Deficiency of dolichyl-P-Man:$Man_7GlcNAc_2$-PP-dolichyl mannosyltransferase causes congenital disorder of glycosylation type Ig

Christian THIEL*[1], Markus SCHWARZ†[1], Martin HASILIK*, Ulrike GRIEBEN‡, Folker HANEFELD§, Ludwig LEHLE†, Kurt VON FIGURA* and Christian KÖRNER*[2]

*Georg-August-Universität zu Göttingen, Abteilung Biochemie II, Heinrich-Düker-Weg 12, D-37073 Göttingen, Germany, †Universität Regensburg, Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universitätsstrasse 31, D-93053 Regensburg, Germany, ‡Otto Heubner-Zentrum für Kinder und Jugendmedizin, SPZ, Neuropädiatrie, Campus Virchow-Klinikum, Charite, D-13344 Berlin, Germany, and §Georg-August-Universität zu Göttingen, Abt. Kinderheilkunde, Schwerpunkt Neuropädiatrie, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany

Deficiency of the endoplasmic reticulum enzyme dolichyl-phosphate mannose (Dol-P-Man):$Man_7GlcNAc_2$-PP-dolichyl mannosyltransferase leads to a new type of congenital disorder of glycosylation, designated type Ig. The patient 1 presented with a multisystemic disorder with microcephaly, developmental retardation, convulsions and dysmorphic signs. The isoelectric focusing pattern of the patient's serum transferrin showed the partial loss of complete N-glycan side chains. In skin fibroblasts from the patient, the activity of Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase was severely reduced leading to the accumulation of $Man_7GlcNAc_2$-PP-Dol, which was transferred to newly synthesized glycoproteins. Sequencing of the Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase cDNA revealed a compound heterozygosity for two point mutations, leading to the exchange of leucine^{158} for a proline residue and a premature translation stop with loss of the C-terminal 74 amino acids. The parents were heterozygous for one of the two mutations. Retroviral expression of the wild-type Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase cDNA in patient's fibroblasts normalized the mannosyltransferase activity.

Key words: inherited disorder, mannosyltransferase, microcephaly, muscular hypotonia, N-glycosylation.

INTRODUCTION

Glycosylation is one of the most common forms of co- or post-translational protein modifications and is ubiquitously found in animals, plants and bacteria. Glycoproteins are present in the cytoplasm of cells, as well as in subcellular organelles, in cellular membranes, and most abundantly in extracellular fluids and matrices. The oligosaccharides linked to glycoproteins affect the folding and the transport of glycoproteins, as well as their biological activity and stability [1]. Glycosylation of proteins involves more than a hundred glycosyltransferases, glycosidases and transport proteins. Inherited defects of glycosylation in humans comprise a rapidly growing group of multisystemic disorders termed congenital disorders of glycosylation (CDG) [2]. The clinical phenotype of these disorders is mostly associated with severe psychomotor and mental retardations. The characteristic biochemical feature of CDG is defective glycosylation of glycoproteins due to mutations in genes required for the biosynthesis of N-linked oligosaccharides. Defects of the assembly of dolichyl-linked oligosaccharides or their transfer on to nascent glycoproteins form the type I (Ia–If) forms of CDG, whereas CDG type II (IIa–IId) comprises all defects of the trimming and elongation of N-linked oligosaccharides [3]. Since 1995, the molecular nature of six CDG-I and four CDG-II types has been described [4–19].

In the present study, we describe the molecular defect in a new type of CDG (CDG-Ig), which affects the transfer of mannosyl residues from dolichyl-phosphate mannose (Dol-P-Man) to Dol-PP-$GlcNAc_2Man_7$ in the endoplasmic reticulum. The deficient enzyme Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase (accession number NP_077010) is encoded by the human orthologue of the yeast asparagine-linked glycosylation (*ALG*)*12* gene. In yeast, the *ALG12* gene product catalyses the addition of the α-1,6 mannosyl residue to Dol-PP-$GlcNAc_2Man_7$, and is therefore one of the mannosyltransferases that are critical for branching and assure the ordered assembly of Dol-PP-$GlcNAc_2Man_9Glc_3$, which serves as a substrate for the oligosaccharyltransferase complex in the endoplasmic reticulum [20].

EXPERIMENTAL

Isoelectric focusing (IEF) and SDS/PAGE of serum transferrin

IEF and SDS/PAGE of serum transferrin was carried out as described previously [6].

Cell lines and cell culture

The fibroblasts from patient 1, her parents and controls were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% fetal calf serum (FCS; PAN Biotech GmbH). The ecotropic packaging cell line ϕNX-Eco (A.T.C.C.) and the amphotropic packaging cell line RetroPack PT67 (ClonTech) were cultured in DMEM containing 10% FCS, which was heat-inactivated at 56 °C for 30 min, at 37 °C under 5% CO_2 unless otherwise stated.

Analysis of dolichol-linked oligosaccharides

Fibroblasts derived from controls and the patient were cultured and metabolically labelled with [2-^{3}H]mannose. Dolichyl- and

Abbreviations used: ALG, asparagine-linked glycosylation; CDG, congenital disorder of glycosylation; DMEM, Dulbecco's modified Eagle's medium; Dol-P-Man, dolichyl-phosphate mannose; DTT, dithiothreitol; FCS, fetal calf serum; IEF, isoelectric focusing.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail ckoerne@gwdg.de).

protein-linked oligosaccharides were extracted and analysed by HPLC as described previously [21].

Enzyme Assay

Dol-PP-GlcNAc$_2$-[^{3}H]Man$_7$ (40 c.p.m.) or Dol-PP-GlcNAc$_2$-[^{3}H]Man$_6$ (40 c.p.m.) and unlabelled Dol-P-Man (1 μM) were dried under N_2 and dispersed in 4.3 mM Na_2HPO_4/citrate buffer, pH 6.5, containing 0.25 % Nonidet P40, 10.1 mM $MnCl_2$, 1.1 mM phosphatidylcholine and 0.15 mg of particulate enzyme in a final volume of 70 μl. Incubations were carried out at 24 °C for 20 min and stopped with 1.8 ml of chloroform/water (2:1, v/v). Further processing and release of oligosaccharides from the lipid by mild acid and their analysis by HPLC was as described previously [22]. [^{3}H]-Labelled Dol-PP-oligosaccharides were isolated from Δ*alg12* and Δ*alg9* yeast, and were metabolically labelled with [^{3}H]mannose as described previously [22]. Dol-P-Man was synthesized *in vitro* as described previously [23]. For preparation of particulate enzyme, fibroblasts were resuspended in 30 mM Tris/HCl, pH 7.5, containing 35 % (v/v) glycerol, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM benzamidine, and were incubated for 15 min on ice and centrifuged at 48000 ***g*** for 20 min. The pellet was homogenized in 10 mM Tris/HCl, pH 7.5, containing 1 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine and centrifuged as above. The pellet was resuspended in 30 mM Tris/HCl, pH 7.5, 35 % glycerol, 3 mM $MgCl_2$, 1 mM DTT and was used as the enzyme source.

Mutation analysis

Total RNA was extracted from control, patient's and parents' fibroblasts and leucocytes using the RNAeasy kit (Qiagen). First-strand cDNA was synthesized from 0.5 μg of total RNA with Omniscript reverse transcriptase (Qiagen) and the primer R1 (5′-GAAACTGGTAGTGATAACAGCTC-3′). In the first round of PCR, the cDNA was amplified using the primers F1 (5′-GGGAGCTGCGGAGCATGCG-3′) and R1 using the HotStarTaq-Polymerase kit (Qiagen) with a preincubation at 95 °C for 15 min followed by 28 cycles with 1 min at 94 °C, 0.5 min at 55 °C and 3 min at 72 °C. Further amplification was carried out with the nested primers F2 (5′-GGAGTGGCAGTGCTAACGGC-3′) and R2 (5′-CCTGGTCCCCCTCAGGACG-3′). Reverse transcriptase-PCR products were run on 1 % agarose gels. The 1549-bp fragment was prepared with the QIAquick PCR purification kit (Qiagen) and subcloned into the pGEM-T-easy vector (Promega). Sequence analyses of the PCR products and the plasmids was carried out by dye-determined cycle sequencing with the primers pUC M13 forward, pUC M13 reverse (Stratagene) F2, F3 (5′-GGACCTCCCCGCTGCTGTG-3′), F4 (5′-GTGCTTTCGCTGTTAGAAATGT-3′), F5 (5′-GCGGGGTCTCTGCTTGTGAT-3′), R2 and R3 (5′-CACAGCAGCGGGGAGGTCC-3′) on an Applied Biosystems model 373A automated sequencer.

Genomic DNA was prepared from control and patient's fibroblasts, as well as from peripheral blood leucocytes of the parents, by standard procedures [24]. PCR was carried out as described above with the primers Int-6 (5′-GCCTGACGTAGCCGTCAGC-3′) and Int-7 (5′-CTCACCTCCCATGGCCAGC-3′) for the T473C mutation resulting in a 1678-bp fragment. Primers Int-14 (5′-GGTGAAGTTGAACAGCAGGTTA-3′) and Int-15 (5′-CATAACACCTGGAATAGTCACC-3′) were used for the C1442G mutation resulting in a 1476-bp fragment. The PCR products were run on a 1 % agarose gel and prepared as described above. Sequence analyses was carried out using primers Int-6 and Int-14.

Site-directed mutagenesis

A 1.5 kb fragment of a wild-type Alg12 cDNA representing the coding sequence (nt −71 to nt 1478) was amplified by PCR using primers F2 and R2. The resulting fragment was purified and cloned into pGEM-T-Easy vector (pGEM-T-Easy–wild-type). The mutation T473C was inserted into the cDNA using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with the primers Mut473-A (5′-GCCTGTAGTCCCGCTGGCCCTC-3′) and Mut473-B (5′-GAGGGCCAGCGGGACTACAGGC-3′) to obtain plasmid pGEM-T-Easy-Pat. Wild-type and patient Alg12-cDNA was subcloned into the MoMuLV-derived vector pLNCX2 (Clon-Tech).

Retroviral complementation

Ecotropic ϕNX-Eco cells (0.5×10^6) were seeded on to dishes (60-mm diameter) 1 day before transfection. Transient transfection by FuGENE6 reagent was carried out according to the manufacturer's protocol (Roche) with 1 μg of LNCX2–vector (mock), LNCX2–wild-type and LNCX2–patient. Further procedures were carried out as described previously [17]. The supernatant with the amphotropic retroviral particles was used to transfect patient's and control fibroblasts. After (24 h) the final infection of the fibroblasts the medium was replaced by DMEM/10 % heat-inactivated FCS with Geneticin (335 μg/ml; Gibco BRL). Selection was carried out for 10 days.

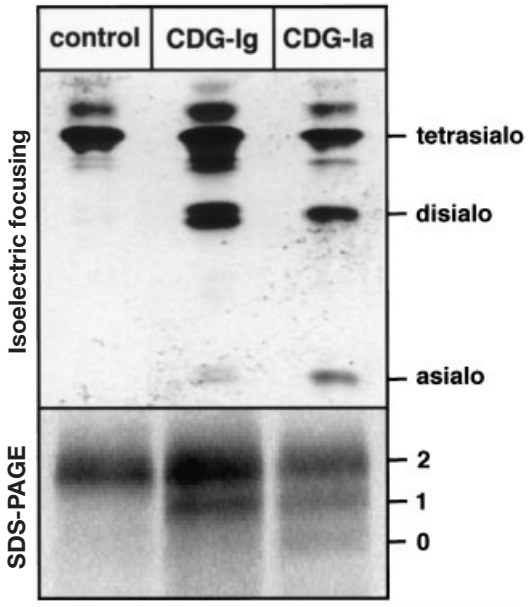


Figure 1 Serum transferrin pattern by IEF and SDS/PAGE

Serum samples from a control, a CDG-Ia reference patient and patient 1 (CDG-Ig) were investigated by IEF, followed by in gel immunodetection of transferrin. Asialo, disialo and tetrasialo indicate transferrin forms carrying either 0, 2 or 4 sialic acid residues (upper panel). Lower panel shows serum samples from a control, a CDG-Ia reference patient and the patient (CDG-Ig) analysed by SDS/PAGE, followed by Western blotting and immunodetection of transferrin. 0, 1 and 2 indicate transferrin forms carrying 0, 1 or 2 N-glycans.

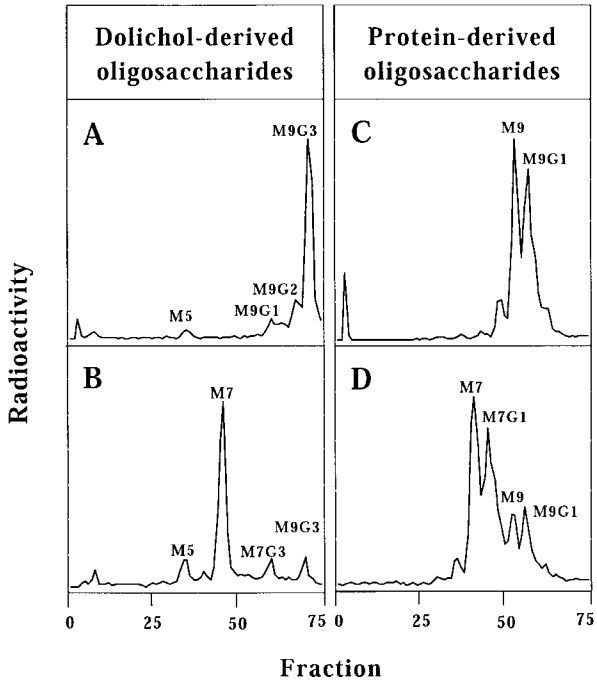


Figure 2 HPLC analysis of dolichyl- and glycoprotein-derived oligosaccharides

Fibroblasts from a control (**A** and **C**) and the patient (**B** and **D**) were metabolically labelled with [^{3}H]mannose for 30 min. For determination of dolichyl-linked oligosaccharides, [^{3}H]-labelled oligosaccharides were released by mild-acid hydrolysis (**A** and **B**). Glycoprotein-bound [^{3}H]-labelled oligosaccharides were released by peptide N-glycosidase F digestion (**C** and **D**). Further size fractionation of [^{3}H]-labelled oligosaccharides was carried out by HPLC followed by liquid scintillation counting of the fractions. The positions of the $GlcNAc_2Man_{5,7,9}$ (M_5, M_7 and M_9) and the $GlcNAc_2Man_9Glc_{1,2,3}$ (M_9G_1, M_9G_2 and M_9G_3) standards are indicated.

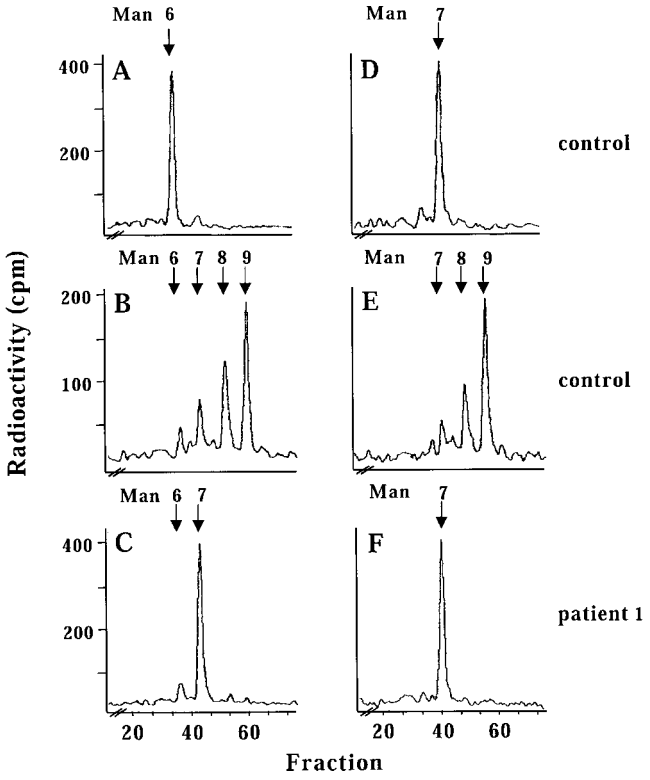


Figure 3 Dolichyl-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase activity

The transfer of mannose residues to Dol-PP-$GlcNAc_2Man_6$ was carried out by incubation of membrane extracts from fibroblasts of controls (**A** and **B**) and the patient (**C**) for 30 min with Dol-PP-$GlcNAc_2$-[^{3}H]Man_6 as acceptor in the absence (**A**) or presence (**B** and **C**) of Dol-P-Man as a donor substrate. Dolichyl-linked oligosaccharides were subsequently extracted. The oligosaccharides moieties were released by mild-acid hydrolysis and size fractionated by HPLC. The positions of $GlcNAc_2Man_{6-9}$ (M_6, M_7, M_8 and M_9) standards are indicated. The transfer of mannose residues to Dol-PP-$GlcNAc_2Man_7$ was carried out by incubation of membrane extracts from fibroblasts of controls (**D** and **E**) and the patient (**F**) for 30 min with Dol-PP-$GlcNAc_2$-[^{3}H]Man_7 as acceptor in the absence (**D**) or presence (**E** and **F**) of Dol-P-Man as donor substrate. Further analysis was proceeded as described above. The positions of $GlcNAc_2Man_{7-9}$ (M_7, M_8 and M_9) standards are indicated.

RESULTS

Clinical phenotype

Patient 1 is a now six-year-old girl of Indian origin, who was born naturally in the 34th week of pregnancy without severe complications. Further mental and motor development of the child was delayed. At the age of 14 months the patient started suffering from convulsions, which were successfully treated with valproic acid (2×150 mg/day) at the age of 4 years. At the age of 3.5 years, the patient presented with a microcephaly, muscular hypotonia, prolonged partial thromboplastin time, supragluteal fat pats and facial dysmorphisms, such as dysplastic ears and a short filtrum. Magnetic resonance spectroscopy revealed a widening of the side ventricles, without giving a hint of a hydrocephalus internus or increased brain pressure.

Diagnosis

Diagnosis of CDG was established by IEF and SDS/PAGE of serum transferrin. In serum from controls, the most abundant form of transferrin carries four sialic acid residues. The IEF pattern of serum transferrin from the CDG-Ig patient showed the presence of transferrin molecules with four, two or no sialic acid residues (Figure 1, upper panel). Such an IEF pattern is typical for CDG-Ia, where transferrin lacks either one or both of its two N-linked glycans each carrying two sialic acid residues [25]. The partial loss of complete N-glycans in CDG-Ig was demonstrated by SDS/PAGE (Figure 1, lower panel) where transferrin from the CDG-Ig patient, as well as from a CDG-Ia patient, showed additional bands with faster mobility, which is compatible with the loss of either one or two glycan chains. The underglycosylation of transferrin from the CDG-Ig patient was less pronounced than in that from the CDG-Ia patient.

HPLC analysis of dolichyl- and protein-derived oligosaccharides

After metabolic labelling of cultured control and patient fibroblasts with 2[^{3}H]mannose for 30 min, oligosaccharides were released from the dolichyl moiety by mild-acid hydrolysis and were size fractionated by HPLC. In control fibroblasts, the major fraction corresponded to a $GlcNAc_2Man_9Glc_3$ standard, whereas the two minor fractions corresponded to $GlcNAc_2Man_9Glc_1$ and $GlcNAc_2Man_9Glc_2$ standards (Figure 2A). In the patient-derived fibroblasts about 95% of the oligosaccharides showed the size of a $GlcNAc_2Man_7$ standard and only a minor fraction of 5% showed a size of $GlcNAc_2Man_9Glc_3$ (Figure 2B), therefore indicating the leaky nature of the deficiency. Trace amounts of $GlcNAc_2Man_5$ were detectable in control and patient cells. An additional peak in the case of the patient's dolichyl-derived oligosaccharides probably corresponds to $GlcNAc_2Man_7Glc_3$ (no standard available).

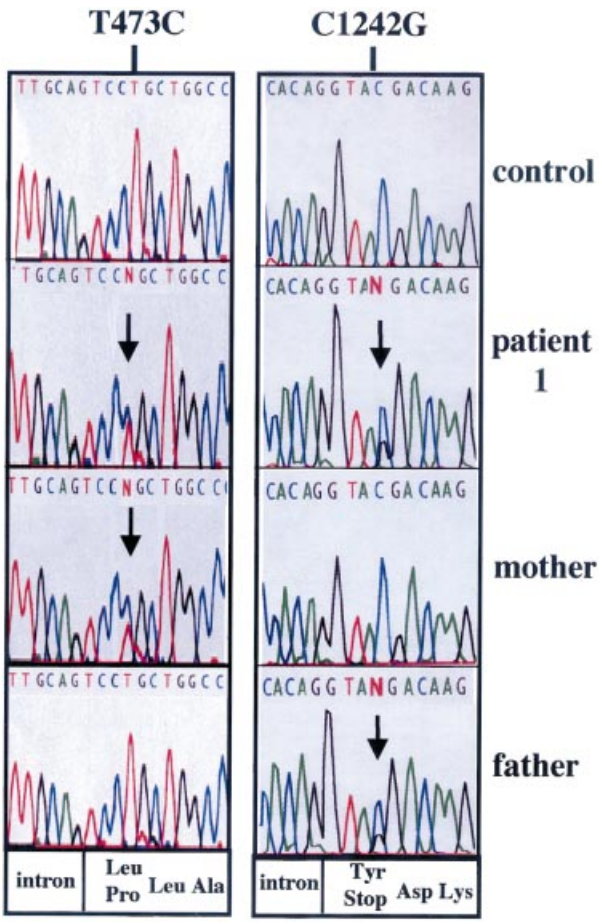


Figure 4 Mutation analysis of genomic DNA

Sequence alignment of PCR-amplified genomic DNA fragments of a healthy control, patient 1 who is compound heterozygous for a T473C transition, leading to substitution of leucine158 with proline, and a C1242G transversion, which changes Tyr414 to a stop codon leading to the loss of the C-terminal 74 amino acids, and the parents, which are heterozygous for the T473C transition (mother) and the C1242G transversion (father).

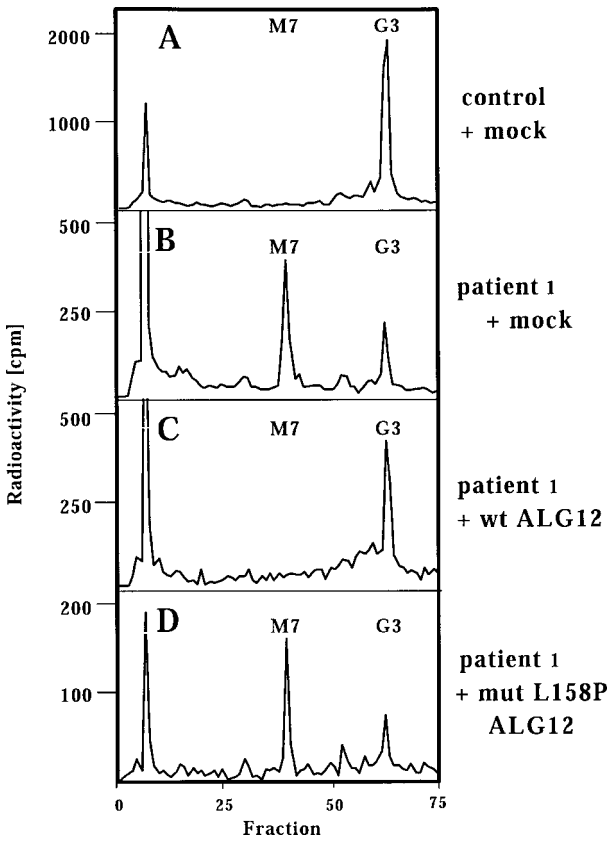


Figure 5 Complementation of the Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase deficiency

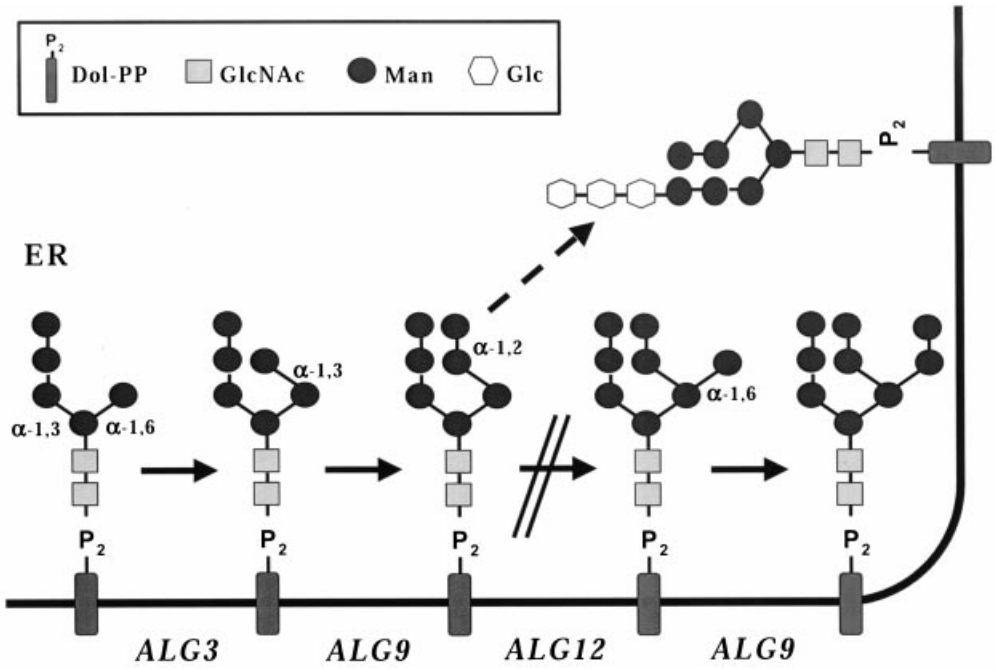
[^{3}H]Mannose-labelled dolichyl-linked oligosaccharides from control and patient 1's fibroblasts expressing the retroviral vector alone (mock), the wild-type or the T473C Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase cDNA were analysed by HPLC. The positions of the $GlcNAc_2Man_7$ (M_7) and $GlcNAc_2Man_9Glc_3$ (G_3) standards are indicated.

In order to determine whether the shortened dolichyl-linked oligosaccharides, which accumulate in the case of the patient's fibroblasts, are transferred on to newly synthesized glycoproteins by oligosaccharyltransferase, 2[^{3}H]mannose-labelled oligosaccharides were released from glycoproteins by peptide N-glycosidase F digestion and were analysed by HPLC. N-glycans from control fibroblasts were eluted mainly at the positions of $GlcNAc_2Man_9Glc_1$ and $GlcNAc_2Man_9$ standards (Figure 2C). In the case of the patient's fibroblasts, N-glycans released from newly synthesized glycoproteins showed two additional species (Figure 2D). The first one eluted at the position of a $GlcNAc_2Man_7$ standard, whereas the second one, which contained one more hexose unit, probably corresponded to $GlcNAc_2Man_7Glc_1$. The latter would be expected if glucosylated $GlcNAc_2Man_7Glc_3$ is transferred on to newly synthesized glycoproteins in CDG-Ig.

Determination of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase activity

The accumulation of Dol-PP-$GlcNAc_2Man_7$ suggested a defect in the mannosyltransferase, which catalyses the transfer of mannosyl residues from Dol-P-Man on to Dol-PP-$GlcNAc_2Man_7$ in the lumen of the endoplasmic reticulum. Therefore we determined the activity of Dol-P-Man: Dol-PP-$GlcNAc_2Man_7$ mannosyltransferase in the absence or presence of Dol-P-Man as mannosyl donor substrate and either Dol-PP-$GlcNAc_2$-[^{3}H]Man_6 or Dol-PP-$GlcNAc_2$-[^{3}H]Man_7 as acceptor substrate (Figure 3). Following incubation with membrane extracts of control and patient fibroblasts, the oligosaccharide moieties were released by mild-acid hydrolysis, were size fractionated by HPLC and the level of radioactivity was measured by liquid scintillation counting.

When membrane extracts from control fibroblasts were incubated with Dol-PP-$GlcNAc_2$-[^{3}H]Man_6 without addition of Dol-P-Man, almost no elongation of the lipid-linked oligosaccharides was observed (Figure 3A), whereas incubation in the presence of Dol-P-Man led to the formation of Dol-PP-$GlcNAc_2$-[^{3}H]Man_{7-9} (Figure 3B). In contrast, incubation of Dol-PP-$GlcNAc_2$-[^{3}H]Man_6 with the patient-derived membrane extract in the presence of Dol-P-Man only allowed synthesis of Dol-PP-$GlcNAc_2$-[^{3}H]Man_7, but showed no further elongation (Figure 3C). This result was confirmed by incubation of control and patient's membrane extracts in the presence of Dol-PP-$GlcNAc_2$-[^{3}H]Man_7 (Figures 3D–3F). The incubation of membrane extracts from control fibroblasts in the absence of Dol-P-Man did not lead to an elongation of the acceptor substrate, whereas the addition of



Scheme 1 Sequential addition of the four mannose residues at the lumenal side of the endoplasmic reticulum

Dol-P-Man led to the synthesis of Dol-PP-$GlcNAc_2$-[^{3}H]Man_{8-9}. Membrane extracts from patient's fibroblasts did not elongate Dol-PP-$GlcNAc_2$-[^{3}H]Man_7 independent of the absence or presence of Dol-P-Man. This points to a deficiency of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase in the patient's fibroblasts.

Genetic analysis of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase

Sequence analysis of the human Dol-P-Man: $Man_7GlcNAc_2$-PP-dolichyl mannosyltransferase cDNA (accession number NM_024105) revealed a compound heterozygosity in case of the patient (Figure 4). A T473C transition causes substitution of leucine^{158} with a proline residue, and a C1242G transversion changes a tyrosine codon to a stop codon, causing the loss of the C-terminal 74 amino acids. Sequence analysis of genomic DNA confirmed the compound heterozygosity of the patient. The mother was heterozygous for the T473C transition and the father was heterozygous for the C1242G transversion.

Complementation for Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase deficiency in CDG-Ig fibroblasts

In order to confirm the deficiency of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase as the cause of the glycosylation defect in the patient, we expressed the wild-type enzyme, as well as the T473C mutant, in patient's fibroblasts utilizing retroviral gene transfer (Figures 5C and 5D). As a control, we investigated whether the retroviral vector alone affects the activity of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase in control and patient's fibroblasts (Figures 5A and 5B). In mock-transfected control fibroblasts a major peak at $GlcNAc_2Man_9Glc_3$ was detected, whereas in mock-transfected fibroblasts from the patient 1 the accumulation of $GlcNAc_2Man_7$ was observed. This illustrates that the retroviral vector alone does not affect the biosynthesis of dolichyl-linked oligosaccharides. Expression of the wild-type Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase in patient's fibroblasts normalized the pattern of dolichyl-linked oligosaccharides (Figure 5C), whereas expression of the T473C mutant did not restore the synthesis of dolichyl-linked oligosaccharides (Figure 5D). These results show that the glycosylation defect in patient 1 is due to the reduced activity of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase and that the T473C mutation is a disease-causing mutation.

DISCUSSION

In a patient who presented clinically with microcephaly, developmental retardation and dysmorphic signs, a deficiency of Dol-P-Man: $Man_7GlcNAc_2$-PP-Dol mannosyltransferase was identified as the molecular defect. The enzyme deficiency impairs the elongation of Dol-PP-$GlcNAc_2Man_7$ in the inner leaflet of the endoplasmic reticulum. The deficiency of Dol-P-Man: $Man_7GlcNAc_2$-PP-dolichyl mannosyltransferase defines a new type of CDG, designated CDG-Ig.

The biosynthesis of Dol-PP-$GlcNAc_2Man_9Glc_3$, from which the oligosaccharide is transferred on to asparagine residues of newly synthesized glycoproteins by the oligosaccharyltransferase complex, is a multistep reaction in which the oligosaccharide is assembled in a strictly ordered manner on the lipid-carrier dolichol-phosphate. The biosynthesis starts with the stepwise addition of two *N*-acetylglucosamine and five mannose residues from the respective nucleotide sugars on the cytoplasmic side of the endoplasmic reticulum. The resulting Dol-PP-$GlcNAc_2Man_5$ intermediate is then transferred to the lumenal side of the endoplasmic reticulum, and is further elongated to Dol-PP-

$GlcNAc_2Man_9Glc_3$ by the stepwise addition of four mannose and three glucose residues, whereby Dol-P-Man and Dol-P-Glc serve as donors [26].

In recent years, two disorders caused by the deficiency of a glycosyltransferase involved in the assembly of Dol-PP-$GlcNAc_2Man_9Glc_3$ have been identified. In the case of CDG-Ic, deficiency of the Dol-P-Glc:$Man_9GlcNAc_2$-PP-Dol glucosyltransferase (human orthologue of yeast *ALG6*) leads to the accumulation of Dol-PP-$GlcNAc_2Man_9$, which cannot be transferred on to newly synthesized glycoproteins by the oligosaccharyltransferase complex. Due to the leaky nature of the defect, a residual N-glycosylation is found in the patient [7,8]. Clinically, CDG-Ic patients presented with a comparably mild psychomotor retardation and only a slight microcephaly [27]. In CDG-Id, a homozygous point mutation in the gene that encodes for Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol mannosyltransferase (human orthologue of yeast *ALG3*) has been identified. This causes an accumulation of Dol-PP-$GlcNAc_2Man_5$, which can be glucosylated and transferred to nascent polypeptide chains [9]. Since it is a poor substrate for the oligosaccharyltransferase [28], only a proportion of the N-glycosylation sites receive oligosaccharides. Clinically, CDG-Id is characterized by the neonatal onset of a severe convulsive disease with almost no psychomotor development and skeletal abnormalities including microcephaly.

In CDG-Ig fibroblasts about 95% of the dolichyl-linked oligosaccharides were represented by Dol-PP-$GlcNAc_2Man_7$. Studies *in vitro* showed a severe loss in the activity of Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase (human orthologue to yeast *ALG12*), the enzyme that catalyses the transfer of a mannosyl residue from Dol-P-Man to Dol-PP-$GlcNAc_2Man_7$ in an α1,6-branching reaction (Scheme 1). A small fraction of the dolichyl-linked oligosaccharides, about 5%, was of the size of Dol-PP-$GlcNAc_2Man_9Glc_3$, which points to a leaky nature of the defect. A compound heterozygosity for a T473C transition, which causes the substitution of leucine158 with proline, and a C1242G transversion, which changes a tyrosine codon to a stop codon thereby causing the loss of the C-terminal 74 amino acids, were identified in Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol mannosyltransferase gene of the patient. The disease-causing nature of the $Leu^{158} \rightarrow Pro$ substitution was demonstrated by the correction of the size of dolichyl-linked oligosaccharides by expressing the wild-type cDNA in the patient's fibroblasts, whereas expression of a cDNA encoding the $Leu^{158} \rightarrow Pro$ mutation did not correct the phenotype. Whether the $Leu^{158} \rightarrow Pro$ mutation or the deletion of the C-terminal 74 residues by the C1242G transversion is associated with some residual mannosyltransferase activity remains to be determined.

Whereas the underglycosylation of transferrin clearly indicates that in hepatocytes glycoproteins are only partially glycosylated (Figure 1, lower panel), in fibroblasts an underglycosylation of glycoproteins was not detectable (results not shown). The analysis of the protein-bound oligosaccharides indicated that only a minor fraction originates from fully assembled dolichyl-linked $GlcNAc_2Man_9Glc_3$ units. The majority is likely to derive from dolichyl-linked $GlcNAc_2Man_7Glc_3$ (see Scheme 1). In fibroblasts, the truncated dolichyl-linked oligosaccharides are apparently sufficient to support N-glycosylation. Also in yeast, deletion of the *ALG12* gene causes only a minor underglycosylation in carboxypeptidase Y [20].

The underglycosylation of serum transferrin, however, clearly demonstrates that in some cell types, such as hepatocytes, the truncated Dol-PP-$GlcNAc_2Man_7Glc_3$ cannot fully substitute for Dol-PP-$GlcNAc_2Man_9Glc_3$, presumably due to its lower affinity for the oligosaccharyltransferase.

This work was supported by the European Commission, (grant number QLG1-CT2000-00047; Euroglycan), the Deutsche Forschungsgemeinschaft, the Graduiertenkolleg 60 and the Fonds der Chemischen Industrie.

REFERENCES

1 Helenius, A. and Aebi, A. (2001) Intracellular functions of N-linked glycans. Science (Washington, D.C.) **291**, 2364–2369
2 Freeze, H. H. (2001) Update and perspectives on congenital disorders of glycosylation. Glycobiology, **11**, 129R–143R
3 Aebi, M., Helenius, A., Schenk, B., Barone, R., Fiumara, A, Berger, E. G., Hennet, T., Imbach, T., Stutz, A., Bjursell, C. et al. (1999) Carbohydrate-deficient glycoprotein syndromes become congenital disorders of glycosylation: an updated nomenclature for CDG. First International Workshop on CDGS. Glycoconj. J. **16**, 669–671
4 van Schaftingen, E. and Jaeken, J. (1995) Phosphomannomutase deficiency is a cause of carbohydrate deficient glycoprotein syndrome type I. FEBS Lett. **377**, 318–320
5 Matthijs, G., Schollen, E., Pardon, E., Veiga-Da-Cunha, M., Jaeken, J., Cassiman, J. and van Schaftingen, E. (1997) Mutations in PMM2, a phosphomannomutase gene on chromosome16p13, in carbohydrate-deficient glycoprotein type I syndrome (Jaeken syndrome). Nat. Genet. **16**, 88–92
6 Niehues, R., Hasilik, M., Alton, G., Körner, C., Schiebe-Sukumar, M., Koch, H. G., Zimmer, K. P., Wu, R., Harms, E., Reiter, K. et al. (1998) Carbohydrate deficient glycoprotein syndrome type Ib: phosphomannose isomerase deficiency and mannose therapy. J. Clin. Invest. **101**, 1414–1420
7 Körner, C., Knauer, R., Holzbach, U., Hanefeld, F., Lehle, L. and von Figura, K. (1998) Carbohydrate deficient glycoprotein syndrome type V: deficiency of dolichyl-P-Glc:$Man_9GlcNAc_2$-PP-dolichyl glucosyltransferase. Proc. Natl. Acad. Sci. U.S.A. **95**, 13200–13205
8 Imbach, T., Burda, P., Kuhner, P., Wevers, R. A., Aebi, M., Berger, E. G. and Hennet, T. (1999) A mutation in the human ortholog of the Saccharomyces cerevisiae *ALG6* gene causes carbohydrate-deficient glycoprotein syndrome type-Ic. Proc. Natl. Acad. Sci. U.S.A. **96**, 6982–6987
9 Körner, C., Knauer, R., Stephani, U., Marquardt, T., Lehle, L. and von Figura, K. (1999) Carbohydrate deficient glycoprotein syndrome type IV: deficiency of dolichyl-P-Man:$Man_5GlcNAc_2$-PP-dolichyl mannosyltransferase. EMBO J. **18**, 6818–6822
10 Imbach, T., Schenk, B., Schollen, E., Burda, P., Stutz, A., Grünewald, S., Bailie, N., King, M., Jaeken, J., Matthijs, G. et al. (2000) Deficiency of dolichol-phosphate-mannose synthase-1 causes congenital disorder of glycosylation type Ie. J. Clin. Invest. **105**, 233–239
11 Kim, S., Westphal, V., Srikrishna, G., Metha, D., Peterson, S., Filiano, J., Karnes, P., Patterson, M. and Freeze, H. (2000) Dolichol phosphate mannose synthase (DPM1) mutations define congenital disorder of glycosylation Ie (CDG-Ie). J. Clin. Invest. **105**, 191–198
12 Schenk, B., Imbach, T., Frank, C. G., Grubenmann, C. E., Raymond, G. V., Hurvitz, H., Raas-Rothschild, A., Luder, A. S., Jaeken, J., Berger, E. G. et al. (2001) Mutations in the Lec35 gene are responsible for a novel form of the congenital disorder of glycosylation (CDG), designated type If. J. Clin. Invest. **108**, 1687–1695
13 Kranz, C., Denecke, J., Lehrman, M. A., Ray, S., Kienz, P., Kreissel, G., Sagi, D., Peter-Katalinic, J., Freeze, H. H., Schmid, T. et al. (2001) A mutation in the human *MPDU1* gene causes Congenital Disorder of Glycosylation Type If (CDG-If). J. Clin. Invest. **108**, 1613–1619
14 Jaeken, J., Schachter, H., Carchon, H., De Cock, P., Coddeville, B. and Spik, G. (1994) Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetyl-glucosaminyltransferase II. Arch. Dis. Child. **71**, 123–127
15 Tan, J., Dunn, J., Jaeken, J. and Schachter, H. (1996) Mutations in the MGAT2 gene controlling complex N-glycan synthesis cause carbohydrate-deficient glycoprotein syndrome type II, an autosomal recessive disease with defective brain development. Am. J. Hum. Genet. **59**, 810–817
16 de Praeter, C., Gerwig, G., Bause, E., Nuytinck, L., Vliegenthart, J., Breuer, W., Kamerling, J., Espeel, M., Martin, J., Paepe, A. et al. (2000) A novel disorder caused by defective biosynthesis of N-linked oligosaccharides due to glucosidase I deficiency. Am. J. Hum. Genet. **66**, 1744–1756
17 Lübke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K. and Körner, C. (2001) Complementation cloning identifies CDG-IIc (LADII), a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency. Nat. Genet. **28**, 73–76
18 Lühn, K., Wild, M., Eckhardt, M., Gerardy-Schahn, R. and Vestweber, D. (2001) The defective gene in leukocyte adhesion deficiency II codes for a putative GDP-fucose transporter. Nat. Genet. **28**, 69–72
19 Hansske, B., Thiel, C., Lübke, T., Hasilik, M., Höning, S., Peters, V., Heidemann, P., Hoffmann, G., Berger, E. G., von Figura, K. and Körner, C. (2002) Deficiency of UDP-galactose: N-acetylglucosamine β-1,4-galactosyltransferase I as cause of the congenital disorder of glycosylation type IId (CDG-IId). J. Clin. Invest. **109**, 725–733

20 Burda, P., Jakob, C. A., Beinhauer, J., Hegemann, J. H. and Aebi, M. (1999) Ordered assembly of the asymmetrically branched lipid-linked oligosaccharide in the endoplasmic reticulum is ensured by the substrate specificity of the individual glycosyltransferases. Glycobiology **9**, 617–625
21 Körner, C., Lehle, L. and von Figura, K. (1998) Abnormal synthesis of mannose-1-phosphate derived carbohydrates in carbohydrate-deficient glycoprotein syndrome type I fibroblasts with phosphomannomutase deficiency. Glycobiology **8**, 165–171
22 Knauer, R. and Lehle, L. (1999) The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*. Isolation of the OST6 gene, its synthetic interaction with OST3, and analysis of the native complex. J. Biol. Chem. **274**, 17249–17256
23 Lehle, L. (1980) Biosynthesis of the core region of yeast mannoproteins. Formation of a glucosylated dolichol-bound oligosaccharide precursor, its transfer to protein and subsequent modification. Eur. J. Biochem. **109**, 589–601
24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25 Yamashita, K., Ideo, H., Ohkura, T., Fukushima, K., Yuasa, I., Ohno, K. and Takeshita, K. (1993) Sugar chains of serum transferrin from patients with carbohydrate deficient glycoprotein syndrome. Evidence of asparagine-N-linked oligosaccharide transfer deficiency. J. Biol. Chem. **268**, 5783–5789
26 Snider, M. D. and Rogers, O. C. (1984) Transmembrane movement of oligosaccharide-lipids during glycoprotein synthesis. Cell (Cambridge, Mass.) **36**, 753–761
27 Hanefeld, F., Körner, C., Holzbach-Eberle, U. and von Figura, K. (2000) Congenital disorder of glycosylation-Ic: case report and genetic defect. Neuropediatrics **31**, 60–62
28 Rearick, J., Fujimoto, K. and Kornfeld, S. (1981) Identification of the mannosyl donors involved in the synthesis of lipid-linked oligosaccharides. J. Biol. Chem. **256**, 3762–3769

Received 20 May 2002/25 June 2002; accepted 2 July 2002
Published as BJ Immediate Publication 2 July 2002, DOI 10.1042/BJ20020794