A New Type of Congenital Disorders of Glycosylation (CDG-II) Provides New Insights into the Early Steps of Dolichol-linked Oligosaccharide Biosynthesis*

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Deficiency of GDP-Man:Man\textsubscript{5}GlcN\textsubscript{4}A\textsubscript{2}-PP-dolichol mannosyltransferase (hALG2), is the cause of a new type of congenital disorders of glycosylation (CDG) designated CDG-II. The patient presented normal at birth but developed in the 1st year of life a multisystemic disorder with mental retardation, seizures, coloboma of the iris, hypomyelination, hepatomegaly, and coagulation abnormalities. An accumulation of Man\textsubscript{4}GlcN\textsubscript{5}Ac\textsubscript{2}-PP-dolichol and Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol was observed in skin fibroblasts of the patient. Incubation of patient fibroblast extracts with Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol and GDP-mannose revealed a severely reduced activity of the mannosyltransferase catalyzing the transfer of mannose residues from GDP-Man to Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol. Because the S. cerevisiae mutant alg2-1 was known to accumulate the same shortened dolichol-linked oligosaccharides as the patient, the yeast ALG2 sequence was used to identify the human ortholog. Genetic analysis revealed that the patient was heterozygous for a single nucleotide deletion and a single nucleotide substitution in the human ortholog of yeast ALG2. Expression of wild type but not of mutant hALG2 cDNA restored the mannosyltransferase activity and the biosynthesis of dolichol-linked oligosaccharides both in patient fibroblasts and in the alg2-1 yeast cells. hALG2 was shown to act as an α1,3-mannosyltransferase. The resulting Manα1,3-ManGlcN\textsubscript{4}A\textsubscript{2}-PP dolichol is further elongated by a yet unknown α1,6-mannosyltransferase.

The attachment of oligosaccharide chains onto newly synthesized proteins is one of the most widespread forms of co- and post-translational modifications and is found in animals, plants, and bacteria. Glycoproteins are located inside cells predominantly in subcellular organelles and in cellular membranes and most abundantly in extracellular fluids and matrices. The oligosaccharide moiety of the glycoproteins can affect their folding, their transport, as well as their biological activity and stability (2, 3). The complex process of protein glycosylation requires more than a hundred glycosyltransferases, glycosidases, and transport proteins. CDG are classified into two groups. Defects of the assembly of lipid-linked oligosaccharides or their transfer onto nascent glycoproteins compose CDG type I, whereas CDG type II includes all defects of trimming and elongation of N-linked oligosaccharides (4). In the past 7 years the molecular nature of eight CDG-I and four CDG-II types could be identified (5–24).

Here we describe for the first time a molecular defect in glycoprotein biosynthesis in man which affects at the cytosolic side of the endoplasmic reticulum the transfer of mannosyl residues from GDP-Man to Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol by the enzyme hALG2. We show that the affected mannosyltransferase is the human ortholog to the yeast ALG2 gene, an enzyme that has so far not been characterized in higher eukaryotes. In the S. cerevisiae alg2-1 mutant a defect caused accumulation of Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol and Man\textsubscript{6}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol pointing to an involvement of ALG2 in mannoside addition (25, 26). However, the precise biochemical defect was not known. The characterization of the human ALG2 deficiency described here has helped to define ALG2, both from man and yeast, as the α1,3-mannosyltransferase that catalyzes the transfer of mannoside residues onto Man\textsubscript{5}GlcN\textsubscript{6}A\textsubscript{2}-PP-dolichol.

MATERIALS AND METHODS

Cell Lines and Cell Culture—The fibroblasts from patient M. S., her father, and the controls were maintained at 37 °C under 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (PAN Biotech GmbH). The ecotropic packaging cell line FNX-Eco (ATCC) and the amphotropic packaging cell line retroPack PT67 (Clontech) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, which was heat-inactivated at 56 °C for 30 min, at 37 °C under 5% CO\textsubscript{2} unless otherwise stated.

Isoelectric Focusing and SDS-PAGE of Serum Transferrin—Isoelectric focusing and SDS-PAGE of serum transferrin was carried out as described previously (7).

Analysis of Dolichol-linked Oligosaccharides—Fibroblasts derived from controls and the patient were cultured and metabolically labeled with [2-\textsuperscript{3}H]mannose for 30 min, and dolichol-linked oligosaccharides...
carrying more than four mannosidases were extracted and analyzed by HPLC as described previously (27). D. G. N. C. α-1,2-manN-PP-dolichol oligosaccharides were extracted with chloroform/methanol (3:2) and analyzed by TLC as described previously (27).

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization–time of flight analysis of oligosaccharides released from dolichol-phosphate by mild acid hydrolysis with 20 μM HCl at 95 °C for 30 min was performed on a Bruker Reflex III (Bruker Daltonik GmbH) as described previously (27).

**Preparation of GlcNAc2-PP-dolichol**—The reaction contained in a final volume of 0.06 ml: Del-P (3.5 μg), UDP-GlcNac (0.05 μCi; specific activity 305 mCi/mmol), 30 mM Tris-HCl, pH 7.5, 19 mM MgCl2, 0.7 mM primers F1 (5’-GGAGCTTGCGCAGAAGACCC-3’) and R1 using the enzyme (equivalent to 0.05 mg of membrane protein). Incubation was performed at 37 °C for 40 min followed by addition of unlabeled UDP-GlcNac (83 μCi final concentration) and incubated for another 10 min. The reaction was stopped by addition of 1 ml of chloroform/methanol (3:2, by volume) and processed by phase partitioning as described (28). As enzyme source a solubilized extract from yeast membranes, prepared according to Ref. 29 was used. Solubilization was carried out by incubation of membranes on ice for 20 min at a protein concentration of 17 mg/ml in the presence of 2.5% Nonidet P-40. The solubilized extract was separated from the insoluble material by centrifugation at 150,000 × g for 40 min.

**Preparation of Man, GlcNAc2-PP-dolichol**—The reaction contained in a final volume of 0.06 ml: 14C-GlcNAc2-PP-dolichol (18,000 cpm), 29 mM Tris-HCl, pH 7.2, 14% NaCl, 14% glycerol, and 0.5% Nonidet P-40, 0.5 mM DTT, 11 mM MgCl2, 1.5 mM GDP-Man, and solubilized enzyme (equivalent to 0.65 mg of membrane protein). Incubation was carried out for 50 min at 24 °C, stopped by addition of 1 ml of chloroform/methanol (3:2, by volume), followed by phase partitioning (28). Both lower and interphase were collected and combined. As enzyme source a solubilized extract from yeast membranes prepared according to Ref. 29 was used. Solubilization was carried out at a protein concentration of 6.5 mg/ml in the presence of 1.5% Nonidet P-40 as described above.

**Preparation of Manα1→6Manα1→4GlcNAc2-PP-dolichol**—Manα1→6Manβ1→4; GlcNAc2-PP-dolichol was produced from 2’-β-HMan,GlcNAc2-PP-dolichol (Manα1→6Manβ1→4; GlcNAc2-PP-dolichol) as described previously (31). The reaction contained in a final volume of 0.015 ml. The reaction was stopped by addition of 1.5% Nonidet P-40 as described above.

**Elongation of Manα1→6Manβ1→4GlcNAc2-PP-dolichol**—The reaction contained the following in a final volume of 0.06 ml: Manα1→6Manβ1→4GlcNAc2-PP-dolichol (0.300 μCi) or 2’-β-HMan,GlcNAc2-PP-dolichol (6.0 mCi) as described previously (31). Solubilization was carried out as described above. The solubilized extract was obtained from a yeast membrance fraction described (23). The solubilized enzyme (equivalent to 0.05 mg of membrane protein). Incubation was performed at 37 °C for the time indicated, stopped with chloroform/methanol to give a ratio of chloroform/methanol/water of 2:1:1 (by volume) and processed further phase separation (28) using an upper phase of chloroform/methanol/water of 1:3:2.8 by volume) and collecting both lower and interphase. The (α1→6)-linkage in the Manα1→6Manβ1→4GlcNAc2-PP-dolichol product formed was determined by incubation of the tetrasccharide, released from the lipid by mild acid hydrolysis (30), with recombinant α1→6-mannosidase (Calbioso) or recombinant Calbioso (Calbioso) and HPLC analysis. The product, released from the lipid in the case of α1→6-mannosidase treatment (1.1 milliunits of enzyme, 3.5 h of incubation), Manα1→6Manβ1→4GlcNAc2 was converted to Manβ1→4GlcNAc2, and no digestion was observed using α1→2-mannosidas (10 units of enzyme, 24 h of incubation). Incubation conditions were as suggested by the manufacturer. Control tests, to verify the correct function of the mannosidas used, were carried out with α1→6-mannobiose, α1→2-mannobiose, and mannose1→3 mannose1→2 mannotriose as substrates, obtained by acetylation from yeast mannan.

**Elongation of Manα1→4GlcNAc2-PP-dolichol and 2’-β-HMan,GlcNAc2-PP-dolichol**—The reactions contained the following in a final volume of 0.06 ml: Manα1→4GlcNAc2-PP-dolichol (0.300 μCi) or 2’-β-HMan,GlcNAc2-PP-dolichol (6.0 mCi) as described previously (31). The reaction was incubated at 24 °C for 40 min followed by addition of unlabeled UDP-GlcNac to a final concentration of 0.06 ml: Del-P (3.5 μg), UDP-GlcNac (0.05 μCi; specific activity 305 mCi/mmol), 30 mM Tris-HCl, pH 7.2, 1.5 mM GDP-Man, and solubilized enzyme (equivalent to 0.05 mg of membrane protein). Incubation was performed at 37 °C for the time indicated, stopped with chloroform/methanol to give a ratio of chloroform/methanol/water of 2:1:1 (by volume) and processed further phase separation (28) using an upper phase of chloroform/methanol/water of 1:3:2.8 by volume) and collecting both lower and interphase. The (α1→6)-linkage in the Manα1→6Manβ1→4GlcNAc2-PP-dolichol product formed was determined by incubation of the tetrasccharide, released from the lipid by mild acid hydrolysis (30), with recombinant α1→6-mannosidase (Calbioso) or recombinant Calbioso (Calbioso) and HPLC analysis. The product, released from the lipid in the case of α1→6-mannosidase treatment (1.1 milliunits of enzyme, 3.5 h of incubation), Manα1→6Manβ1→4GlcNAc2 was converted to Manβ1→4GlcNAc2, and no digestion was observed using α1→2-mannosidas (10 units of enzyme, 24 h of incubation). Incubation conditions were as suggested by the manufacturer. Control tests, to verify the correct function of the mannosidas used, were carried out with α1→6-mannobiose, α1→2-mannobiose, and mannose1→3 mannose1→2 mannotriose as substrates, obtained by acetylation from yeast mannan.

**Sequence analysis of the PCR products and the plasmids was done by dye-terminated cycle sequencing with the primers pUC M13 forward, pUC M13 reverse (Stratagene Europe), F2, Ex1-O (5’-TCCTGGGCGCT- TACGTCGGTT-3’), Ex2-L (5’-GGAAGATGTGGACCAATCTA CAGG-3’), R2 on a Applied Biosystems model 373A automated sequencer.**

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age of 5 months, a cranial magnetic resonance tomography showed a severely retarded myelinization. A follow-up MRT at the age of 8 months showed that myelin formation had come to a standstill and that the volume of white matter was markedly reduced. Mental and motor development were both severely delayed, and tendon reflexes were brisk without distinct spasticity. Hearing was not impaired. With the exception of a coccygeal dimple, a faint cardiac murmur, and borderline enlargement of the liver, the remainder of physical findings were unremarkable.

Extensive laboratory investigations failed to reveal any significant metabolic or hematological abnormality with the exception of a prolonged activated partial thromboplastin time.

Because abnormalities of these parameters had been described in cases of CDG, investigations on the glycosylation state of serum transferrin by isoelectric focusing and SDS-PAGE were carried out.

Isoelectric Focusing and SDS-PAGE of Serum Transferrin—The isoelectric focusing of serum transferrin, the standard diagnostic procedure for CDG, showed an increased amount of di- and asialo-transferrin at the expense of tetrasialo-transferrin, a pattern characteristic of CDG-I (Fig. 2, upper panel). The activity of phosphomannomutase and phosphomannose isomerase, which are missing in two of the most common forms of CDG, CDG-Ia and Ib, were found to be normal.

Analysis of Protein- and Dolichol-derived Oligosaccharides—To determine whether the loss of complete N-glycan chains in transferrin molecules of the patient was due to a reduced transfer of the oligosaccharide Glc3Man9GlcNAc2 from dolichol-PP onto newly synthesized glycoproteins in the endoplasmic reticulum by the oligosaccharyltransferase, [2-3H]mannose-labeled oligosaccharides were released from the total glycoprotein fraction by peptide:N-glycosidase F digestion and analyzed by HPLC. N-Glycans from control and patient fibroblasts eluted mainly at the positions of Glc3Man9GlcNAc2 and Man9GlcNAc2 standards, respectively (Fig. 3, A and D). In the patient, the amount of 3H radioactivity in N-glycans was consistently reduced to about 70% of controls, although the oligosaccharyltransferase activity as well as the activities of N-acetylgalactosaminyltransferase I and II and dolichol-P-mannose synthase were not significantly altered (data not shown). Furthermore, the size of the oligosaccharides in the N-glycan fraction released from newly synthesized glycoproteins was normal (Fig. 3D).

Further investigations focused on the analysis of dolichol-linked oligosaccharides, which so far have been observed to be truncated in all known CDG-I types, except CDG-Ib (7). Fibroblasts from control and patient were metabolically labeled with [2-3H]mannose, and the dolichol-linked oligosaccharides were extracted with a mixture of chloroform/methanol/water (10:10:3). The glycan moiety was released by mild acid hydrolysis and analyzed by HPLC. The main peak fraction in control and the patient cells eluted with a Glc3Man9GlcNAc2 standard (Fig. 3, A and C), although the amount of Glc3Man9GlcNAc2 was slightly reduced in case of the patient.

In order to examine the more hydrophobic dolichol-linked saccharides with short sugar chains that in part may have escaped the extract with chloroform/methanol/water (10:10:3), we also analyzed the chloroform/methanol (3:2) extract of [2-3H]mannose-labeled fibroblasts by thin layer chromatography (Fig. 4). Besides radioactivity that comigrated with dolichol-P-mannose, only non-migrating material at the origin, which was supposed to be Man9GlcNAc2-PP-dolichol and Man9GlcNAc2-PP-dolichol. For further characterization of the dolichol-linked oligosaccharides from the chloroform/methanol (3:2) extract, the oligosaccharide moieties were released by mild acid hydrolysis and analyzed by HPLC (Fig. 5). In case of the patient two [2-3H]mannose-labeled oligosaccharides were observed. Their masses, 917.2 and 1079.2 Da, as determined by
Deficiency of GDP-mannose:Man1GlcNAc2-PP-dolichol ManT Causes CDG-II

Fig. 3. Analysis of dolichol- and protein-derived oligosaccharides in CDG-II. Fibroblasts from a control person (A) and the patient (C) were metabolically labeled with [2-3H]mannose for 30 min. Short lipid-linked oligosaccharides were released from the dolichol-PP moiety by mild acid hydrolysis and size-fractionated by HPLC. \( M_1G_1 \) refers to the position of a Glc,Man,GlcNAc, standard. Glycoprotein-derived oligosaccharides from the control (B) and the patient (D) were prepared after metabolic labeling with [2-3H]mannose for 30 min. Short linkage was inferred from the glycoprotein fraction by peptide:N-glycosidase F digestion and size-fractionated by HPLC. \( M_2G_2 \) and \( M_1G_1 \) refer to the positions of Man, G and Glc,Man, G and GlcNAc, standards, respectively.

Fig. 4. Thin layer chromatography analysis of short dolichol-linked oligosaccharides. Fibroblasts derived from a control person and patient M. S., respectively, were metabolically labeled for 30 min with [2-3H]mannose. Short lipid-linked oligosaccharides were extracted with chloroform/methanol (3:2) and further analyzed by TLC in a running buffer containing chloroform/methanol/H2O (65:25:4). The position of the origin, a [3H]mannose-P-dolichol standard, the assumed positions of [3H]Man,GlcNAc,PP-dolichol, and [3H]Man,GlcNAc,PP-dolichol are indicated.

Fig. 5. High performance liquid chromatography and mass spectrometric analysis of short dolichol-linked [3H]mannose oligosaccharides. Short dolichol-linked oligosaccharides were extracted from control and patient fibroblasts after metabolic labeling with [2-3H]mannose for 30 min. The oligosaccharide moieties were released by mild acid hydrolysis, separated by HPLC, and subsequently analyzed by liquid scintillation counting. The peak fractions were further investigated by mass spectrometry. The values beside the HPLC peaks indicate the detected masses. The \( \alpha-1,3 \)-linkage of Man,GlcNAc, is inferred from the enzymatic studies presented in Fig. 6. N-acetyl-glucosamine; mannose.

Deficiency of GDP-mannose:Man1GlcNAc2-PP-dolichol \( \alpha-1,3 \)-Mannosyltransferase in Patient-derived Fibroblasts—The accumulation of Man,GlcNAc,PP-dolichol and Man,GlcNAc,PP-dolichol observed in skin fibroblasts of the patient resembled the phenotype described previously for the temperature-sensitive \( S. \ cervisiae \) alg2-1 mutant (25). We therefore investigated microsomal extracts from control and patient-derived fibroblasts for their ability to elongate Man,GlcNAc,PP-dolichol and Man,GlcNAc,PP-dolichol. When cell extracts from controls were incubated with GDP-mannose and Man, [14C]GlcNAc,PP-dolichol, a time-dependent extension up to Man, [14C]GlcNAc,PP-dolichol was observed (Fig. 6C). In contrast, when using the microsomal extract from the patient as an enzyme source, hardly any elongation was detectable (Fig. 6A) indicating that in the patient the biosynthesis of dolichol-linked oligosaccharides is defective at the step adding the second mannose residue.

The elongation of Man,GlcNAc,PP-dolichol to Man,GlcNAc,PP-dolichol involves the addition of either an \( \alpha-1,3 \)- or an \( \alpha-1,6 \)-mannosyl residue (Fig. 7). In order to define the sequence in which the \( \alpha-1,3 \)- and \( \alpha-1,6 \)-mannosyl residues are added, [3H]Man,(\( \alpha-1,6 \))
Man-GlcNAc$_2$-PP-dolichol was prepared by partial enzymatic digestion from $[^3H]$Man$_5$GlcNAc$_2$-PP-dolichol and used as acceptor substrate. As shown in Fig. 6B a microsome extract from patient fibroblasts fails to elongate Man$\alpha$_1,6Man-GlcNAc$_2$-PP-dolichol in the presence of GDP-mannose, whereas a microsome extract from control fibroblasts elongates the acceptor to Man$_5$GlcNAc$_2$-PP-dolichol (Fig. 6D). The rate of elongation of the Man$_2$GlcNAc$_2$ acceptor, however, is much slower than that of the Man$_1$GlcNAc$_2$ acceptor (compare Fig. 6, C and D, note the different incubation times). These results demonstrate that Man$_1$GlcNAc$_2$-PP-dolichol is the preferred substrate of the $\alpha$1,3-mannosyltransferase and that it is this activity which is deficient in the patient.

Genetic Analysis of hALG2—Due to the similar glycosylation phenotypes in the patient fibroblasts and the $\text{alg2-1}$ strain of $S.\ \text{cerevisiae}$, we performed an NCBI Blast search for the human ortholog of the yeast ALG2 gene (accession number NP_011450). A human homolog (accession number CAC07999.1) with 37% sequence identity to the yeast Alg2 protein was identified. The hALG2 gene is located on chromosome 9q22 and encodes a polypeptide of 416 amino acids.

Sequencing of the ALG2 gene of the patient revealed heterozygosity for a $\Delta$1040G deletion and a single nucleotide substitution (G393T). The $\Delta$1040G mutation causes a frameshift with altering the sequence after amino acid 346 of hALG2 and a premature translation stop after amino acid 372 (Fig. 8). At the level of RNA the patient was homozygous for this mutation indicating that the transcript carrying the G393T substitution is unstable. The mother was heterozygous for the $\Delta$1040G mutation on the genomic and the RNA level. The father was heterozygous for the G393T mutation at the genomic level, whereas no transcripts with the G393T substitution were detectable.

Complementation for hALG2 Activity in CDG-II Fibroblasts—To confirm the deficiency of hALG2 as the primary cause of the glycosylation defect in the patient, we expressed the human ALG2 wild type enzyme as well as the $\Delta$1040G hALG2 cDNA. Further analysis was carried out by TLC. wt, wild type; transf, transfected. The positions of Man$_1$GlcNAc$_2$-PP-dolichol and Man$_1$GlcNAc$_2$-PP-dolichol as well as the origin are indicated.
Complementation of the Glycosylation and Growth Defects in a S. cerevisiae alg2-1 Mutant Strain by hALG2—To demonstrate that the human hALG2 gene is the ortholog of yeast ALG2 and to further confirm that the Δ1040G deletion is the disease causing mutation in our patient, we introduced the cDNAs encoding wild type hALG2 or the Δ1040G mutant into patient fibroblasts (Fig. 9, lane 5). These results indicate that the accumulation of shorted dolichol-linked oligosaccharides in the patient is caused by the reduced activity of the truncated hALG2 and that expression of wild type hALG2 restores the biosynthesis of dolichol-linked oligosaccharides.

Complementation of the Glycosylation and Growth Defects in a S. cerevisiae alg2-1 Mutant Strain by hALG2—To demonstrate that the human hALG2 gene is the ortholog of yeast ALG2 and to further confirm that the Δ1040G deletion is the disease causing mutation in our patient, we introduced the cDNAs encoding wild type hALG2 or the Δ1040G mutant into the temperature-sensitive alg2-1 yeast strain. Only transformation with the wild type but not with the mutant form of hALG2 cDNA restored the formation of lipid-linked oligosaccharides in alg2-1 cells (Fig. 10A). The glycosylation status of the vacuolar glycoprotein carboxypeptidase Y in the alg2-1 strain carboxypeptidase Y is underglycosylated at the non-permissive temperature of 36 °C because of a reduced transfer of truncated oligosaccharides to the protein (Fig. 10B). We also assessed the glycosylation status of the vacuolar glycoprotein carboxypeptidase Y. In the alg2-1 strain carboxypeptidase Y is underglycosylated at the non-permissive temperature of 36 °C because of a reduced transfer of truncated oligosaccharides to the protein (Fig. 10C). Transformation of the alg2-1 strain with the wild type but not with the Δ1040G hALG2 cDNA normalized the glycosylation of carboxypeptidase Y. Altogether these results demonstrate that the hALG2 gene is the ortholog of the yeast ALG2 gene.

**DISCUSSION**

A new disorder affecting one of the early steps of dolichol-linked oligosaccharide biosynthesis was identified in a patient who was born healthy but developed in the 1st year of life a multisystemic disorder with mental retardation, defective myelinization, seizures, coloboma of the iris, hepatomegaly, and coagulation abnormalities. The molecular defect in this disorder, termed CDG-II, is a deficiency of hALG2 that catalyzes the elongation of Man₅GlcNAc₂-PP-dolichol.

**Biosynthesis of N-glycans starts at the cytoplasmic side of the endoplasmic reticulum membrane with the stepwise addition of two N-acetylglucosamine and five mannose residues onto the lipid carrier dolichol-phosphate. Donors are the respective nucleotide sugars. The product is Man₅GlcNAc₂-PP-dolichol, which is translocated to the luminal face of the endoplasmic reticulum membrane by a reaction that involves RFT1.
Whereas in the nature resemble each other closely, there is one difference. Blasts and of yeast types. Although the biochemical phenotype of the patient fibroblasts and yeast alg2-1 aspartylproteinase carboxypeptidase Y were normalized. Growth behavior, and the glycosylation of the yeast vacuolar membrane proteins predicted to have 8-13 transmembrane helices (37).

The accumulation of the short dolichol-linked oligosaccharides Man,GlcNAc2-PP-dolichol and Man,GlcNAc2-PP-dolichol gave the first hint for identification of the defect. The accumulation pattern in patient-derived fibroblasts resembled that of the S. cerevisiae alg2-1 strain at the restrictive temperature (25, 28).

Two different mutations were detected in the human ortholog of the yeast ALG2 gene: a substitution mutation inherited from the father, which did not give rise to stable transcripts, and a single nucleotide deletion inherited from the mother, which did not affect the stability of the transcripts. The disease-causing nature of the mutations in the hALG2 gene could be demonstrated by complementation of the mannosylation defect in patient-derived fibroblasts by transduction of the wild type hALG2 cDNA, whereas the hALG2 cDNA carrying the maternal mutation did not restore the defect.

The orthologous nature of hALG2 and yeast ALG2 was further substantiated by expression of the hALG2 cDNA in the yeast alg2-1 strain that led to phenotypic complementation. The biosynthesis of dolichol-linked oligosaccharides, the growth behavior, and the glycosylation of the yeast vacuolar aspartylproteinase carboxypeptidase Y were normalized. Again, transfection with a hALG2 cDNA carrying the maternal single nucleotide deletion did not complement the alg2 pheno-types. Although the biochemical phenotype of the patient fibroblasts and of yeast alg2-1 strain at the non-permissive temperature resemble each other closely, there is one difference. Whereas in the alg2-1 strain Man,GlcNAc2-PP-dolichol and Man,GlcNAc2-PP-dolichol are transferred from dolichol to newly synthesized glycoproteins (38), no shortened oligosaccharides could be detected in newly synthesized proteins of the patient. A possible explanation might be a somewhat different substrate specificity of the oligosaccharyltransferase in yeast and man.

The elongation of Man,GlcNAc2-PP-dolichol can occur by the addition of either an α,3- or an α,6-linked mannose. Both reactions are likely to be catalyzed by two different mannosyltransferases (see Fig. 7). As shown here the hALG2 deficiency is associated with an inability to elongate both Manβ,1,GlcNAc2-PP-dolichol and Manα,1,6-Manβ,1,GlcNAc2-PP-dolichol. This clearly demonstrates that the mannosyltransferase encoded by hALG2 has an α,3-linkage specificity. The comparison of the elongation rate of Manβ,1,GlcNAc2-PP-dolichol and Manα,1,6-Manβ,1,GlcNAc2-PP-dolichol in extracts from control fibroblasts, which contain both the hALG2 encoded α,1,3-mannosyltransferase and the α,1,6-mannosyltransferase encoded by a gene of unknown function, provides evidence for the order of the α,3- and the α,1,6-mannosylation steps. The preferred reaction sequence is the addition of the α,1,3-linked mannose residue to Man1,αβ,GlcNAc2-PP-dolichol by ALG2 followed by elongation of the Man1,αβ,Man2GlcNAc2-PP-dolichol by an α,1,6-mannosyltransferase (Fig. 7). This result supports previous data of the dolichol-linked oligosaccharide biosynthesis in Chinese hamster ovary cells (39). The fact that hALG2 complements the yeast alg2-1 mutation indicates also that the yeast ALG2 gene encodes the α,1,3-mannosyltransferase.

Under the conditions of the in vitro assay, the α,1,6-mannosyltransferase was inactive toward Manβ,1,GlcNAc2-PP-dolichol. Fibroblasts of the patient, however, accumulated also Manβ,1,GlcNAc2-PP-dolichol. This indicates that under conditions such as ALG2 deficiency, some of the accumulating Manβ,1,GlcNAc2-PP-dolichol can be elongated by the α,1,6-mannosyltransferase.

The normal size pattern of dolichol-linked oligosaccharides in the chloroform/methanol/H2O (10:10:3) extract of fibroblasts and the residual N-glycosylation clearly demonstrate that the defect in the patient is leaky as observed in other CDG-type I forms. Moreover, the defect would have been missed, if the analysis of dolichol-linked oligosaccharides would have been restricted to the chloroform/methanol/H2O (10:10:3) extract. The early intermediates such as Manβ,1,GlcNAc2-PP-dolichol are enriched in the chloroform/methanol (3:2) extract. Analysis of the latter fraction should therefore routinely be performed and may help to elucidate further defects among the group of CDG type I patients still awaiting molecular identification (40).

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A New Type of Congenital Disorders of Glycosylation (CDG-Ii) Provides New Insights into the Early Steps of Dolichol-linked Oligosaccharide Biosynthesis

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