Guanosine diphosphate-mannose: $GlcNAc_2$-PP-dolichol mannosyltransferase deficiency (congenital disorders of glycosylation type Ik): five new patients and seven novel mutations

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ABSTRACT

Background In type I congenital disorders of glycosylation (CDG I), proteins necessary for the biosynthesis of the lipid-linked oligosaccharide (LLO) required for protein N-glycosylation are defective. A deficiency in guanosine diphosphate-mannose: $GlcNAc_2$-PP-dolichol mannosyltransferase-1 (MT-1) causes CDG Ik (OMIM 608540), and only five patients, with severe multisystemic clinical presentations, have been described with this disease.

Objective To characterise genetic, biochemical and clinical data in five new CDG Ik cases and compare these findings with those of the five previously described patients.

Methods LLO biosynthesis was examined in skin biopsy fibroblasts, mannosyltransferases were assayed in microsomes prepared from these cells, and *ALG1*-encoding MT-1 was sequenced at the DNA and complementary DNA levels. Clinical data for the five new patients were collated.

Results Cells from five patients with non-typed CDG I revealed accumulations of $GlcNAc_2$-PP-dolichol, the second intermediate in the biosynthesis of LLO. Assay of MT-1, -2 and -3, the first three mannosyltransferases required for extension of this intermediate, demonstrated only MT-1 to be deficient. DNA sequencing of *ALG1* revealed nine different mutations, seven of which have not been previously reported. Clinical presentations are severe, with dysmorphias, CNS involvement and ocular disturbances being prevalent.

Conclusions 5 patients with CDG Ik are described, and their identification reveals that in France, this disease and CDG Ib (mannose phosphate isomerase deficiency: OMIM 602579) are the most frequently diagnosed CDG I after CDG Ia (phosphomannomutase 2 deficiency: OMIM 601785) and substantiate previous observations indicating that this disease presents at the severe end of the CDG I clinical spectrum.

Type I congenital disorders of glycosylation (CDG I) are rare autosomal recessive metabolic disorders affecting protein N-glycosylation.[1] About 1000 cases have been identified worldwide. The diseases present with multisystemic signs, and their biochemical hallmark is the presence of hypoglycosylated serum glycoproteins.[2] The underlying deficits in CDG I have been shown to affect steps in the biosynthesis of the lipid-linked oligosaccharide (LLO) precursor that is required for N-glycosylation.[3] LLO is generated by the successive additions of one residue of GlcNAc-P, one residue of GlcNAc, nine residues of mannose and three residues of glucose to dolichol phosphate to generate $Glc_3Man_9GlcNAc_2$-PP-dolichol. The oligosaccharide moiety of this LLO is transferred onto nascent polypeptides to yield N-glycosyl glycoproteins and dolichol pyrophosphate (dolichol-PP). The so-called dolichol cycle is then completed by the regeneration of dolichol-P from dolichol-PP.[4] Many proteins are required for dolichol recycling. So far, mutations in genes encoding 15 of them have been shown to underlie CDG I. CDG I is subtyped according to the defective protein.[5] Phosphomannomutase 2 deficiency (CDG Ia) is the commonest form of CDG I with >600 cases described worldwide, and other CDG I subtypes appear to be much rarer.[6] Identification of the molecular bases of type I CDG is important because first, it enables the generation of antenatal tests for the affected families; second, mannose phosphate isomerase deficiency (CDG Ib) is treatable; and third, the establishment of potential genotype/phenotype relationships for the different CDG I subtypes could potentially facilitate future diagnostic procedures.

Here we report that skin biopsy fibroblasts from five patients with severe type I CDG-like clinical presentations reveal abnormal accumulations of the immature LLO intermediate $GlcNAc_2$-PP-dolichol and display <10% normal guanosine diphosphate (GDP)-mannose:$GlcNAc_2$-PP-dolichol mannosyltransferase-1 (MT-1) activity. Overall, nine mutations in *ALG1*-encoding MT-1 were identified. Seven novel mutations are described, and the clinical presentations of these five MT-1-deficient (CDG Ik) patients are compared with those of the five previously described cases.[7–10]

MATERIALS, METHODS AND PATIENTS

Patients

Three girls (patients P1, P2 and P4) and two boys (patients P3 and P5) were diagnosed as having type I CDG of unknown molecular origin at the ages of 4, 16, 10 and 18 months, and 3 years and 7 months, respectively. The diagnosis was made after Western blot of serum proteins using blood samples collected onto paper as previously described.[11] After parental consent was obtained, a skin biopsy was performed on the forearm of each child. For gene studies, signed informed consent protocols were obtained from all parents.

Culture and metabolic radiolabelling of cultured skin biopsy fibroblasts

Skin biopsy fibroblasts from two control subjects and the five patients were prepared and cultivated[12] as previously described. Cells were metabolically radiolabelled for 30 min in RPMI 1640 (Invitrogen, Cergy Pontoise, France) medium containing 0.5 mM glucose and 2% dialysed fetal calf serum with either [2-^{3}H] mannose (21.5 Ci/mmol; Perkin Life Sciences, France) or [6-^{3}H] glucosamine (37.7 Ci/mmol; Perkin Life Sciences, Zaventem, Belgium).

Recovery of metabolically radiolabelled LLO

Subsequent to radiolabelling, LLOs were extracted from cells as previously described[12] except that the chloroform ($CHCl_3$) fractions from the $CHCl_3$/methanol/water extracts were pooled with the 10/10/3 fractions before analysis.

Analysis of LLOs

Lipid extracts were dried down and subjected to acid hydrolysis (20 mM HCl), and released oligosaccharides were resolved by thin layer chromatography (TLC) using either system A (silica-coated plastic sheets; (Merck KGaA, Darmstadt, Denmark) developed for 18 h in *n*-propanol:acetic acid:water, 3/2/1) or system B (cellulose-coated plastic sheets; (Merck KGaA, Darmstadt, Denmark) developed in ethyl acetate:pyridine:water: acetic acid, 5/5/3/1, for 8–18 h). Radioactive components were visualised by fluorography after spraying the chromatograms with En^{3}Hance spray (Perkin Life Sciences). Radiolabelled di-*N*-acetylchitobiose ([^{3}H]$GlcNAc_2$; BIOTREND GmbH, Cologne, Denmark) was used as a standard.

Mutation analysis

Genomic DNA was extracted from blood. RNA was isolated from fibroblasts or from fresh blood cells. Sequencing was performed with the BigDye terminator kit (Applied Biosystems, Foster City, California, USA) and analysed on an ABI PRISM 3130 sequencer (Applera, Foster City, California, USA). The *ALG1* gene (NM_019109.4) was first sequenced on genomic DNA. Primers were designed to amplify all 13 coding exons and flanking intronic sequences, with selected 3′ ends matching the correct *ALG1* sequence and not those of the *ALG1* pseudogenes. At the RNA level, primers were designed to amplify complementary DNA (cDNA) in five fragments from exon 1 to the 3′ untranslated region. Primer sequences are available on request.

To exclude common polymorphisms, 82 unrelated healthy individuals who served as control subjects were sequenced in the region of each of the missense mutations (164 alleles). The PolyPhen (http://coot.embl.de/PolyPhen), PANTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp), SIFT2 (http://blocks.fhcrc.org/sift/ SIFT) and SNPs3D (http://www.snps3d.org/) algorithms were used to evaluate the potential impact of the missense mutations on protein structure and function. The mutation nomenclature is based on the Human Genome Variation Society recommendations[13]; for cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence of the GenBank (NM) accession number, and for protein, the initiation codon is codon 1.

Microsatellite analysis

Haplotype analysis was performed on CDG Ik families with the c.773C→T (p.Ser258Leu) (n=3), c.1263G→A (p.Cys396X) (n=2) and c.826C→T (p.Arg276Trp) (n=2) mutations. Three highly polymorphic microsatellite markers close to *ALG1* were selected: 31GT (UCSC hg18: 5 045 841–5 046 102) at −0.16MB, 16GT (UCSC hg18: 5 144 906–5 145 138) at +0.67 MB and D1S3134 (UCSC hg18: 5 164 462–5 164 691) at +0.87 MB. Primer sequences were obtained from the Genome Database. Fragments were analysed on an ABI PRISM 3100 with GeneMapper v4.0 software (Applera).

Preparation of $GlcNAc_2$-PP-dolichol from the MT-1-deficient yeast strain *alg1-1*

$GlcNAc_2$-PP-dolichol was generated in the temperature-sensitive yeast strain *alg1-1* (kindly donated by Professor L Lehle, Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Regensburg, Germany) deficient in MT-1 activity.[14] After extraction and removing of organic solvents, LLOs were taken up in $CHCl_3$/methanol/water, 10/10/3, and subjected to anion exchange chromatography on DE-52 cellulose (acetate form).[15] LLO was washed twice with 2 ml H_2O, and $GlcNAc_2$-PP-dolichol was quantitated by hydrolysing the LLO with 20 mM HCl and assaying the released disaccharide using bovine galactosyltransferase and UDP-[^{14}C]galactose.[15]

Assay of MT-1 activity

Microsomes prepared from the different fibroblast lines[16] were incubated at 37°C for 20 min in 50 µl 50 mM Tris HCl, pH 8.0 containing protease inhibitors (SIGMA–Aldrich SARL, St Quentin Fallavier, France),1 µM $GlcNAc_2$-PP-dolichol, 1 µM GDP-[^{14}C]mannose (275 mCi/mmol; GE Healthcare, Aulnay Sous Bois, France), 10 mM $MgCl_2$, 0.1% Triton-X100, and 2–20 µg microsomal protein. Reactions were stopped by addition of 150 µl ice-cold H_2O, 400 µl methanol and 600 µl $CHCl_3$. After vigorous shaking, the tubes were centrifuged to generate two phases. The lower $CHCl_3$ phase was taken for scintillation counting. Radioactive components recovered from the $CHCl_3$ phase were further characterised after hydrolysis with 20 mM HCl, and released sugars were examined using TLC system B.

Assay of MT-2 and -3 activities

Microsomes generated from HepG2 cells[16] were incubated with 1 µM $GlcNAc_2$-PP-dolichol and GDP-[^{14}C]mannose for 10 min, and the resulting [^{14}C]LLO were extracted and purified as described previously then incubated with 20 µg microsomal proteins derived from a control subject (Ctrl 2) and the five patients in either the absence or presence of 20 µM GDP-Man (Sigma) as described previously. After extraction of reaction mixtures with organic solvents, radioactive components recovered from the $CHCl_3$ phase were further characterised after hydrolysis with 20 mM HCl. Released sugars were examined using TLC system A.

RESULTS

Patients and clinic

Clinical presentations of the five patients are detailed in table 1. Pregnancies associated with patients P4 and P5 were uneventful, whereas pregnancy-induced maternal hypertension developed with those associated with P2 and P3, and fetal growth was retarded for P1 and P3. At birth, neurological signs were noted: central hypotonia and psychomotor delay were present. All patients presented with at least one episode of epilepsy, ranging in severity from a unique treatable episode for patient P2 to multiple intractable seizures in patient P4. Exploration of neurological occurrences by electroencephalography revealed more or less serious abnormalities for all children except P2. Magnetic resonance imaging (MRI) revealed that P2 and P5 presented normally at the time of examination. After MRI examinations at 18 days and 18 months, patient P1 revealed a progressive cerebellar

Table 1 Comparison of the clinical presentations of five new cases of CDG Ik with those of the previously described cases

	P1	P2	P3	P4	P5		Case summaries published*
Date of birth	2001	2006	2004	2005	2001		
Age at CDG I diagnosis	4 months	1 year 4 months	1 year 6 months	10 months	3 years 7 months		
Sex	F	F	M	F	M		
Complications during pregnancy	Fetal growth retardation	Pregnancy-induced hypertension	Pregnancy-induced hypertension Fetal growth retardation	No	Fetal growth retardation	4/5	1/5
Postdelivery complications	Hypotonia	Low blood pressure Vomiting	Hypotonia Absence of ocular contact	Hypotonia	Not reported	4/5	
Feeding difficulties	Yes	Yes	No	Yes	No	3/5	
Central hypotonia	Yes	Yes	Yes	Yes	Yes	5/5	3/5
Psychomotor retardation	Yes	Yes	Yes	Yes	Yes	5/5	
Epilepsy	Multifocal epilepsy	Once, treatable	Multifocal epilepsy	Intractable seizure	Multifocal epilepsy	5/5	5/5
MRI	Evolutive cerebellar hypoplasia	Normal	Cortical atrophy Demyelination	Cortical and sub cortical atrophy	Normal	3/5	2/5
EEG	Abnormal	Normal	Abnormal	Abnormal	Abnormal	4/5	
Dysmorphias	Thin lips Small triangular chin Turned-up nose	Large cup-shaped ears Temporal narrowing of forehead Depressed nasal bridge Small upturned nose Thick lower eyelids Short neck	No	Triangular face Almond-shaped eyes	Thin lips Large forehead Large mouth Epicanthus Long smooth filtrum	4/5	4/5
Microcephaly	Yes		Yes		Yes	3/5	2/5
Ocular manifestations	Abnormal VEP† test results Poor visual contact	Normal	Abnormal VEP test results				
Partially blind	Abnormal VEP test results Absence of ocular pursuit	Abnormal VEP test results Strabismus				4/5	3/5
Fatal outcome	Yes					1/5	4/5
Maternal allele	p.Cys396X/p.Arg438Trp‡	p.Met377Val	p.Ser150Arg	p.Gly145Asp	p.Cys396X		
Paternal allele		p.Met377Val	p.Ala211_Arg247del	p.Ser258Leu	p.Arg276Trp		

CDG, congenital disorders of glycosylation; EEG, electroencephalography; F, female; M, male, MRI, magnetic resonance imaging.
*Data taken from references 7–9 and 17.
†Visually evoked potential test.
‡Deduced from messenger RNA.

hypoplasia. Patients P1, P3 and P4 presented with cortical atrophy. Finally, dysmorphias were found to be present to differing degrees in all children with microcephaly (−2.5 for P5) being noted for 4/5 patients. With the exception of P2 who presented with less severe neurological signs, it is notable that the other children presented with ocular problems ranging from simple strabismus to partial blindness. Liver and kidney functions appeared normal in all children, and when explored, haematological complications and coagulopathy were absent. Only patient P1 has died (respiratory insufficiency at 4 years and 9 months). These clinical pictures are compatible with those noted for type I CDG in which hypoglycosylation of serum glycoproteins is a hallmark.

Clinical biochemistry

Western blot analysis of the serum glycoproteins transferrin, haptoglobin, orosomucoid and α1-antitrypsin revealed the presence of hypoglycosylated glycoforms in all patients (figure 1A). In order to identify the molecular origins of this phenomenon, phosphomannomutase activity, known to be depressed in CDG Ia, the commonest CDG I subtype,[17] was measured in cell extracts from these patients and in all cases found to be normal.

Metabolic radiolabelling of LLOs

In order to identify potential blocks in the biosynthesis of the LLO precursor required for N-glycosylation, skin biopsy fibroblasts derived from the five patients and two control subjects were first metabolically radiolabelled with [2-^{3}H]mannose. The use of this radioisotope allows detection of all LLO intermediates containing mannose ($Man_1GlcNAc_2$-PP-dolichol–$Glc_3Man_9GlcNAc_2$-PP-dolichol). Analysis of [^{3}H-Man]oligosaccharides released from LLO by mild acid hydrolysis revealed no significant differences between the distribution of these components generated in cells from either the patients or control subjects (figure 1B, lower panel)

Blocks in the second and third steps in LLO production potentially lead to the accumulation of GlcNAc-PP-dolichol and $GlcNAc_2$-PP-dolichol, and these intermediates can be detected after metabolic radiolabelling of cells with [^{3}H]glucosamine. Examination of [^{3}H]GlcNAc-labelled oligosaccharides released from LLO by mild acid hydrolysis reveals a disaccharide that comigrates with standard di-*N*-acetylchitobiose ($GlcNAc_2$) in pathological but not normal cells (figure 1B, upper panel). An accumulation of $GlcNAc_2$-PP-dolichol has previously been shown to be indicative of a deficiency in GDP-mannose:$GlcNAc_2$-PP-dolichol MT-1 that adds the first mannose residue onto LLO.[8–10]

Mutation analysis of ALG1

MT-1 is encoded by *ALG1*, and the 13 exons and intron–exon boundaries of genomic *ALG1* were sequenced using primers designed to discriminate between this gene and its documented pseudogenes. Where appropriate, cDNA was also sequenced. Nine

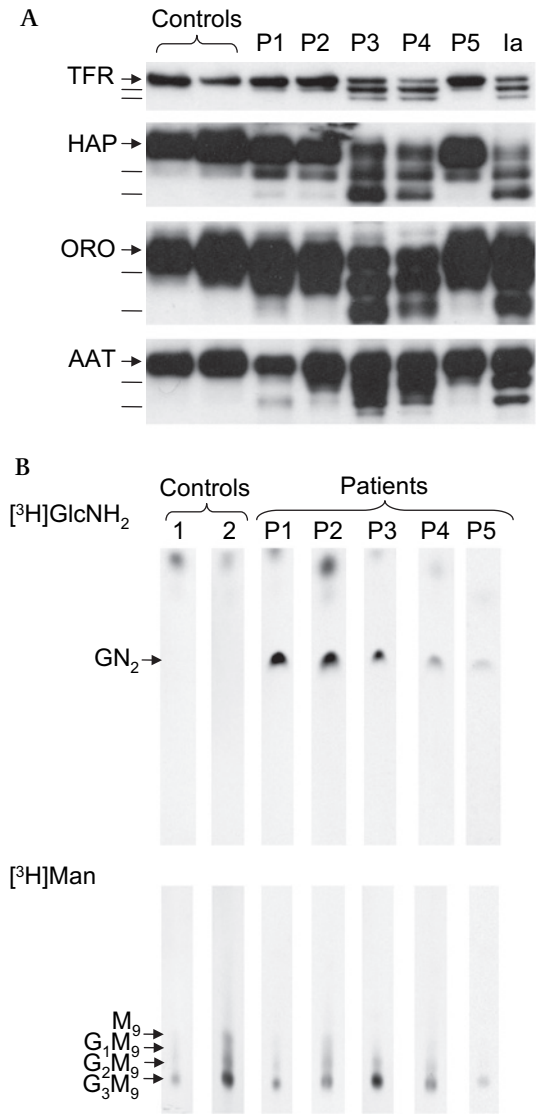

Figure 1 Serum glycoprotein hypoglycosylation and defective lipid-linked oligosaccharide (LLO) biosynthesis in five patients with type I congenital disorders of glycosylation (CDG). (A) Western blot analysis of serum proteins derived from control subjects (Controls), patients P1–5 and a patient diagnosed as having CDG Ia (Ia). Migration positions of normal transferrin (TFR), haptoglobin (HAP), orosomucoid (ORO) and alpha 1 antitrypsin (AAT) are indicated by arrowheads, whereas hypoglycosylated isoforms of these glycoproteins are indicated by lines. (B) Fibroblasts derived from control subjects 1 and 2 (different from above control subjects) and the five patients (P1–5) were radiolabelled with either [6-^{3}H]glucosamine ([^{3}H]$GlcNH_2$, upper panel) or [2-^{3}H] mannose ([^{3}H]Man, lower panel), and oligosaccharides released from LLO by mild acid hydrolysis were examined by TLC using system B (upper panel) or system A (lower panel). The radioactive components that migrate faster than di-*N*-acetylchitobiose were not further characterised. $G_{1-3}M_9$ indicates $Glc_{1-3}Man_9GlcNAc_2$; GN_2, di-N-acetylchitobiose.

mutations were encountered in the genomic DNA prepared from the five patients (figure 2 and table 1). Patient P3 harbours a previously described disease causing missense mutation c.450C→G (p.Ser150Arg[8]) and a 36-amino acid deletion (p.Ala211_Arg247del) caused by skipping of exon 6. Exon skipping is potentially caused by one or both of two mutations on the same allele: the last base of exon 6 and +5 from the donor site (c.740G→T; c.740+5G→A). Patient P4 revealed compound heterozygosity with respect to the false sense mutations c.434G→A (p.Gly145Asp) and c.773C→T (p.Ser258Leu). Whereas the latter mutation has been demonstrated to be deleterious,[8–10] the former is predicted to be deleterious to protein function by four in silico software packages that were used to evaluate the impact of the missense mutations on protein structure and function (see supplementary table). In addition, a synonymous mutation (c.765G→A, p.Thr255Thr: not found in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?locusId=56052)) in exon 7 was found to be associated with the maternally inherited c.434G→A (p.Gly145Asp) mutation in patient 4. Patients P1 and P5 have the same splicing mutation of the last base of exon 12 (c.1263G→A) resulting in a premature stop codon (p.Cys396X) and loss of exons 12 and 13 on one allele. In conjunction with this mutation, patients P1 and P5 harbour the c.1312C→T (p.Arg438Trp) and c.826C→T (p.Arg276Trp) mutations, respectively, on the other allele. Patient P2 is homozygous with respect to the c.1129A→G (p.Met377Val) mutation that is predicted in silico to be deleterious. Allelic inheritance was confirmed in all cases by analysis of DNA obtained from the parents or RNA in the case of patient P1.

Haplotype studies revealed a possible founder effect for the frequent p.Ser258Leu mutation observed in the three non-related families. In contrast, no specific common haplotype was associated with p.Arg276Trp and the c.1263G→A splicing mutations, that were each observed in two families (data not shown).

In vitro assays of microsomal GDP-Man mannosyltransferases

The mutations described previously are not all predicted to perturb protein function, so MT-1 activity was measured in the fibroblast cell lines. To this end, microsomes were prepared from cells from the five patients and incubated with GDP-[^{14}C]Man in either the absence or presence of $GlcNAc_2$-PP-dolichol. Microsomes derived from the control subject are able to incorporate substantial amounts of radioactivity into lipid components only when $GlcNAc_2$-PP-dolichol is added to the incubation mixtures (figure 3). By contrast, the microsome preparations generated from cells derived from the patients manifested <10% of control $GlcNAc_2$-PP-dolichol-dependent synthesis of radioactive lipid components (figure 3A,B). In order to examine the radioactive products generated by microsomes derived from the five patients under our assay conditions, LLO-derived oligosaccharides and monosaccharides were resolved by TLC as shown in figure 4A–C. When incubated with $GlcNAc_2$-PP-dolichol and GDP-[^{14}C]Man, control microsomes yielded substantial quantities of components migrating as $Man_{1-5}GlcNAc_2$ (figure 4A), consistent with the capacity of MT-1, GDP-mannose:$Man_1GlcNAc_2$-PP-dolichol MT-2 and GDP-mannose:$Man_3GlcNAc_2$-PP-dolichol MT-3 to generate [^{14}C]$Man_{1-5}GlcNAc_2$-PP-dolichol in a $GlcNAc_2$-PP-dolichol-dependent manner. Microsomes derived from cells of the five patients generate reduced amounts of all the [^{14}C]$Man_{1-5}GlcNAc_2$-PP-dolichol species (figure 4A). These results are compatible with but do not prove MT-1 deficiency. In order to rule out a general reduction in GDP-Man requiring mannosyltransferase activity in these microsome preparations, MT-2 and MT-3 that act subsequent to MT-1 in the dolichol cycle were examined (figure 4B). Microsomes were incubated with exogenously added [^{14}C]$Man_{1-2}GlcNAc_2$-PP-dolichol in either the absence or presence of GDP-Man. Results demonstrate that control and patient microsome preparations are similarly capable of elongating exogenously added [^{14}C]$Man_{1-2}GlcNAc_2$-PP-dolichol to yield predominantly [^{14}C]$Man_5GlcNAc_2$-PP-dolichol (figure 4B). The ensemble of these assays indicates that only MT-1 is defective in the five patients.

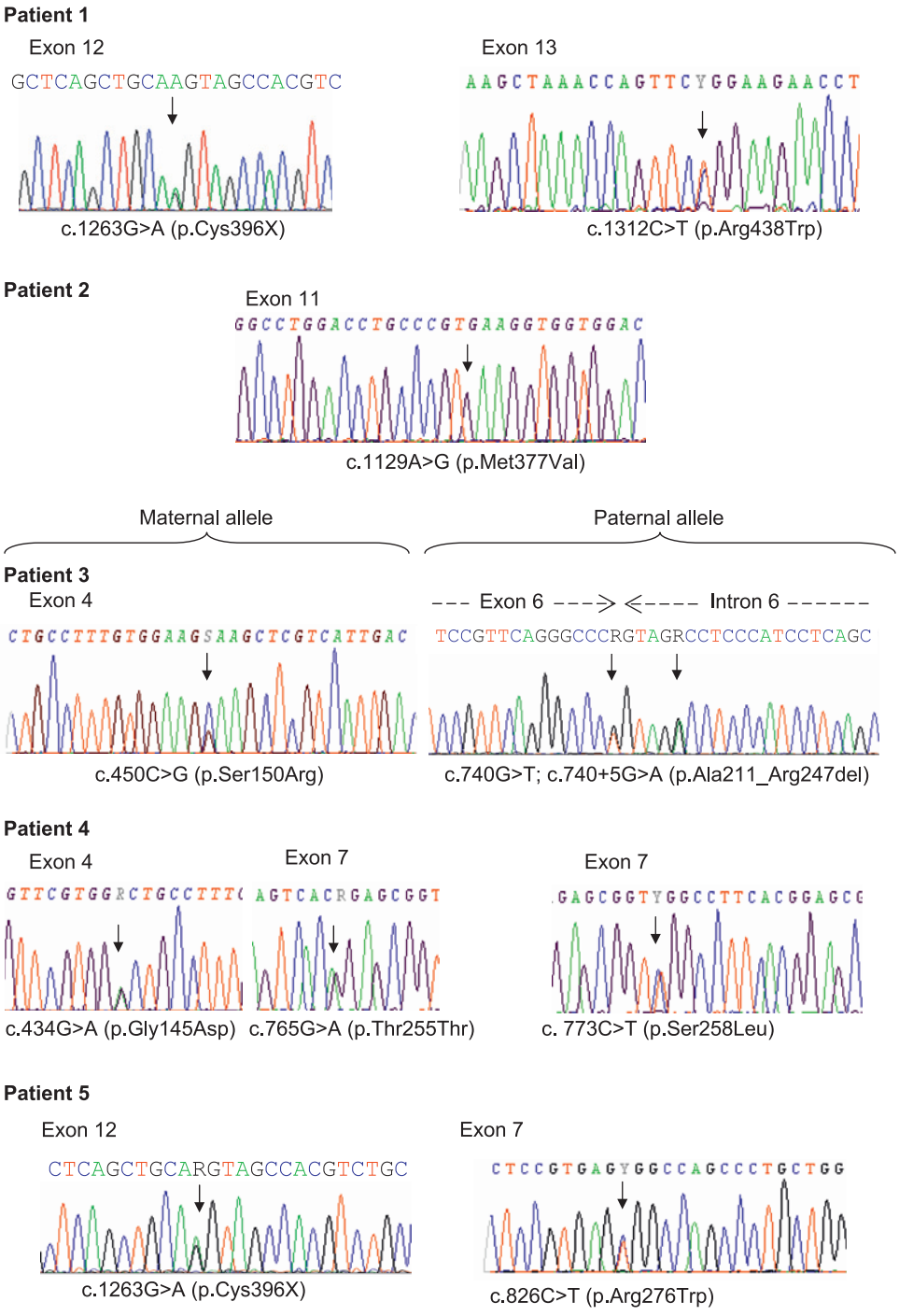

Figure 2 Mutations found in genomic DNA corresponding to the exons and intron/exon junctions of the human ALG1 gene derived from patients. Genomic and/or complementary DNA were prepared from blood leucocytes or cultured skin biopsy fibroblasts as described in Materials, methods and patients. Apart from the maternally inherited synonymous mutation in exon 7 detected in patient 4, the genotypes of patients P1–5 are given in table 1. Where possible (P2–P5), allelic inheritance is indicated.

Finally, the origin of the high $GlcNAc_2$-PP-dolichol-independent incorporation of radioactivity into lipids by microsomes originating from patient P4 (figure 3A) was investigated. Data shown in figure 4C demonstrate that these microsomes generate substantially more dolichol-P-[^{14}C]Man than the other microsome preparations, and further experiments are being conducted in order to examine the origin of this phenomenon.

DISCUSSION

Here we report upon five CDG I patients whose skin biopsy fibroblasts manifest <10% normal MT-1 activity. Sequencing of *ALG1* revealed nine mutations, two of which (p.Ser150Arg[8] and p.Ser258Leu[8–10]) have been demonstrated to be pathogenic. The c.1263G→A mutation leads to loss of exons 12 and 13 and a premature stop codon that causes a 69-amino acid truncation of the MT-1 protein. Interestingly, the underlying mutation in ALG1 of the temperature-sensitive *alg1-1 Saccharomyces cerevisiae* strain[18] also causes a C-terminal truncation, and it has been suggested that this region of the protein is important for its interactions with yeast MT-2 and MT-3.[18] One or both of two novel mutations (c.740G→A/c.740+5G→A) at the exon6/intron6 splice site junction leads to skipping of exon 6 and generation of an enzyme with a 36-amino acid deletion. This deleted region contains two amino acids (Asp 216 and Phe 222) that are conserved in yeast and mammals. Finally, four novel point mutations have been identified. The p.Met377Val mutation is predicted to be deleterious by all four of the pathogenicity software packages (supplementary table). Met 377 is conserved in yeast and mammals and lies between two similarly conserved aspartic acid residues whose substitution affects yeast MT-1

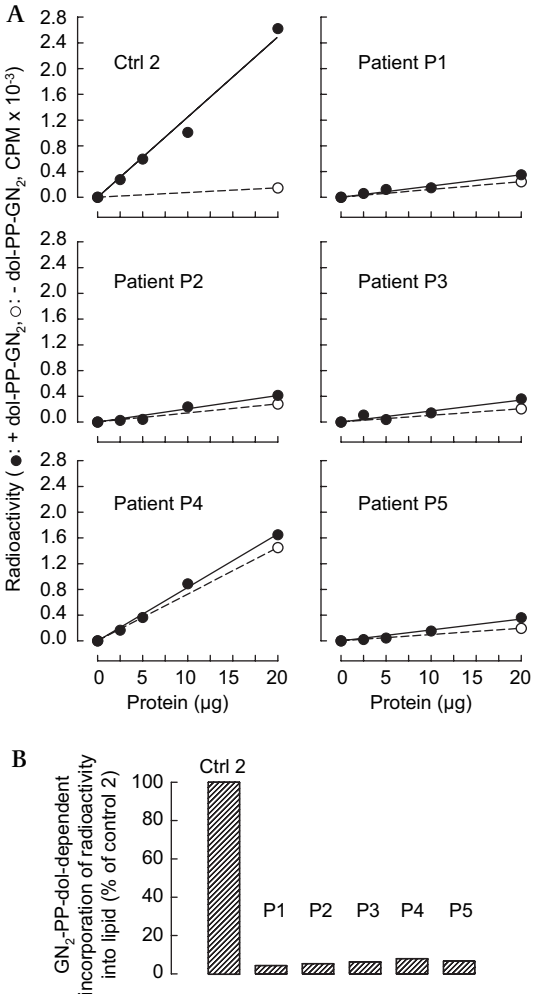

Figure 3 In vitro lipid-linked oligosaccharide (LLO) biosynthesis in microsomes derived from fibroblasts of a control subject and patients. (A) Increasing amounts of microsomes derived from control subject 2 (Ctrl 2) and the five patients (P1–5) were incubated with GDP-[^{14}C]Man in either the absence (−) or presence (+) of $GlcNAc_2$-PP-dolichol (GN_2-PP-dol) for 20 min. After cessation of the reactions by the addition of organic solvents, [^{14}C]LLO was quantitated by scintillation counting. The results were obtained from a single experiment. (B) The capacity of microsomes to incorporate radioactivity into lipid components in a $GlcNAc_2$-PP-dolichol-dependent manner was calculated from the 20 µg data point shown in A and expressed as a percentage of the control (Ctrl2).

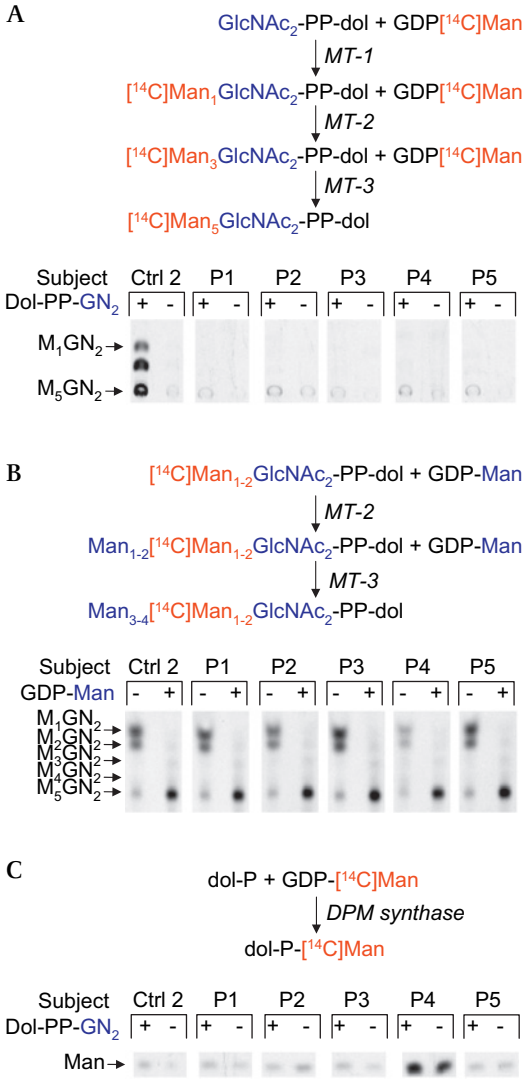

Figure 4 Further evaluation of the mannosyltransferase activities in microsomes derived from cells of patients with type 1 congenital disorders of glycosylation (CDG I). (A) Microsomal MT-1, MT-2 and MT-3 mannosyltransferases generate [^{14}C]$Man_5GlcNAc_2$-PP-dolichol using exogenously added $GlcNAc_2$-PP-dolichol and GDP-[^{14}C]Man. With the use of lipid fractions derived from the incubations described in figure 3, lipid-linked oligosaccharides (LLOs) were subjected to mild acid hydrolysis, and liberated oligosaccharides were resolved by TLC using system B. (B) To assay MT-2 and MT-3, a preparation of [^{14}C]Man-labelled LLO enriched in the MT-1 product $Man_1GlcNAc_2$-PP-dolichol was generated and incubated with the different microsome preparations in the presence or absence of unlabelled GDP-Man. Oligosaccharides liberated from LLO were resolved using TLC system A. (C) Dolichol-P-mannose synthase (*DPM synthase*) generates dolichol-P-[^{14}C]mannose (dol-P-[^{14}C]Man) from GDP-[^{14}C]Man. The different microsome preparations were incubated with GDP-[^{14}C]Man in either the absence or presence of $GlcNAc_2$-PP-dolichol (GN_2-PP-dol) as described in figure 3. The resulting radiolabelled lipid-linked sugars were examined using TLC system B. The region of the chromatogram corresponding to the migration position of mannose (Man) is shown. M_1GN_2 indicates $Man_1GlcNAc_2$; M_5GN_2, $Man_5GlcNAc_2$; $M_{1-5}GN_2$, $Man_{1-5}GlcNAc_2$.

activity.[18] Pathogenicity predictions for the p.Arg438Trp substitution are ambiguous, but Arg 438 is adjacent to the C-terminal region that, in the yeast enzyme, affects interactions with MT-2 and MT-3 as described previously. Likewise, pathogenicity predictions for the p.Arg276Trp and p.Gly145Asp substitutions are ambiguous, but these residues are situated adjacent to the previously described p.Ser258Leu and p.Ser150Arg pathogenic mutations, respectively.

Five patients with CDG Ik were described in 2004,[7–10] and because no further case reports have appeared, the five patients described here double the descriptions of patients with this disease. CDG Ik seems to be associated with a severe clinical picture.[7–10] Four of the five originally described patients died in the first year of life. Of the five patients described here, only one has died. By contrast, serious neurological complications are a constant feature with central hypotonia (4/5 in our patients and 5/5 in originally described patients), with epilepsy (5/5 and 5/5 cases, respectively) being particularly prominent. Ocular complications ranging from absence of visual contact (2/5 and 1/4) to blindness (1/5 and 1/4) are noteworthy. With the exception of microcephaly described in 3of the 5 patients described here, dysmorphias are not constant, and when present, they appear to

be variable. Recurrent infections and/or complications of the immune system were only noted in one of the presently described patients compared with 4 of the 5 originally described cases. Another notable difference between the originally described cases and those described herein is the absence of hepatic and renal complications in the latter patients. The 773 C→T (p.Ser258Leu) mutation is present in all the previously described cases, and in two, homozygosity is associated with fatal outcome. In our study, this mutation was only found in patient P4 and occurred in the heterozygous state. All other factors being equal, it might be that the p.Ser258Leu mutation may be more severe than the p.Met377Val mutation, present in the homozygous state in P2. Despite these observations, with only nine patients harbouring 11 different mutations, it is difficult to establish potential genotype/phenotype relationships. All CDG Ik descriptions include severe neurological presentations that are also observed in other CDG I subtypes with the exception of CDG Ib where only visceral hepatointestinal manifestations are observed.[19] In CDG Ih (ALG8 deficiency: OMIM 608104) and CDG Im (DK1 deficiency: OMIM 610768), neurological signs are accompanied by hepatointestinal[20] and skin/heart[21] complications, respectively.

In our French experience of CDG diagnosis, 131 families have now been demonstrated to be affected by CDG I. The majority of patients (81 families, 62%) are affected by CDG Ia, and the next commonest forms of the disease are CDG Ib and CDG Ik with eight families each, (6%), closely followed by CDG Ic (ALG6 deficiency: OMIM 603147; five families, 4%). We have also diagnosed three families as having CDG Ie (DPM1 deficiency: OMIM 603503), three families as having CDG Ig (ALG12 deficiency: OMIM 607143) and one family each as having CDG Ih and CDG Im. The 20 other families remain to be subtyped (CDG Ix: 15%). These statistics suggest that CDG Ik should be given more consideration when diagnostic strategies are prioritised according to apparent CDG I subtype frequencies.

To summarise, five patients with severe type I CDG-like clinical presentations possessing an underlying MT-1 deficiency are described. The identification of these patients reveals that in France, CDG Ik and CDG Ib are the most frequently diagnosed type I CDG after CDG Ia.

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REFERENCES

1. **Jaeken J,** Matthijs G. Congenital disorders of glycosylation: a rapidly expanding disease family. *Annu Rev Genomics Hum Genet* 2007;**8**:261—78.
2. **Freeze HH.** Congenital disorders of glycosylation: CDG-I, CDG-II, and beyond. *Curr Mol Med* 2007;**7**:389—96.
3. **Aebi M,** Hennet T. Congenital disorders of glycosylation: genetic model systems lead the way. *Trends Cell Biol* 2001;**11**:136—41.
4. **Burda P,** Aebi M. The dolichol pathway of N-linked glycosylation. *Biochim Biophys Acta* 1999;**1426**:239—57.
5. **Jaeken J,** Hennet T, Freeze HH, Matthijs G. On the nomenclature of congenital disorders of glycosylation (CDG). *J Inherit Metab Dis* 2008;**31**:669—72.
6. **Grunewald S,** Matthijs G, Jaeken J. Congenital disorders of glycosylation: a review. *Pediatr Res* 2002;**52**:618—24.
7. **de Koning TJ,** Toet M, Dorland L, de Vries LS, van den Berg IE, Duran M, Poll-The BT. Recurrent nonimmune hydrops fetalis associated with carbohydrate-deficient glycoprotein syndrome. *J Inherit Metab Dis* 1998;**21**:681—2.
8. **Grubenmann CE,** Frank CG, Hulsmeier AJ, Schollen E, Matthijs G, Mayatepek E, Berger EG, Aebi M, Hennet T. Deficiency of the first mannosylation step in the N-glycosylation pathway causes congenital disorder of glycosylation type Ik. *Hum Mol Genet* 2004;**13**:535—42.
9. **Kranz C,** Denecke J, Lehle L, Sohlbach K, Jeske S, Meinhardt F, Rossi R, Gudowius S, Marquardt T. Congenital disorder of glycosylation type Ik (CDG-Ik): a defect of mannosyltransferase I. *Am J Hum Genet* 2004;**74**:545—51.
10. **Schwarz M,** Thiel C, Lubbehusen J, Dorland B, de Koning T, von Figura K, Lehle L, Körner C. Deficiency of GDP-Man:GlcNAc2-PP-dolichol mannosyltransferase causes congenital disorder of glycosylation type Ik. *Am J Hum Genet* 2004;**74**:472—81.
11. **Seta N,** Barnier A, Hochedez F, Besnard MA, Durand G. Diagnostic value of western blotting in carbohydrate-deficient glycoprotein syndrome. *Clin Chim Acta* 1996;**254**:131—40.
12. **Chantret I,** Dupre T, Delenda C, Bucher S, Dancourt J, Barnier A, Charollais A, Heron D, Bader-Meunier B, Danos O, Seta N, Durand G, Oriol R, Codogno P, Moore SE. Congenital disorders of glycosylation type Ig is defined by a deficiency in dolichyl-P-mannose:Man7GlcNAc2-PP-dolichyl mannosyltransferase. *J Biol Chem* 2002;**277**:25815—22.
13. **den Dunnen JT,** Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 2000;**15**:7—12.
14. **Huffaker TC,** Robbins PW. Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. *J Biol Chem* 1982;**257**:3203—10.
15. **Chantret I,** Dancourt J, Barbat A, Moore SE. Two proteins homologous to the N- and C-terminal domains of the bacterial glycosyltransferase Murg are required for the second step of dolichyl-linked oligosaccharide synthesis in *Saccharomyces cerevisiae*. *J Biol Chem* 2005;**280**:9236—42.
16. **Dancourt J,** Vuillaumier-Barrot S, de Baulny HO, Sfaello I, Barnier A, le Bizec C, Dupre T, Durand G, Seta N, Moore SE. A new intronic mutation in the DPM1 gene is associated with a milder form of CDG Ie in two French siblings. *Pediatr Res* 2006;**59**:835—9.
17. **Matthijs G,** Schollen E, Pardon E, Veiga-Da-Cunha M, Jaeken J, Cassiman JJ, Van Schaftingen E. Mutations in PMM2, a phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type I syndrome (Jaeken syndrome). *Nat Genet* 1997;**16**:88—92.
18. **Gao XD,** Nishikawa A, Dean N. Physical interactions between the Alg1, Alg2, and Alg11 mannosyltransferases of the endoplasmic reticulum. *Glycobiology* 2004;**14**:559—70.
19. **de Lonlay P,** Seta N. The clinical spectrum of phosphomannose isomerase deficiency, with an evaluation of mannose treatment for CDG-Ib. *Biochim Biophys Acta* 2009;**1792**:841—3.
20. **Schollen E,** Frank CG, Keldermans L, Reyntjens R, Grubenmann CE, Clayton PT, Winchester BG, Smeitink J, Wevers RA, Aebi M, Hennet T, Matthijs G. Clinical and molecular features of three patients with congenital disorders of glycosylation type Ih (CDG-Ih) (ALG8 deficiency). *J Med Genet* 2004;**41**:550—6.
21. **Kranz C,** Jungeblut C, Denecke J, Erlekotte A, Sohlbach C, Debus V, Kehl HG, Harms E, Reith A, Reichel S, Grobe H, Hammersen G, Schwarzer U, Marquardt T. A defect in dolichol phosphate biosynthesis causes a new inherited disorder with death in early infancy. *Am J Hum Genet* 2007;**80**:433—40.

Corrections

Dupré T, Vuillaumier-Barrot S, Chantret I, *et al*. Guanosine diphosphate-mannose: GlcNAc2-PP-dolichol mannosyltransferase deficiency (congenital disorders of glycosylation type Ik): fivenew patients and seven novel mutations. *J Med Genet* 2010;47:729–35. doi:10.1136/jmedgenet-2009-072504. The third author's name has been corrected to H Sadou Yayé.

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