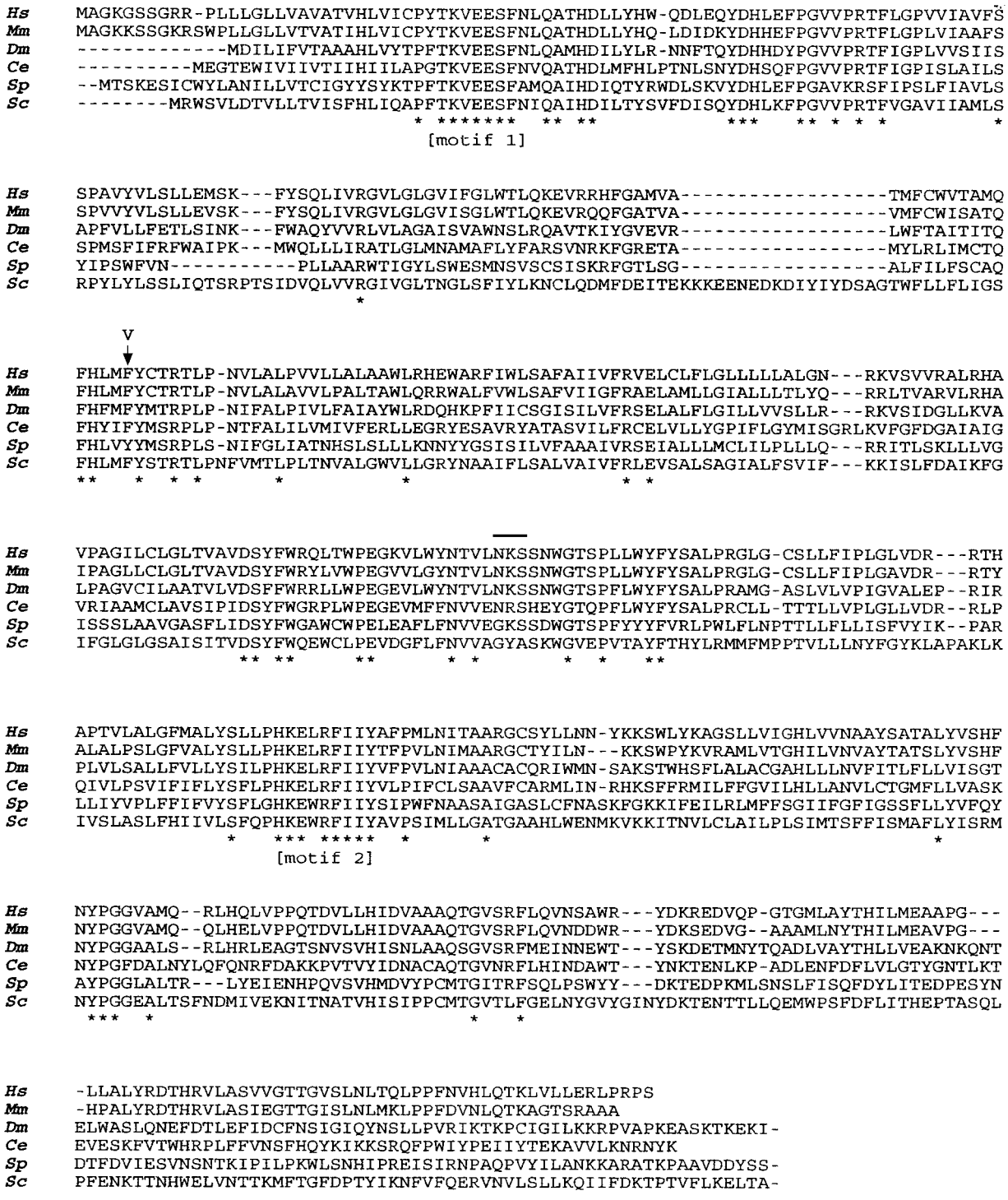

FIG. 3. **The human (*Hs*) *ALG12* peptide sequence corresponding to the ORF of the nucleotide sequence reported here (GenBank™ accession number AJ303120) is shown aligned with its *Mus musculus* (*Mm*, SWALL accession number CAD22101), *Drosophila melanogaster* (*Dm*, SPTREMBL accession number Q9VH78), *Caenorhabditis elegans* (*Ce*, 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.

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. 6*A*) shows that control fibroblasts yield predominantly $Man_9GlcNAc$ 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 $Glc_1Man_7GlcNAc$ and $Man_7GlcNAc$ 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 $Man_7GlcNAc$ intermediate commonly found associated with glycoproteins. Triglucosylated oligosaccharides are more efficiently transferred from dolichol onto polypep-

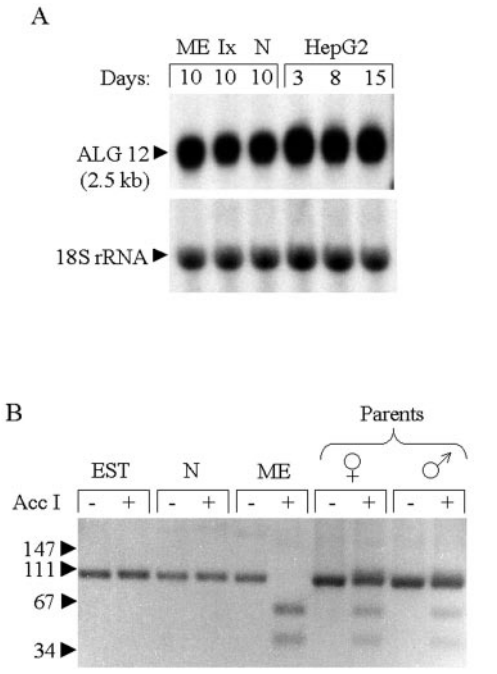

FIG. 4. **Expression and genetic analysis of *hALG12* alleles.** *A*, Northern blot analysis of total RNA from control, CDG Ix, and ME fibroblasts. Total RNA was extracted from pre-confluent ME, CDG Ix, and normal fibroblasts and from HepG2 hepatocellular carcinoma cells at different days of culture. The radiolabeled probe was generated by ^{32}P labeling of the double digestion (*Sal*I and *Not*I) fragment generated from the EST. 18 S rRNA was visualized as a gel loading control. *B*, restriction digestion of PCR products generated from ME and parental cDNA. The PCR products obtained from the EST template or from the cDNA templates derived from normal (*N*) or ME fibroblasts (*ME*) or from parental lymphocytes were incubated either in the absence (−) or presence (+) of the *Acc*I restriction enzyme.

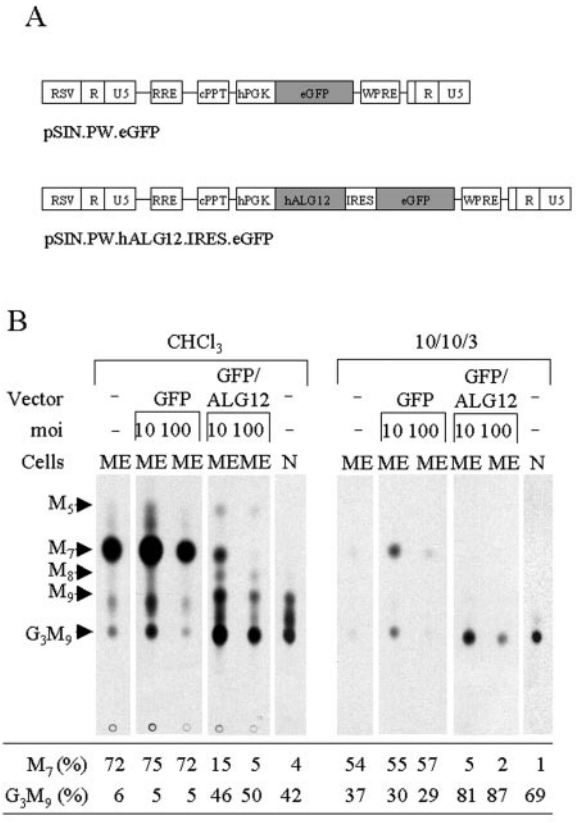

FIG. 5. **Transduction of ME fibroblasts with an HIV-1-derived lentiviral vector harboring wild type *hALG12*.** *A*, HIV-1-derived lentiviral vectors containing DNA encoding enhanced GFP (*eGFP*) alone or both eGFP and *hALG12* DNA in a bicistronic arrangement were constructed as described under "Experimental Procedures" (abbreviations are defined under "Experimental Procedures"). Fibroblasts from patient ME were immortalized and subsequently transduced with either 10 or 100 multiplicities of infection (*moi*) of each of the vector particles. 48 h after transduction the cells were radiolabeled. *B*, after mild acid hydrolysis LLO was resolved by TLC, and after elution and scintillation counting of the radioactive components, the percentages of total structures occurring as $Man_7GlcNAc_2$ or $Glc_3Man_9GlcNAc_2$ were computed and are indicated *below* each lane. LLOs derived from a representative pulse radiolabeling of normal fibroblasts (*N*) are also shown. The abbreviations are as for Fig. 1*B*.

tides; however, the direct transfer of $Man_7GlcNAc_2$ 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 $Man_{9-7}GlcNAc$ species and reduced the amount of $Man_7GlcNAc$, it did not lead to the disappearance of *N*-linked $Glc_1Man_7GlcNAc$. When similar 30-min pulse radiolabeling experiments were conducted in the presence of CST and the Golgi mannosidase I inhibitor, kifunensin, there was little change to the patterns of *N*-linked structures observed in either cell population (results not shown). The inability of CST to inhibit the appearance of $Glc_{0-1}Man_7GlcNAc$ species in ME cells demonstrates that $Man_7GlcNAc_2$ can be transferred from dolichol onto protein. The inability of CST to inhibit the generation of *N*-linked $Glc_1Man_7GlcNAc$ in ME fibroblasts can be accounted for by UDP-glucose:glycoprotein glucosyltransferase-mediated monoglucosylation of $Man_7GlcNAc_2$ *N*-linked to ER-situated misfolded glycoproteins (3). We tested this hypothesis by pretreating cells with the protein synthesis inhibitor, cycloheximide, and tunicamycin, an inhibitor of LLO biosynthesis (49). Under these conditions, as shown in Fig. 6*B*, 2-[^{3}H]mannose is not incorporated into newly synthesized glycoproteins. By contrast, when cells are incubated with [^{3}H]galactose, which is incorporated into UDP-glucose via the action of UDP-glucose epimerase on UDP-galactose, oligosaccharides released from glycoproteins by endo H are radiolabeled irrespective of the presence or absence of the two inhibitors. These results are compatible 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 $Glc_1Man_{9-7}GlcNAc$ (Fig. 6*B*). Although ME fibroblasts also generated the same $Glc_1Man_{9-8}GlcNAc$ species, two additional reglucosylated *N*-linked structures were found. A $Glc_1Man_7GlcNAc$ structure was observed that, in contrast to its counterpart observed in [^{3}H]galactose-labeled normal fibroblasts, comigrated with the $Glc_1Man_7GlcNAc$ observed in 2-[^{3}H]mannose-labeled ME fibroblasts. In addition, a species that migrated as $Glc_1Man_6GlcNAc$ was observed in [^{3}H]galactose-labeled ME fibroblasts (Fig. 6*B*).

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; $Glc_3Man_9GlcNAc$, $Glc_3Man_7GlcNAc$, and $Man_7GlcNAc$. Furthermore, the *N*-linked $Man_7GlcNAc$ structure that is observed in the cells from patient ME can be glucosylated by UDP-glucose:glycoprotein glucosyltransferase.

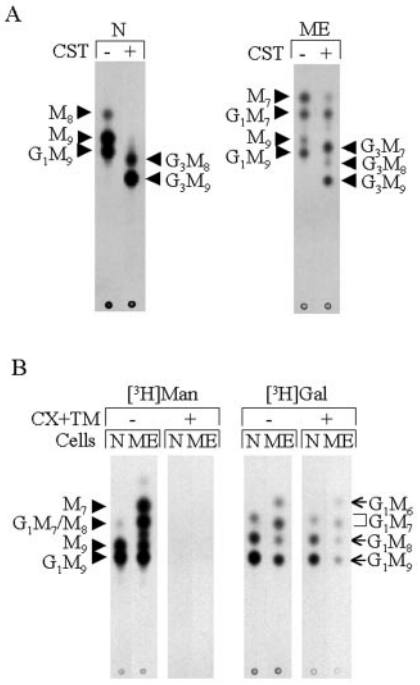

FIG. 6. **Truncated oligosaccharides are transferred onto glycoproteins in ME fibroblasts.** *A*, normal (*N*) and CDG I (*ME*) cells were radiolabeled in either the absence or presence of castanospermine (*CST*). Pronase-digested cellular glycoproteins were incubated with endo H. The resulting oligosaccharides were resolved by TLC. The migration positions of the standard oligosaccharides, M_8, $Man_8GlcNAc$; M_9, $Man_9GlcNAc$; G_1M_9, $Glc_1Man_9GlcNAc$; G_3M_9, $Glc_3Man_9GlcNAc$; G_3M_8, $Glc_3Man_8GlcNAc$, are shown. The species, labeled M_7, $Man_7GlcNAc$; G_1M_7, $Glc_1Man_7GlcNAc$; G_3M_7, $Glc_3Man_7GlcNAc$, were assigned structures based on their behavior during both TLC and ConA-Sepharose chromatography and upon examining their susceptibility to acetolysis. *B*, where appropriate, EBV-transformed lymphoblasts were pretreated with cycloheximide (*CX*) and tunicamycin (*TM*) prior to radiolabeling with either [^{3}H]galactose or 2-[^{3}H]mannose. The migration positions of the 2-[^{3}H]mannose-labeled species are indicated to the *left* by *solid arrowheads*, and the *arrows* to the *right* indicate the migration positions of the [^{3}H]galactose-labeled components.

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 $Man_7GlcNAc_2$ that was found to accumulate in fibroblasts from this patient revealed the same isomeric 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 to the growing oligosaccharide-lipid (50), an abnormal $Man_7GlcNAc_2$ 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:$Man_7GlcNAc_2$-PP-dolichyl α6-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:$Man_5GlcNAc_2$-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 (GenBank™ accession number BC001729). Interestingly, we noted the presence of a consensus glycosylation site (N(K/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. 5*B*), 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 $Man_7GlcNAc_2$-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 NH_2 terminus of ALG12p. In fact, in all species for which sequence data are available, we noted that the position of the replaced Phe residue in the ALG12p is invariably occupied by the aromatic amino acids Phe or Tyr (*Schizosaccharomyces pombe*) and that this position is next to a completely conserved Tyr residue. At present the role of this 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.[2] 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 $Man_7GlcNAc_2$-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 $Glc_3Man_9GlcNAc_2$ from dolichol onto nascent polypeptides in the fibroblasts of the patient. We also observed that *N*-linked $Glc_3Man_7GlcNAc_2$ and $Man_7GlcNAc_2$ 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

polypeptide suggests the following: first, the lipid-linked $Man_7GlcNAc_2$ is poorly glucosylated; second, the small amount of lipid-linked $Glc_3Man_7GlcNAc_2$ that is formed can be more efficiently transferred to protein than its non-glucosylated counterpart. The observation that substantial amounts of non-glucosylated $Man_7GlcNAc_2$ can be transferred from lipid onto protein was surprising considering that human skin biopsy fibroblasts deficient in dolichol-P-Glc:$Man_9GlcNAc_2$-PP-dolichol transferase do not appear to be able to transfer $Man_9GlcNAc_2$ 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 $Man_7GlcNAc_2$ 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 $Man_7GlcNAc_2$ 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" isomers of $Man_7GlcNAc$ (generated by ER mannosidases) that are substrates for UDP-glucose:glycoprotein glucosyltransferase have only 15% of the acceptor activity of $Man_9GlcNAc$ (54). However, we show here that the "biosynthetic" $Man_7GlcNAc$ structure that is transferred directly from dolichol onto protein in cells from patient ME can be glucosylated 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 $Man_7GlcNAc_2$-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 22q13.3 contains the *ALG12* gene.

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Congenital Disorders of Glycosylation Type Ig Is Defined by a Deficiency in Dolichyl-P-mannose:Man $_7$GlcNAc$_2$-PP-dolichyl Mannosyltransferase

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