Deficiency of GDP-Man:GlcNAc$_2$-PP-Dolichol Mannosyltransferase Causes Congenital Disorder of Glycosylation Type Ik

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The molecular nature of a severe multisystemic disorder with a recurrent nonimmune hydrops fetalis was identified as deficiency of GDP-Man:GlcNAc$_2$-PP-dolichol mannosyltransferase, the human orthologue of the yeast *ALG1* gene (MIM 605907). The disease belongs to the group of congenital disorders of glycosylation (CDG) and is designated as subtype CDG-Ik. In patient-derived serum, the total amount of the glycoprotein transferrin was reduced. Moreover, a partial loss of N-glycan chains was observed, a characteristic feature of CDG type I forms. Metabolic labeling with [6-^{3}H]glucosamine revealed an accumulation of GlcNAc$_2$-PP-dolichol and GlcNAc$_1$-PP-dolichol in skin fibroblasts of the patient. Incubation of fibroblast extracts with [^{14}C]GlcNAc$_2$-PP-dolichol and GDP-mannose indicated a severely reduced activity of the β1,4-mannosyltransferase, elongating GlcNAc$_2$-PP-dolichol to Man$_1$GlcNAc$_2$-PP-dolichol at the cytosolic side of the endoplasmic reticulum. Genetic analysis of the patient's *hALG1* gene identified a homozygous mutation leading to the exchange of a serine residue to leucine at position 258 in the hALG1 protein. The disease-causing nature of the *hALG1* mutation for the glycosylation defect was verified by a retroviral complementation approach in patient-derived primary fibroblasts and was confirmed by the expression of wild-type and mutant hALG1 in the *Saccharomyces cerevisiae alg1-1* strain.

Introduction

Glycosylation of proteins is one of the most widespread forms of co- and posttranslational modification that has been found in eukaryotes, as well as in prokaryotes. In eukaryotes, glycoproteins are located inside cells predominantly in membranes of the secretory and endocytic pathway. Moreover, they are abundant in extracellular fluids and matrices. Glycan moieties attached to glycoproteins were shown to affect their folding and their transport, as well as their biological activity and stability (Spiro et al. 2002). The process of protein glycosylation is essential for viability and normal development and requires >100 glycosyltransferases, glycosidases, and transport proteins for sugar nucleotides (Helenius and Aebi 2001; Trombetta 2003). So far, 14 different molecular causes for inherited deficiencies in this complex metabolic pathway have been described in humans; these are termed "congenital disorders of glycosylation" (CDG) (Jaeken 2003; Thiel et al. 2003; Wu et al. 2003).

CDG comprise a group of multisystemic diseases with mostly severe psychomotor and mental retardation. Biochemically, the disorders are characterized by defective glycosylation of proteins caused by mutations in genes required for the biosynthesis of N-linked oligosaccharides. CDG are subdivided into two groups. CDG type I (CDG-I) comprise defects that affect biosynthesis of dolichol-linked oligosaccharides in the cytosol or the endoplasmic reticulum (ER), as well as defects involving the transfer of oligosaccharides onto nascent glycoproteins. CDG type II (CDG-II) encompass all defects of further trimming and elongation of N-linked oligosaccharides in the ER and the Golgi (Aebi et al. 1999).

Here we describe a molecular defect in glycoprotein biosynthesis that results in a new type of CDG-I in humans (termed "CDG-Ik"). The defective biosynthesis is due to the faulty transfer of mannosyl residues from GDP-Man to GlcNAc$_2$-PP-dolichol, which is catalyzed by the enzyme hALG1 at the cytosolic side of the ER.

Methods

Patient

The patient described here is the second child (P.B.) in the original case report (de Koning et al. 1998). Ultrasound analysis at the 30th wk of pregnancy revealed fetal hydrops and hepatosplenomegaly. Pregnancy was

interrupted at the 35th wk because of bradycardia. The severely hydropic boy showed multiple dysmorphic features with a large fontanelle, hypertelorism, micrognathia, hypogonadism, contractures, areflexia, cardiomyopathy, and multifocal epileptic activity. The patient died at 2 wk of age. Isoelectric focusing (IEF) of serum transferrin revealed a typical CDG-I pattern with the appearance of disialo- and asialotransferrin. Normal activity of phosphomannomutase excluded CDG-Ia.

IEF and SDS/PAGE of Serum Transferrin

IEF and SDS/PAGE of serum transferrin were performed as described elsewhere (Niehues et al. 1998).

Cell Lines and Cell Culture

Fibroblasts from the patient and the controls were maintained at 37°C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM [Gibco BRL]), which contained 10% fetal calf serum (FCS [PAN Biotech]). The ecotropic packaging cell line FNX-Eco (ATCC) 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- and Protein-Derived Oligosaccharides

Fibroblasts derived from the controls and the patient were grown and metabolically labeled with [2-³H]mannose for 30 min at 37°C. Dolichol- and protein-linked oligosaccharides were extracted, released, and analyzed by high-performance liquid chromatography (HPLC), as described elsewhere (Körner et al. 1998).

TLC Analysis of Short Dolichol-Linked Oligosaccharides

Prior to the experiment (60 h), 3.6×10^6 control- or patient-derived fibroblasts were plated onto 100-mm cell-culture dishes. Cells were labeled for 60 min in the presence of 1 mCi [6-³H]glucosamine (29.0 Ci/mmol [Amersham Biosciences]) in DMEM containing 2% dialyzed FCS, 0.5 mM glucose, and 0.5 mM mannose. Fibroblasts were scrapped in methanol, and the short dolichol-linked oligosaccharides were extracted and separated by thin-layer chromatography (TLC), as described elsewhere (Körner et al. 1998).

Enzyme Assays

To determine *ALG1*-encoded mannosyltransferase activity, GlcNAc₂-PP-Dol was used as the glycosyl acceptor. Analysis of subsequent mannose elongation steps was performed with Man₁GlcNAc₂-PP-Dol as the ac-

ceptor. The reactions contained the following, in a final volume of 0.06 ml: [¹⁴C]GlcNAc₂-PP-Dol (4,000 cpm) or Man₁[¹⁴C]GlcNAc₂-PP-Dol (4,000 cpm), 0.13% Nonidet P40, 10 mM $MgCl_2$, 0.9 mM DTT, 0.14 mM Na-EDTA, 19 mM Tris-HCl (pH 7.2), 1 mM GDP-Man, and solubilized enzyme (equivalent to 0.05 mg membrane protein). Incubations were performed at 37°C for 12 min. The reaction was stopped by the addition of chloroform:methanol to give a ratio of chloroform:methanol:water of 2:1:1 (by volume) and was processed further by phase separation (Sharma et al. 1982) by use of an upper phase of chloroform:methanol:water of 1:32:48 (by volume) and by collecting both lower phase and inter phase. The solubilized extract was obtained from a particulate fibroblast fraction, prepared as described elsewhere (Knauer and Lehle 1994; Thiel et al. 2002), except that membranes were suspended in 20 mM Tris (pH 7.2), 10 mM $MgCl_2$, and 1 mM DTT. Solubilization was performed at a protein concentration of 7 mg/ml and 1% Nonidet NP40. Preparation of [¹⁴C]GlcNAc₂-PP-dolichol and Man₁[¹⁴C]GlcNAc₂-PP-dolichol glycosyl acceptors was performed as described elsewhere (Thiel et al. 2003).

Mutation Analysis

Total RNA was extracted from fibroblasts and leukocytes from the controls, the patient, and the patient's parents by use of 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′-CAAAGCTTCCCGGGTCACAG-3′). In the first round of PCR, the cDNA was amplified using the primers F1 (5′-GATCCCAATACAGTAACAGC-TTT-3′) and R1 by use of the HotStar *Taq* 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 performed with the nested primers F2 (5′-GGGAACCG-CGTCCACTTCTC-3′) and R2 (5′-CACTGGGAGGT-GCTGCTCG-3′). Reverse transcriptase PCR products were run on 1% agarose gels. The 1,593-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 were performed by dye-determined cycle sequencing with the primers pUC M13 forward and pUC M13 reverse (Stratagene), F2, R2, R3 (5′-CTCAG-GAGTTTCTGGTCCGGT-3′), and R4 (5′-GTGGAGC-CGGAGGTCGGTC-3′) on an Applied Biosystems model 373A automated sequencer.

Genomic DNA was prepared from control and patient fibroblasts, as well as from blood leukocytes of the parents, by use of standard procedures (Maniatis 1989). PCR was performed with the primers gen-F1 (5′-GATG-
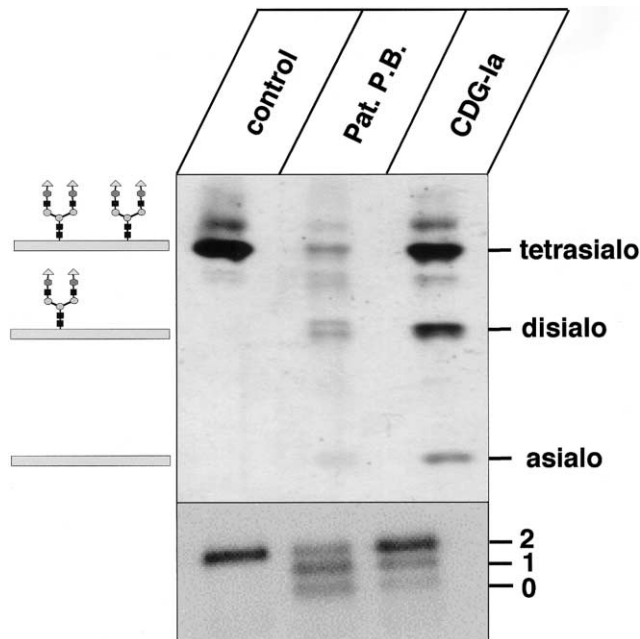
Figure 1 IEF pattern and SDS-PAGE of serum transferrin. Sera from a control, patient P.B., and a patient with CDG-Ia were analyzed by IEF (*upper panel*) and SDS-PAGE, followed by western blotting (*lower panel*) and immunodetection of transferrin. "Tetrasialo," "disialo," and "asialo" on the upper panel indicate transferrin forms with four, two, or no sialic acid residues. The numerals "2," "1," and "0" in the lower panel indicate transferrin forms with two, one, or zero oligosaccharide chains.

TGGCTGGGCACCCCA-3′) and gen-R1 (5′-CTGGCA-GGGGTGAGGAGAAC-3′), as described above. Nested PCR was performed using the primers gen-F2 (5′-GGG-AGCCTGCAGGCCTCG-3′) and gen-R2 (5′-AGGTGC-CCGTCACACCAACC-3′), resulting in a 301-bp fragment. The PCR products were analyzed on a 1% agarose gel and prepared as described above. Sequence analyses were performed using primers gen-F3 (5′-GGAGATGC-CTCTCCTGGGTC-3′).

Site-Directed Mutagenesis

A 1.6-kb fragment of a wild-type *ALG1*-cDNA representing the coding sequence (nt 107–nt 1486) was amplified by PCR, by use of primers F2 and R2. The resulting fragment was purified and cloned into the pGEM-T-Easy vector (pGEM-T-Easy–wild-type). The mutation C773T was inserted into the cDNA by use of the QuickChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, with the primers Mut773-A (5′-GGAGCGGTTGGCCTTC-ACG-3′) and Mut773-B (5′-CGTGAAGGCCAACCG-CTCC-3′) to obtain plasmid pGEM-T-Easy-Pat. Wild-type and patient *ALG1* cDNA was subcloned into the MoMuLV-derived vector pLNCX2 (Clontech).

Retroviral Complementation

Ecotropic FNX-Eco cells (5×10^5) were seeded onto dishes (60-mm diameter) 1 d before transfection. Transient transfection by FuGENE6 reagent was performed according to the manufacturer's protocol (Roche), with 1 μg of LNCX2 vector (mock), LNCX2 wild type, and LNCX2 patient. Further procedures were performed as described elsewhere (Thiel et al. 2002). The supernatant with the amphotropic retroviral particles was used to transfect patient and control fibroblasts. After infection of the fibroblasts, the medium was replaced by DMEM, containing 10% heat-inactivated FCS with geneticin (335 μg/ml [Gibco BRL]). Selection was performed for 10 d.

Yeast Genetics

The cDNAs of the human *alg1* mannosyltransferases from the controls and the patient, cloned into the p-LNCX2 vector (Clontech), were isolated as *Not*I 1.4-kbp fragments and ligated into the *Not*I site of the yeast shuttle vector pNEV-N, under the control of the *PMA1* promotor, to give pNEV-*hALG1*-Wt and pNEV-*ALG1*-Pat, respectively. Plasmids were transformed into the *alg1-1* yeast strain (MATα *ura3-52*) by use of standard techniques (Gietz and Schiestl 1991). Yeast cells were grown in YNBD medium (0.67% yeast nitrogen dropout ura, 2% glucose).

Growth Complementation of alg1 *Yeast Cells*

Transformants were grown in selective liquid medium overnight at 25°C, and 3 μl of serial 10-fold dilutions were spotted on agar plates, starting at 10^4 cells, and were incubated at 25°C or 36°C for 4 d.

In Vivo Labeling of Dolichol-Linked Oligosaccharides in Yeast

Yeast cells were grown at 25°C to midlogarithmic phase in selective medium and were shifted to 36°C for 40 min. Subsequent labeling with [2-³H]mannose at 36°C, extraction, and analysis of dolichol-linked oligosaccharides were performed as described elsewhere (Knauer and Lehle 1999).

Metabolic Labeling of Carboxypeptidase Y (CPY)

Yeast cells were grown at 25°C in selective medium to midlogarithmic phase. Cells were harvested, resuspended in fresh medium, adapted to 25°C or 36°C for 30 min, and labeled with 75 μCi [³⁵S] methionine/cysteine (Pro-mix [Amersham Biosciences]) for 45 min. Preparation of cell lysates, immunoprecipitation of CPY, and analysis by SDS-PAGE were performed as described elsewhere (Knauer and Lehle 1999).
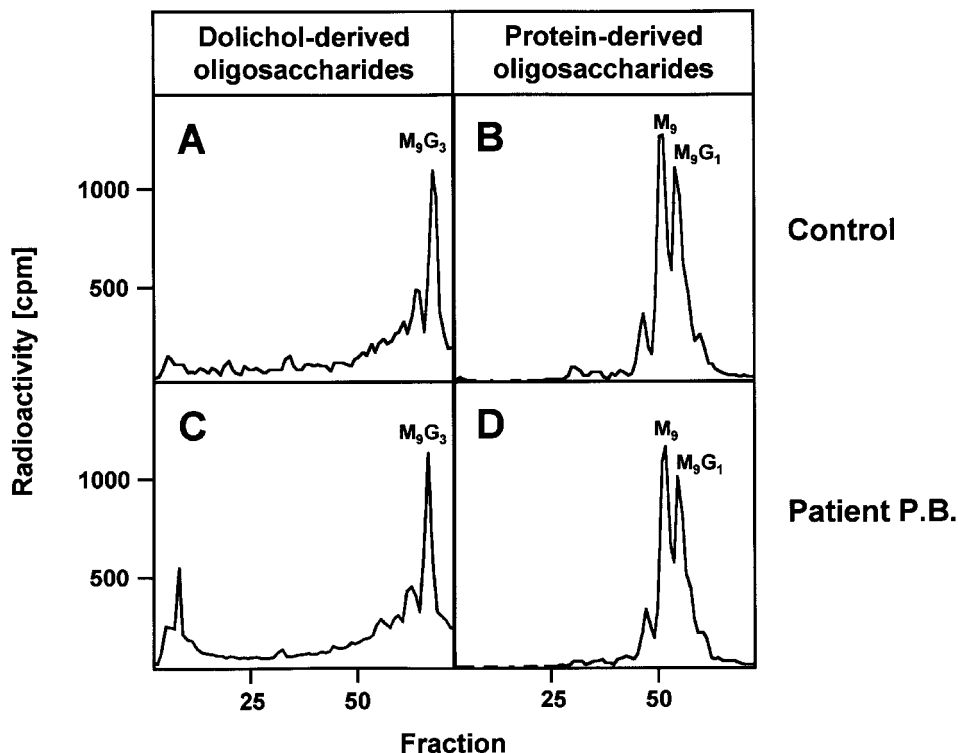
Figure 2 Analysis of protein- and dolichol-derived oligosaccharides in CDG-Ik. Fibroblasts of a control (*B*) and the patient (*D*) were metabolically labeled with [2-^{3}H]mannose for 30 min, [2-^{3}H]glycans were released from equal amounts of the glycoprotein fraction by PNGase F digestion and were size fractionated by HPLC. M_9 and M_9G_1 refer to the positions of $GlcNAc_2Man_9$ and $GlcNAc_2Man_9Glc_1$ standards, respectively. Control- (*A*) and patient-derived (*C*) fibroblasts (in equal amounts) were metabolically labeled with [2-^{3}H]mannose for 30 min. The [2-^{3}H]oligosaccharides were released from the dolichol-PP moiety by mild acid hydrolysis and were size fractionated by HPLC. M_9G_3 refers to the position of a $GlcNAc_2Man_9Glc_3$ standard.

Results

Partial Lack of Entire N-Linked Oligosaccharide Side Chains in Serum Transferrin

IEF of serum transferrin is the standard diagnostic procedure for CDG. Previous data for the index patient revealed a partial loss of sialic acids leading to an increase of di- and asialotransferrin at the expense of tetrasialotransferrin, indicating a case of CDG-I. To exclude a diagnosis of CDG-Ia, which is the most frequent type of CDG-I, the activity of phosphomannomutase 2, the enzyme deficient in CDG-Ia, was determined and was found to be normal (de Koning et al. 1998).

Our analysis of the transferrin IEF confirmed the data published for patient P.B. (de Koning et al. 1998) and demonstrated, moreover, that the total amount of transferrin was reduced in the serum of the patient, compared with serum of a control person and a CDG-Ia patient (fig. 1, *upper panel*). The mass of the protein was determined by SDS-PAGE (fig. 1, *lower panel*). The presence of faster-migrating transferrin forms indicated the loss of one or both of the two N-linked oligosaccharide

side chains, which are normally present in transferrin. Deficiency of phosphomannose isomerase, which leads to a comparable IEF pattern of transferrin in CDG-Ib, was excluded, since enzyme activity determined in patient fibroblasts was normal (data not shown).

Accumulation of Shortened Dolichol-Linked Oligosaccharides in Patient-Derived Fibroblasts

First, to investigate the molecular mechanism of the glycosylation defect, we attempted to study the transfer of $GlcNAc_2Man_9Glc_3$ moieties from dolichol onto newly synthesized glycoproteins, which is catalyzed by the oligosaccharyltransferase complex. Control- and patient-derived fibroblasts were metabolically labeled with [2-^{3}H]mannose, followed by extraction of total glycoproteins. N-glycan chains from equal amounts of glycoproteins were released by peptide:N-glycosidase F (PNGase F) treatment and subsequently were analyzed by HPLC (fig. 2*B* and 2*D*). The oligosaccharides released from newly synthesized glycoproteins of controls and the patient eluted mainly at positions corresponding to $GlcNAc_2Man_9Glc_1$ and $GlcNAc_2Man_9$ standards, re-
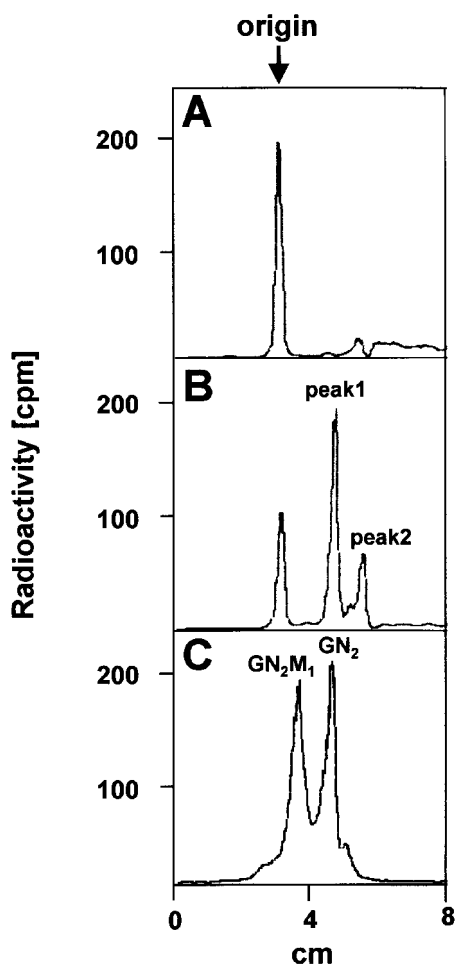
Figure 3   TLC analysis of [6-^{3}H]glucosamine-labeled short dolichol-linked oligosaccharides. Fibroblasts from a control (*A*) and from patient P.B. (*B*) were metabolically labeled for 60 min with [6-^{3}H]glucosamine. After extraction of the short lipid-linked oligosaccharide fraction with chloroform:methanol (3:2), further analysis was performed by TLC on silica gel 60 plates with chloroform:methanol:water (65:25:4) as solvent. The position of the origin and the positions of a [^{14}C]GlcNAc$_2$-PP-dolichol standard and a Man$_1$[^{14}C]GlcNAc$_2$-PP-dolichol standard are indicated as GN$_2$ and GN$_2$M$_1$, respectively (*C*).

acid hydrolysis, and subsequent size fractionation of the oligosaccharides by HPLC. The majority of dolichol-linked oligosaccharides from the control and the patient eluted at the position of a GlcNAc$_2$Man$_9$Glc$_3$ standard (fig. 2*A* and 2*C*), indicating the formation of full-length dolichol-linked oligosaccharides. Radioactivity eluting in fractions 1–6 (e.g., fig. 2*C*) represents [2-^{3}H]mannose that is occasionally carried over into the chloroform:methanol:water (10:10:3) fraction during extraction of dolichol-linked oligosaccharides.

For CDG-Ii [MIM 607906], it was shown (Thiel et al. 2003) that a defect in one of the early steps of dolichol-linked oligosaccharide biosynthesis leads to an accumulation of short dolichol-linked oligosaccharides that escape the extraction procedure described above. Moreover, defects in the initiating steps of dolichol-linked oligosaccharide biosynthesis, such as the addition of the two glucosamine residues and the first mannose residue, are missed by metabolic labeling with [2-^{3}H]mannose. Therefore, we labeled fibroblasts from a control and the patient for 60 min in the presence of [6-^{3}H]glucosamine, extracted the cells with chloroform:methanol (3:2), and analyzed the extract by TLC (fig. 3). Besides nonmigrating radioactivity at the origin in the control (fig. 3*A*) and the patient (fig. 3*B*) extracts, we observed two additional peaks in the case of the patient (fig. 3*B*; peak 1 and peak 2). Peak 1 migrated with GlcNAc$_2$-PP-dolichol from a standard mixture containing GlcNAc$_2$-PP-dolichol and Man$_1$GlcNAc$_2$-PP-dolichol (fig. 3*C*). Peak 2 is supposed to be dolichol-PP-GlcNAc$_1$, which migrates faster due to its more hydrophobic nature.

Deficiency of GDP-Mannose:GlcNAc$_2$-PP-Dolichol β1,4-Mannosyltransferase (hAlg1) in Patient-Derived Fibroblasts

The analysis of dolichol-linked oligosaccharides revealed a predominant accumulation of GlcNAc$_2$-PP-dolichol and a minor accumulation of GlcNAc$_1$-PP-dolichol in fibroblasts of the patient. A comparable accumulation pattern of biosynthetic intermediates of dolichol-linked oligosaccharides has been described for the temperature-sensitive *alg1-1* mutant from *Saccharomyces cerevisiae*, in which the GDP-mannose:GlcNAc$_2$-PP-dolichol β1,4-mannosyltransferase is defective at the restrictive temperature (Huffacker and Robbins 1982). To determine whether elongation of GlcNAc$_2$-PP-dolichol is impaired in the patient, an in vitro assay was established by incubating microsomal extracts from control and patient fibroblasts with GDP-mannose and [^{14}C]GlcNAc$_2$-PP-dolichol. An extension of the newly synthesized glycan up to Man$_5$[^{14}C]GlcNAc$_2$-PP-dolichol was observed in presence of the microsomal extract from control fibroblasts but not from fibroblasts of patient P.B. (fig. 4*A* and 4*B*).
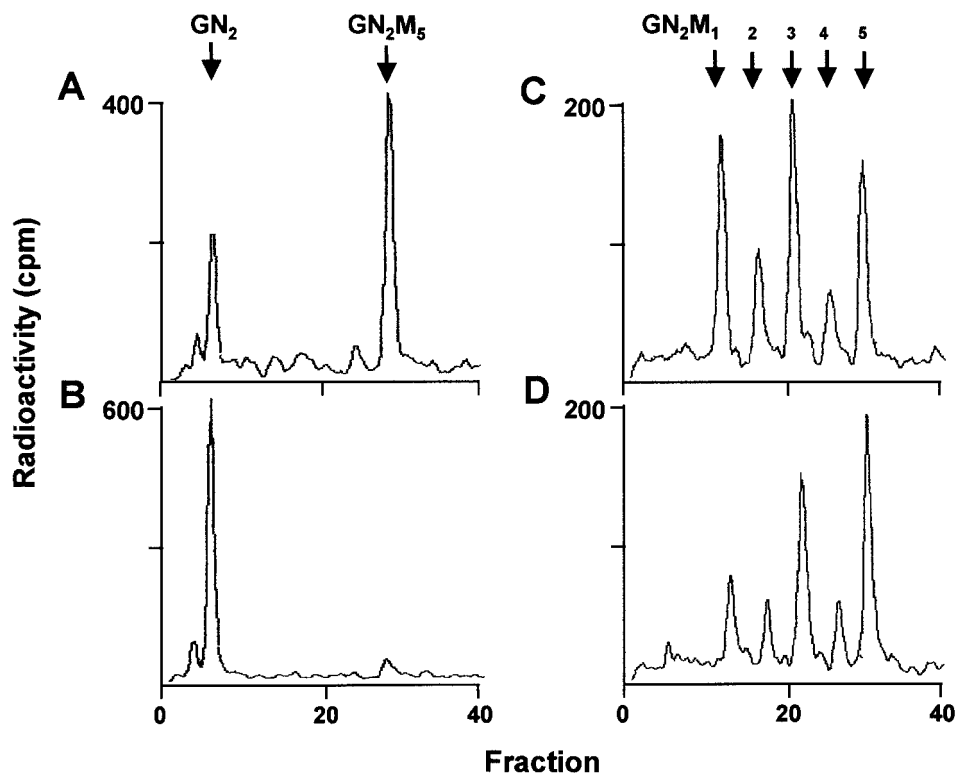
spectively. In four independent experiments, no significant difference in the amount of labeled oligosaccharides was observed between the patient and the controls. These results indicate that, at least under the experimental conditions, sufficient dolichol-linked oligosaccharides in patient fibroblasts are transferred onto newly synthesized glycoproteins with a normal activity of oligosaccharyltransferase.

Next, we investigated the size of dolichol-linked oligosaccharides by metabolic labeling of fibroblasts from a control and the patient with [2-^{3}H]mannose for 30 min, followed by extraction with chloroform:methanol:water (10:10:3), release of the glycan moieties by mild

Figure 4 In vitro determination of hALG1 and hALG2 activity. Microsomal extracts from fibroblasts of a control (*A, C*) and the patient (*B, D*) were incubated for 10 min with either [^{14}C]GlcNAc$_2$-PP-dolichol (*A, B*) or with Man$_1$[^{14}C]GlcNAc$_2$-PP-dolichol (*C, D*), respectively, in the presence of GDP-mannose. Dolichol-linked oligosaccharides were extracted from the incubation mixture and were treated by mild acid hydrolysis, and the released oligosaccharides were separated by HPLC. The positions of a GlcNAc$_2$-standard (GN$_2$) and Man$_{1-5}$GlcNAc$_2$ (GN$_2$M$_{1-5}$)-standards are marked by arrows.

Next, we investigated the ability of control and patient cell extracts to elongate Man$_1$GlcNAc$_2$-PP-dolichol, which is catalyzed by the *hALG2*-encoded α1,3 mannosyltransferase. The reaction catalyzes the transfer of the second mannosyl residue in the dolichol-linked oligosaccharide assembly. Fibroblast extracts from a control and from patient P.B. were incubated in the presence of GDP-mannose and Man$_1$[^{14}C]GlcNAc$_2$-PP-dolichol. We found that both cell extracts were able to elongate the oligosaccharide chain up to Man$_5$[^{14}C]GlcNAc$_2$-PP-dolichol (fig. 4*C* and 4*D*). These findings demonstrate that the transfer of the first mannosyl residue to GlcNAc$_2$-PP-dolichol, which is catalyzed by hALG1, is impaired in case of the patient.

Detection of a Homozygous Missense Mutation in the hALG1 *Gene*

Sequencing of the *hALG1* cDNA (accession number BAA90748 [NCBI Entrez Database]) revealed homozygosity for a C773T transition in the case of the patient (data not shown). The C773T transition causes the substitution of serine residue 258 with a leucine residue. The mutation was confirmed on the level of genomic

DNA. Both parents were shown to be heterozygous for the C773T transition mutation on the cDNA, as well as on the genomic level (data not shown).

Retroviral Expression of Wild-Type hALG1 Complements for GDP-Man:GlcNAc$_2$-PP-Dolichol Mannosyltransferase Deficiency in the Patient

To confirm that the C773T transition mutation in the *hALG1* gene is the disease-causing mutation in the patient, we expressed *hALG1* wild-type cDNA (fig. 5*B*), as well as the *hALG1* cDNA carrying the C773T mutation (fig. 5*C*) in patient-derived fibroblasts, by use of a retroviral expression system. The effect of the retroviral vector alone was investigated in patient fibroblasts, as well as control fibroblasts (fig. 5*A* and 5*D*). TLC analysis of extracts from [6-^{3}H]glucosamine-labeled patient fibroblasts expressing the retroviral vector alone (fig. 5*A*) showed a major peak corresponding to a Dol-PP-GlcNAc$_2$ standard and a second peak that is supposed to be Dol-PP-GlcNAc$_1$, which is not present in control fibroblasts (fig. 5*D*). Retroviral transduction of the wild-type *hALG1* cDNA, but not of the C773T mutant *hALG1* cDNA, led to normalization of dolichol-

linked oligosaccharide biosynthesis in the patient, demonstrating that the hALG1 deficiency causes the biochemical defect in the patient and that the C773T mutation causes inactivity of hALG1.

Wild-Type hALG1 Complements for the Defect in an S. Cerevisiae alg1 Mutant Strain

To confirm the complementation results in patient fibroblasts, we introduced cDNAs encoding wild-type *hALG1* or the *hALG1* C773T transition mutation into the temperature-sensitive *alg1-1* yeast strain. We found that only transformation with the wild type but not with the mutant *hALG1* cDNA led to normalization of dolichol-linked oligosaccharide biosynthesis in *alg1-1* cells (fig. 6A), when analyzed by metabolic labeling with [2-^{3}H]mannose.

Complementation was also evident when the growth behavior in the *alg1-1* strain was investigated (fig. 6B). At the permissive temperature of 25°C, *alg1-1* cells that were transformed with either the vector alone, the *hALG1* C773T mutant cDNA, or the *hALG1* wild-type cDNA showed comparable growth (fig. 6, *left panel*). At the nonpermissive temperature of 36°C, correction of the growth phenotype occurred only by transformation with wild-type *hALG1* cDNA, but not with the *hALG1* C773T mutant (fig. 6, *right panel*).

Next, we analyzed the glycosylation of the vacuolar glycoprotein CPY (fig. 6C). At the permissive temperature, the glycosylation state of CPY was comparable between *alg1-1* cells either transformed with the vector alone (*lane 3*), the *hALG1* C773T cDNA (*lane 2*), or the *hALG1* wild-type cDNA (*lane 1*). The glycosylation state of CPY is severely reduced in the *alg1-1* strain at the nonpermissive temperature of 36°C because of a reduced transfer of truncated oligosaccharides onto the newly synthesized protein (fig. 6C, *lane 6*). Again, only transformation of the *alg1-1* strain with the wild type (*lane 4*), but not with the C773T *hALG1* cDNA (*lane 5*), led to normalization of CPY glycosylation, as occurring in yeast wild-type cells (*lane 7*). Taken together, these results confirm, first, that the C773T transition in the *hALG1* gene is the disease-causing mutation in CDG-Ik and, second, that the isolated gene is indeed the human orthologue to the yeast *ALG1* gene.

Discussion

In a patient suffering from multiple dysmorphic features, hypertelorism, micrognathia, hepatosplenomegaly, hypogonadism, contractures, areflexia, cardiomyopathy, and multifocal epileptic activity (de Koning et al. 1998), we determined a new molecular defect in the biosynthesis of N-linked glycans. The defect in this new disorder, termed "CDG-Ik," affecting an early step of dol-
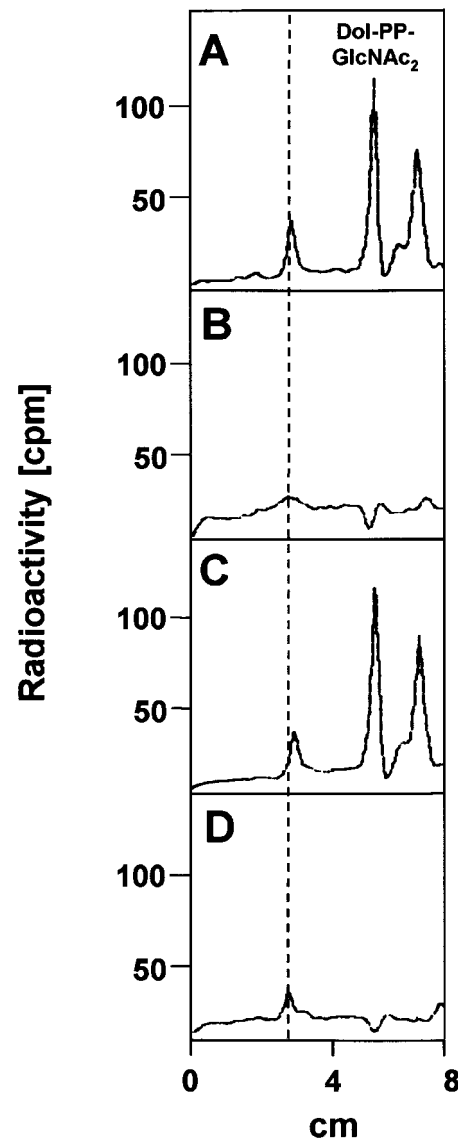

Figure 5 Retroviral transduction of patient fibroblasts with wild-type *hALG1* cDNA leads to complementation of the hALG1 deficiency. Dolichol-PP-[^{3}H]GlcNAc$_2$ and dolichol-PP-[^{3}H]GlcNAc$_1$ were extracted with chloroform:methanol (3:2) from [6-^{3}H]glucosamine-labeled control fibroblasts expressing the retroviral vector alone (*D*) and from patient fibroblasts, which were either transduced with the retroviral vector alone (*A*), the wild-type cDNA (*B*), or the C773T h*ALG1* cDNA (*C*). Further analysis was performed by TLC. The elution position of a [^{14}C]GlcNAc$_2$-PP-dolichol standard and the origin (*dotted line*) are indicated.

ichol-linked oligosaccharide biosynthesis catalyzed by the *ALG1*-encoded mannosyltransferase gene, was independently identified in two other patients with CDG (T. Marquardt, personal communication).

Dolichol-linked oligosaccharide biosynthesis comprises a series of reactions by which, in a stepwise, ordered manner, two GlcNAc and five mannose residues
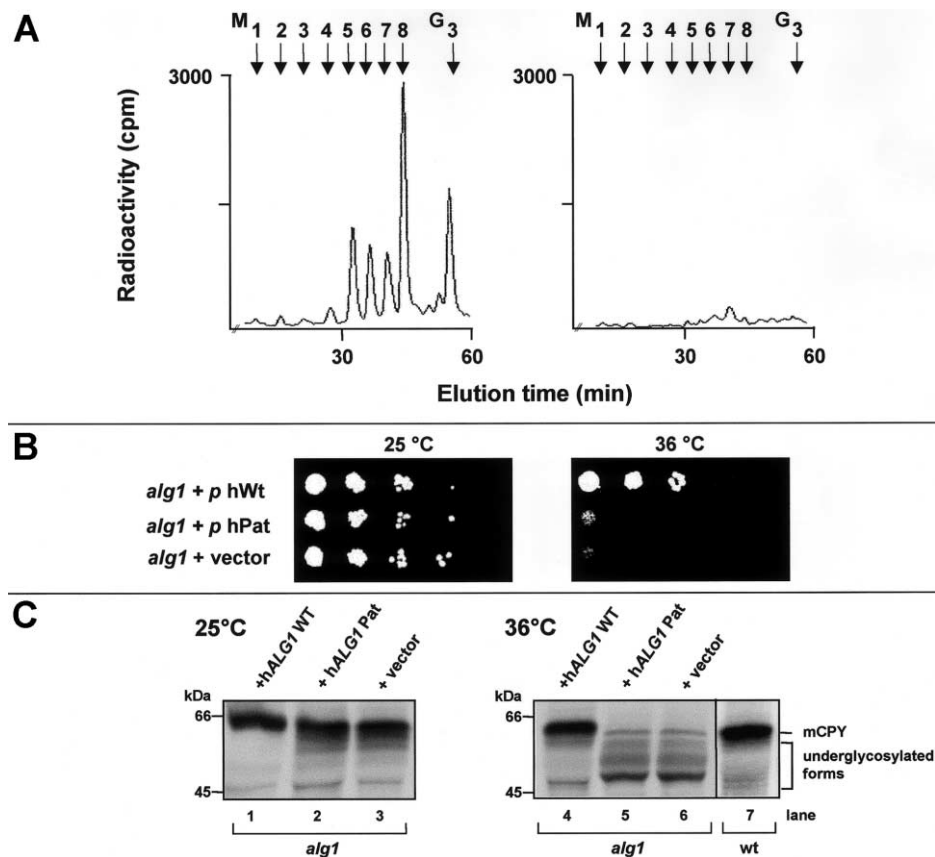
Figure 6 Defects in dolichol-linked oligosaccharide biosynthesis (*A*), growth (*B*), and CPY glycosylation (*C*) in the alg1-1 yeast mutant are complemented by expression of wild-type *hALG1*. *A,* Biosynthesis of dolichol-linked oligosaccharides was investigated in an *alg1-1* strain transformed with the wild-type h*ALG1* cDNA (*left panel*) or the *hALG1* cDNA encoding the C773T mutation (*right panel*). Yeast cells were metabolically labeled with [2-^{3}H]mannose for 30 min, [2-^{3}H] oligosaccharides were released from the dolichol moiety by mild acid hydrolysis and further analyzed by HPLC. M_1–M_8 and G_3 refer to $Man_{1-8}GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$ standards, respectively. *B,* Growth of yeast *alg1-1* cells either transformed with wild-type *hALG1* cDNA, the C773T *hALG1* or the expression vector was investigated under permissive (25°C, *left panel*) and nonpermissive temperature (36°C, *right panel*). *C,* The glycosylation status of CPY is shown at the permissive temperature (25°C, *left panel*) and at the nonpermissive temperature (36°C, *right panel*). Yeast cells were metabolically labeled with ^{35}S-methionine for 30 min, and CPY was immunoprecipitated and analyzed by SDS-PAGE. The position of the mature form of CPY in wild-type yeast cells (mCPY) and in the complemented *alg1-1* cells are indicated on the right. Molecular weight standards are indicated on the left.

are added from nucleotide sugar donors to dolichol-phosphate by glycosyltransferases on the cytosolic side of the ER. After the translocation of $Man_5GlcNAc_2$-PP-dolichol into the lumen of the ER, the oligosaccharide moiety is further elongated to $Glc_3Man_9GlcNAc_2$-PP-dolichol, with Dol-P-Man and Dol-P-Glc as donor substrates (Snider and Rogers 1984). The molecular defect of the CDG-Ik patient affects the *ALG1*-encoded mannosyltransferase, catalyzing the transfer of the first β1,4-linked mannose residue onto the dolichol-bound chitobiose residue at the outer leaflet of the ER, causing the accumulation of $GlcNAc_2$-PP-dolichol. Accumulation of $GlcNAc_1$-PP-dolichol, the biosynthetic precursor of $GlcNAc_2$-PP-dolichol, was also observed. This is probably caused by feedback inhibition of the preceding reaction resulting from the accumulation of $GlcNAc_2$-PP-dolichol, which interferes with a so-far-unknown enzyme catalyzing the elongation of $GlcNAc_1$-PP-dolichol. A comparable accumulation of $GlcNAc_2$-PP-dolichol and $GlcNAc_1$-PP-dolichol for the temperature-sensitive *S. cerevisiae alg1-1* strain has been described elsewhere. In yeast, deficiency of *ALG1* leads to underglycosylation of glycoproteins and cell death (Huffacker and Robbins 1982). The cDNA for the human orthologue of the yeast *ALG1* encodes a transmembrane protein with 464 amino acids containing several regions highly conserved between yeast and humans (Takahashi et al. 2000). In the case of the patient with CDG-Ik, we identified homozygosity for a C773T transition mutation in *hALG1* at the mRNA and genomic DNA levels, whereas the patient's parents were heterozygous carriers of the mutation. The mutation leads to replacement of a serine

by a leucine residue at position 258 in the hALG1 protein. To prove the disease-causing nature of the C773T mutation in the *hALG1* gene, we expressed the wild-type and the mutant *hALG1* cDNA in patient-derived fibroblasts and in an *S. cerevisiae alg1-1* strain, respectively. In both cases, only the wild-type cDNA complemented for the *ALG1* deficiency, but not the C773T mutation.

The serine residue at position 258 in the hAlg1 protein is conserved in the mouse and is replaced by a threonine residue in *Drosophila melanogaster,* as well as in *S. cerevisiae,* indicating the importance of the hydroxyl group. Computer-assisted protein structure analysis (TMpred) did not predict phosphorylation or O-glycosylation of serine 258. The hALG1 protein is predicted to have four transmembrane domains. Serine 258 is part of a loop, 222 amino acids in length, located in the lumen of the ER. This would indicate that the mutation does not directly affect either the binding sites for the substrates or the catalytic sites that are supposed to reside in the cytosolic domain. Instead, the Ser258Leu mutation causes a conformational change of the hAlg1 protein in the patient.

The comparable HPLC size patterns of dolichol-linked oligosaccharides in the chloroform:methanol:water (10:10:3) fraction in control and patient fibroblasts metabolically labeled with $[2\text{-}^3H]$mannose points to the leaky nature of the hALG1 deficiency in the patient. Manifestation of the defect may be restricted to tissues, such as liver or pancreas, with high rates of glycoprotein biosynthesis.

The biosynthesis of dolichol-linked oligosaccharides is routinely investigated by metabolic labeling of cells with $[2\text{-}^3H]$mannose and analysis of the glycan moieties in the fraction extracted with chloroform:methanol:water (10:10:3). Similar to the metabolic defect in patients with CDG-Ii, in which the Alg2-mediated elongation of the dolichol-linked $Man_1GlcNAc_2$ trisaccharide is deficient, the metabolic defect in patients with CDG-Ik would have escaped detection if just the routine protocol for dolichol-linked oligosaccharide analysis had been applied. Early biosynthetic intermediates of dolichol-linked oligosaccharides are seldom (and poorly) labeled with $[2\text{-}^3H]$mannose. Moreover, they are extracted with chloroform:methanol (3:2) preceding extraction with chloroform:methanol:water (10:10:3). Therefore, we recommend that, in the growing number of patients with a CDG-I IEF pattern for transferrin of unknown origin, the fibroblasts should be metabolically labeled with $[6\text{-}^3H]$glucosamine and the chloroform:methanol (3:2) extract should be analyzed for dolichol-linked sugar residues.

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

National Center for Biotechnology Information (NCBI) Entrez Database, http://www.ncbi.nlm.nih.gov/Entrez/ (for *hALG1* cDNA [accession number BAA90748])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *ALG1* and CDG-Ii)

References

Aebi M, Helenius A, Schenk B, Barone R, Fiumara A, Berger EG, Hennet T, 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

Gietz RD, Schiestl RH (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single stranded nucleic acids as carrier. Yeast 7:253–263

Helenius A, Aebi A (2001) Intracellular functions of N-linked glycans. Science 291:2364–2369

Huffaker T, Robbins P (1982) Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. J Biol Chem 257:3203–3210

Jaeken J (2003) Congenital disorders of glycosylation (CDG): it's all in it! J Inherit Metab Dis 26:99–118

Knauer R, Lehle L (1994) The N-oligosaccharyltransferase complex from yeast. FEBS Letters 344:83–86

Knauer R, 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

Körner C, Lehle L, 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

de Koning TJ, Toet M, Dorland L, de Vries LS, van den Berg IET, Duran M, Poll-The BT (1998) Recurrent nonimmune hydrops fetalis associated with carbohydrate-deficient glycoprotein syndrome. J Inher Metab Dis 21:681–682

Maniatis NT, Fritsch EF, Sambrook J (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Niehues R, Hasilik M, Alton G, Körner C, Schiebe-Sukumar M, Koch, HG, Zimmer KP, Wu R, Harms E, Reiter K, von Figura K, Freeze HH, Harms HK, Marquardt T (1998) Carbohydrate-deficient glycoprotein syndrome type 1b: phosphomannose isomerase deficiency and mannose therapy. J Clin Invest 101:1414–1420

Sharma CB, Lehle L, Tanner W (1982) Solubilization and characterization of the initial enzymes of the dolichol pathway from yeast. Eur J Biochem 126:319–325

Snider MD, Rogers OC (1984) Transmembrane movement of

oligosaccharide-lipids during glycoprotein synthesis. Cell 36:753–761

Spiro R (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 12:43R–56R

Takahashi T, Honda R, Nishikawa Y (2000) Cloning of the human cDNA which can complement the defect of the yeast mannosyltransferase I-deficient mutant *alg1*. Glycobiology 10:321–327

Thiel C, Schwarz M, Hasilik M, Grieben U, Hanefeld F, Lehle L, von Figura K, Körner C (2002) Congenital disorder of glycosylation-Ig is caused by deficiency of dolichyl-P-Man:Man$_7$ GlcNAc$_2$-PP-dolichyl mannosyltransferase. Biochem J 367:195–201

Thiel C, Schwarz M, Peng J, Grzmil M, Hasilik M, Braulke T, Kohlschütter A, von Figura K, Lehle L, Körner C (2003) A new type of congenital disorders of glycosylation (CDG-Ii) provides new insights into the early steps of dolichol-linked oligosaccharide biosynthesis. J Biol Chem 278:22498–22505

Trombetta S (2003) The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. Glycobiology 13:77R–91R

Wu R, Rush J, Karaoglu D, Krasnewich D, Lubinsky M, Waechter C, Gilmore R, Freeze H (2003) Deficiency of UDP-GlcNAc:dolichol-phosphate N-acetylglucosamine-1 phosphate transferase (DPAGT1) causes a novel congenital disorder of glycosylation type Ij. Hum Mutat 22:144–150