Congenital Disorder of Glycosylation Type Ik (CDG-Ik): A Defect of Mannosyltransferase I

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This study describes the discovery of a new inherited disorder of glycosylation named "CDG-Ik." CDG-Ik (congenital disorder of glycoslyation type Ik) is based on a defect of human mannosyltransferase I (MT-I [MIM 605907]), an enzyme necessary for the elongation of dolichol-linked chitobiose during N-glycan biosynthesis. Mutations in semiconserved regions in the corresponding gene, *HMT-1* (yeast homologue, *Alg1*), in two patients caused drastically reduced enzyme activity, leading to a severe disease with death in early infancy. One patient had a homozygous point mutation (c.773C→T, S258L), whereas the other patient was compound heterozygous for the mutations c.773C→T and c.1025A→C (E342P). Glycosylation and growth of *Alg1*-deficient PRY56 yeast cells, showing a temperature-sensitive phenotype, could be restored by the human wild-type allele, whereas only slight restoration was observed after transformation with the patients' alleles.

Introduction

Mannosyltransferase I (MT-I [MIM 605907]) catalyzes the first mannosylation reaction in the biosynthesis of dolichol-linked oligosaccharides, forming a β1,4-linkage with the adjacent N-acetylglucosamine (GlcNAc) residue of chitobiose (Couto et al. 1984). In *Saccharomyces cerevisiae*, the transferase is encoded by *asparagine-linked glycosylation 1* (*Alg1*). The coding sequence of the human *MT-I* gene, *HMT-1* (also named "*HMAT-1*" [*human mannosyltransferase 1*]; GenBank accession number AB019038), was found by homology search by use of the yeast gene (Takahashi et al. 2000). *HMT-1* encodes an unglycosylated 52.5-kDa type II transmembrane protein consisting of 464 amino acids. Its amino acid identity to the yeast homologue is 36%. The human protein was found to complement the growth phenotype of the yeast *ALG1* mutant (Takahashi et al. 2000).

In yeast, the gene defect results in hypoglycosylation of glycoproteins (Huffaker and Robbins 1982, 1983). $GlcNAc_2$-PP-dolichol and, to a lesser extent, GlcNAc-PP-dolichol are accumulated at the restrictive temperature (36°C), but not at the permissive temperature (26°C) (Huffaker and Robbins 1982, 1983). The accumulated $GlcNAc_2$-PP-dolichol must be able to translocate across the membrane of the endoplasmic reticulum (ER), since it can be transferred to newly synthesized proteins in ALG1 cells (Cueva et al. 1998). The ALG1 phenotype is largely corrected by increasing the GDP-mannose pool by use of overexpression of GDP-mannose synthase (GDP-mannose pyrophosphorylase, Mpg1p), whereas the addition of 10 mM mannose to the culture medium had no effect (Janik et al. 2003).

In this study, we describe a new inherited metabolic disease, which is caused by a defect of *HMT-1*. The disease belongs to the rapidly growing group of disorders affecting glycan biosynthesis named "congenital disorders of glycosylation" (CDG). According to the current nomenclature, *HMT-1* deficiency is named "CDG-Ik." The clinical phenotype of the first two identified patients with CDG-Ik analyzed in this study was unusually severe, with death in early infancy.

Material and Methods

Patients

Skin biopsies were taken from two patients (NK, GM) after informed consent of the parents, and fibroblasts were cultivated in minimal essential medium (MEM). EDTA blood samples were taken from the parents to extract genomic DNA to confirm their heterozygous carrier status.

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Yeast Strains

The yeast strains that were used for the experiments are listed in table 1. (For reference, see the *Saccharomyces* Genome Database Web site.)

Yeast Culture

Yeast cells were grown in YPD medium containing 1% yeast extract, 2% peptone, and 1% glucose (pH 6.5). Selection was done in YNB medium (Difco yeast nitrogen base [BD Biosciences]) with 2% glucose. Finally, 2% agar was added for plates.

Labeling of Human Fibroblasts and Yeast Cells

Fibroblasts were labeled for 30 min or 1 h with 100 μCi [2-^{3}H]mannose (specific activity 10–20 Ci/mmol) or 500 μCi [6-^{3}H]glucosamine (specific activity 15–35 Ci/mmol) per ml of Dulbecco's Modified Eagle Medium without glucose (1.2:1) (50 μM mannose) and were washed with PBS. Yeast cells were labeled for 20 min with 2 mCi [2-^{3}H]mannose or [6-^{3}H]glucosamine per ml YP medium (1% yeast extract, 2% peptone [pH 6.5]). Before labeling, the cells had been grown overnight at 30°C in YP medium with 1% glucose. Of this preculture, 10% were used to inoculate the main culture, which grew for 4 h in log phase until cells were harvested and labeled.

Extraction of Lipid-Linked Oligosaccharides (LLO) and Protein-Derived Oligosaccharides (PDO)

For LLO and PDO isolation, cells were extracted three times with chloroform:methanol (2:1). The pellet was dried under nitrogen and extracted several times with water. Dolichylpyrophosphate-linked oligosaccharides were predominantly recovered from the subsequent chloroform:methanol:water (10:10:3) extract and released by mild acid hydrolysis for 20 min at 100°C in n-propanolol:0.1 N HCl (1:2). The pellet obtained by the chloroform:methanol:water extraction was digested overnight at 37°C with PNGase F (New England BioLabs) after denaturation for 10 min at 100°C to release the protein-linked oliogsaccharides.

For thin-layer chromatography (TLC), dolichol-linked oligosaccharides were extracted with chloroform:methanol (3:2) and were separated on Silica IB2 plates (Mallinckrodt Baker) by use of chloroform:methanol:water (65:25:4) as solvent. For each lane, ~300,000 dpm were transferred to the plate. For the detection of radioactivity, films were exposed for 4 d at −80°C to dried TLC plates treated with EN^{3}HANCE (NEN Life Science Products). Spots were scraped from the TLC plate and counted in a scintillation counter.

Table 1

Yeast Strains Used in Study

Yeast Type	Yeast Strain
Control strains:	
AH22	*MATa, leu2 his4, Cir*$^{+/-}$
BY4741	*MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
Mutant strains:	
pRY55	*MATa, alg1-1 ura3-52*
pRY56	*MATa, alg1-1 ura3-52*
pRY59	*MATa, alg1-1 ura3-52*

Mutation Analysis

mRNA was transcribed by reverse transcriptase (1st Strand cDNA Synthesis Kit [Roche]), and the coding sequence of the human *MT-I* was amplified by PCR. Primers were HMT-1f 5′-GTACGGATCCGGCGGGCCAGCCAAGATGGC-3′ and HMT-1r 5′-GATCGTCGACCAACGTGGACACACTCAGTT-3′ (Takahashi et al. 2000). The samples were incubated at 94°C for 3 min. After 35 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 2 min, a final incubation was performed at 72°C for 10 min. Proof-reading polymerase Pfx (Invitrogen) was used for the generation of amplicons for expression cloning. For sequencing, PCR products were cloned by TA-cloning and sequenced with standard primers by use of an automated DNA sequencer (Applied Biosystems).

Since several pseudogenes for *HMT-1* exist in the human genome, only primers with 3′-ends matching the correct *HMT-1* sequence were selected. For confirmation of the mutations at the genomic level, exons 7 and 10 were amplified using the primers HMT-1-Ex7-f 5′-GCCACGGCAGGAGATGCCTC-3′, HMT-1-Ex7r 5′-CAGCTCACAGGCCCTCCCG-3′, HMT-1-Ex10-f 5′-ACACACCCCTGCAGGTCTCA-3′ and HMT-1-Ex10-r 5′-ACCCCTTGGTAAACGGCCC-3′. The exon 7 PCR was incubated at 94°C for 3 min, and the samples were cycled 35 times: 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min. Final incubation was at 72°C for 10 min. The same conditions were used for the exon 10 PCR, with an annealing temperature of 62°C and the addition of Q-Solution (Qiagen) for the incubations.

To exclude common polymorphisms, 100 alleles of healthy donors of white European background were sequenced in each case. In this investigation, the published sequences of human *HMT-1* at the genomic level (GenBank accession number NT_010552) and of the coding region (GenBank accession numbers AB019038 and NM_019109) have been used and confirmed.

Mannosyltransferase Assays

The reactions contained the following in a final volume of 0.06 ml: [^{14}C]GlcNAc$_2$-PP-dolichol (4,000 cpm)

or Man_1[^{14}C]$GlcNAc_2$-PP-dolichol (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 50 μg membrane protein). Incubations were performed at 37°C for the durations indicated. Reactions were stopped by addition of chloroform:methanol to give a ratio of chloroform:methanol:water of 2:1:1 (by vol) and were processed further by phase separation (Sharma et al. 1982) by use of an upper phase of chloroform:methanol:water of 1:32:48 (by vol) and by collecting both lower phase and interphase. 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 P40. Preparation of [^{14}C]$GlcNAc_2$-PP-dolichol and Man_1[^{14}C]$GlcNAc_2$-PP-dolichol acceptors was performed using as enzyme source a solubilized extract from yeast membranes, as described elsewhere (Thiel et al. 2003).

Construction of the Expression Vector

The cDNA generated by the PCR reaction by use of HMT-1f and HMT-1r (described above) was digested with *Bam*HI and *Sal*I (New England BioLabs) and cloned into the expression vector pYEX-BX (Clontech) that had been digested with the same enzymes. After amplification in bacteria (TOP10F′ (Invitrogen]), the vector constructs were purified and the sequence of the coding region was verified by sequencing by use of an automated DNA sequencer and standard procedures (Applied Biosystems). Induction of the yeast expression from pYEX-BX was done by the addition of 0.4 mM $CuSO_4$ to the culture medium. Transformation was done using standard procedures (Gietz and Woods 2002).

Results

Patients

Two patients (NK, GM) with a defect of β1,4-mannosyltransferase were identified and analyzed in this study. Both patients had a type I isoelectric focusing (IEF) pattern of serum transferrin with a hypoglycosylation much more pronounced than that found in the most common CDG, CDG-Ia (fig. 1). In early infancy, the children presented with recurrent seizures refractory to anticonvulsive therapy, rapidly developing microcephaly, and severe coagulation abnormalities. Patient NK was incapable of visual fixation. Seizures started at 5 mo of age. General cerebral atrophy was revealed by MRI. Frequent episodes of unexplained fever occurred. The boy developed progressive stupor and died at 10 mo of age. Patient GM developed seizures already 2 h after birth. At 1 mo of age, severe muscular hypotonia was present and MRI revealed cerebral atrophy. Nephrotic syndrome was present and a severe decrease of circulating B-cells with a complete absence of immunoglobulin G (IgG) in the blood was noticed. Severe seizures and progressive stupor developed, and the boy died from respiratory failure at 11 wk of age. More details about the clinical phenotype and the hypoglycosylation of serum glycoproteins in this disorder will be described elsewhere (Marquardt et al., unpublished data).

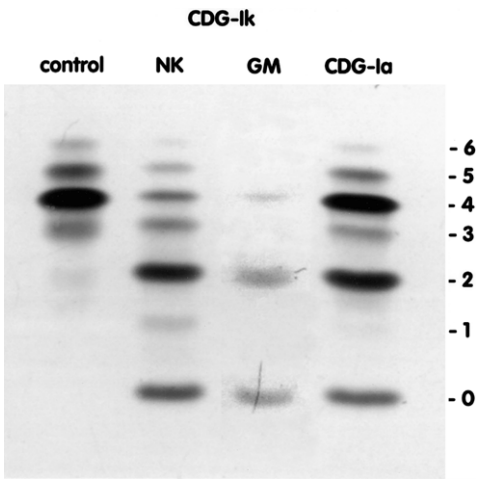

Figure 1 IEF of serum transferrin. The numbers on the right describe the numbers of sialic acids. Transferrin has two branched carbohydrate side chains, each bearing two sialic acids at the end. Fully glycosylated transferrin has four sialic acids; higher branching leads to increased numbers of sialic acids in a small fraction of the protein. Patients with CDG-Ia have either one or both carbohydrate side chains missing in about half of the transferrin population. The hypoglycosylation in CDG-Ik is much more severe.

Metabolic Labeling of Fibroblasts

Subconfluent fibroblasts of the two patients were labeled with 100 μCi [2-^{3}H]mannose and 10 μCi [^{35}S]methionine per ml for 30 min, as described elsewhere (Kranz et al. 2001). LLO were subjected to high-performance liquid chromatography (HPLC) analysis and showed a major oligosaccharide peak at the position of $Glc_3Man_9GlcNAc_2$, indicating that LLO after mannose labeling were structurally intact (not shown). To determine the amount of newly synthesized LLO, the radioactivity incorporated into LLO was determined and related to the amount of total cellular protein. On average, the amount of radioactivity incorporated after [2-^{3}H]mannose labeling into newly synthesized LLO was reduced to 29.1% in patient NK and to 39.9% in patient

GM (table 2). Labeling with [^{3}H]glucosamine revealed an increased incorporation (table 2). The results demonstrated that the patients' cells synthesized a reduced amount of mature LLO and that glucosamine incorporation was not diminished, whereas mannose incorporation was greatly reduced. Since no truncated oligosaccharides were observed in the HPLC analysis of mannose-labeled LLO, we concluded hat the cells must have an early defect in the incorporation of mannose into LLO, most likely in the incorporation of the first mannose.

Accumulation of $GlcNAc_2$-PP-Dolichol in Fibroblasts

Fibroblasts were labeled with 500 μCi/ml [^{3}H]glucosamine for 1 h and LLO were extracted. Next, 300,000 dpm of the labeled sample was placed on a TLC plate and separated, as described above. Wild-type and ALG1 yeast were labeled at the same time as controls. Three different ALG1 mutants (PRY55, PRY56, and PRY59), known to produce mainly $GlcNAc_2$-PP-dolichol (Huffaker and Robbins 1982, 1983), accumulated the radioactive label in a band migrating further than in the two different wild-type yeast strains (fig. 2, *row 2*). Thus, dolichol-linked oligosaccharides extracted from labeled ALG1 yeast cells were used as controls. This band was not found after labeling with [^{3}H]mannose, demonstrating that the band contained only glucosamine and no mannose (not shown).

The radioactive label in control fibroblasts was mainly found in higher–molecular-weight dolichol-linked oligosaccharides, as demonstrated by HPLC. These dolichol-linked oligosaccharides migrated on TLC plates to the same position as the one isolated from wild-type yeast cells. CDG-Ik fibroblasts also synthesized higher–molecular-weight oligosaccharides, as found in their wild-type counterparts, but, in addition, they showed an accumulation at the position of the band with higher mobility found in ALG1 yeast known to accumulate $GlcNAc_2$-PP-dolichol. Quantification of the bands by scintillation counting revealed that both CDG-Ik fibroblast cultures accumulated approximately three times more label at the $GlcNAc_2$-PP-dolichol position than healthy controls. In contrast to ALG1 yeast cells, the majority of the label in CDG-Ik cells was recovered at the wild-type position, indicating that the defect must be leaky in the two patients.

Table 2

Mannose and Glucosamine Incorporation

	Radioactivity (%) Incorporated into LLO after Labeling with	
Subject	[^{3}H]mannose	[^{3}H]glucosamine
Control	100	100
Patient NK	29.1 (n = 5)	185.0 (n = 3)
Patient GM	39.9 (n = 4)	119.6 (n = 3)

Note.—Fibroblasts were labeled for 30 min with 100 μCi [2-^{3}H]mannose or [6-^{3}H]glucosamine per ml labeling medium and dolichol-linked oligosaccharides were extracted as described. Total cellular protein amount was determined, and the ratio of the incorporated label to the total cellular protein was calculated. Controls were set to 100.

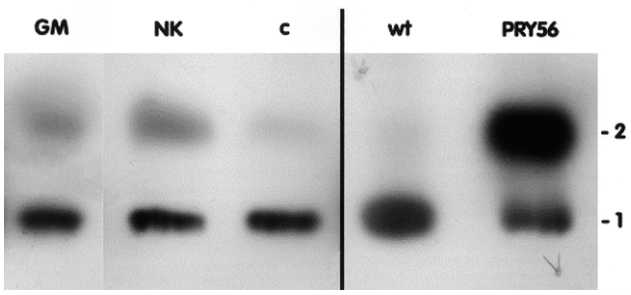

Figure 2 TLC of dolichol-linked oligosaccharides. Fibroblasts were labeled with [^{3}H]glucosamine for 1 h, and dolichol-linked oligosaccharides were extracted with chloroform:methanol (3:2), as described. Lanes 1–3 show fibroblasts (GM, NK = CDG-Ik fibroblasts; c = control fibroblasts). Lanes 4–5 show yeast fibroblasts (wt = wild type; PRY56 = ALG1 mutant). 1 = migration of higher–molecular-weight dolichol-linked oligosaccharides, 2 = migration of the dolichol-linked oligosaccharides accumulated in the ALG1 mutant.

Elongation of $GlcNAc_2$-PP-Dolichol in the Presence of Cell Extracts

The substrate for the MT-I reaction, [^{14}C]-labeled $GlcNAc_2$-PP-dolichol, was incubated in the presence of detergent-solubilized membrane extracts from control and patient fibroblasts. Incubations were done for 12 min in the presence of 1 mM GDP-mannose. The reactions were stopped by the addition of chloroform:methanol, and dolichol-linked oligosaccharides were analyzed by HPLC.

In control extracts, nearly half of the substrate was elongated, mainly to $Man_5GlcNAc_2$-PP-dolichol (fig. 3*A*). In fibroblast extracts from both patients, only a small fraction of the substrate was elongated (about 10% of controls), confirming the deficiency of the elongation reaction for the $GlcNAc_2$ substrate.

To demonstrate that the subsequent mannosylation reaction was unaffected, Man_1[^{14}C]$GlcNAc_2$-PP-dolichol was used as substrate in the presence of GDP-mannose (fig. 3*B*). In the control, the substrate (fig. 3*B.a*) was elongated after 4 min incubation to Man_{3-5}[^{14}C]$GlcNAc_2$-PP-dolichol (fig. 3*B.b*). In both patients with CDG-Ik, the elongation that occurred was the same as in the control (fig. 3*B.c* and 3*B.d*), demonstrating a specific deficiency

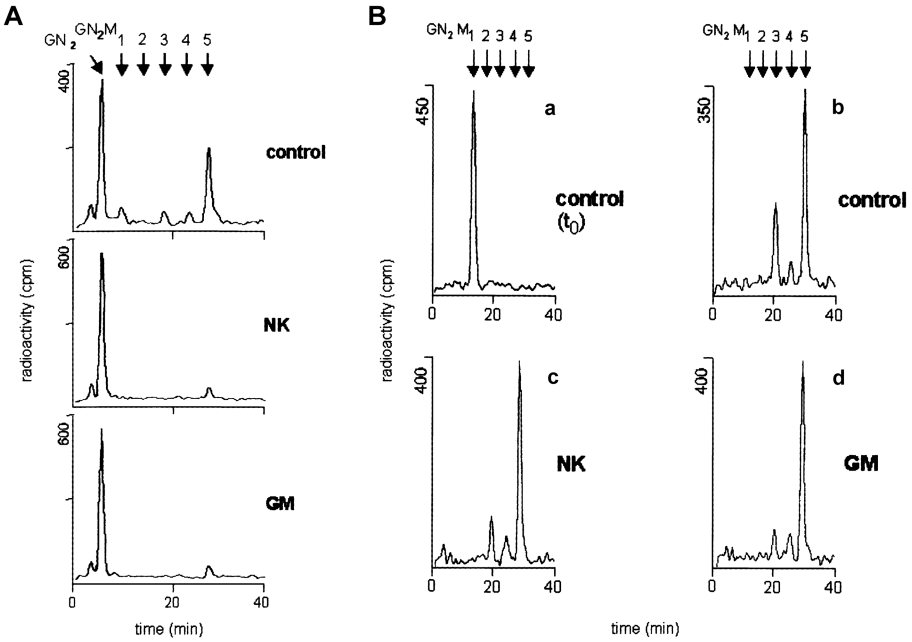

Figure 3 In vitro elongation of $GlcNAc_2$-PP-dolichol (*A*) or $Man_1GlcNAc_2$-PP-dolichol (*B*). NK and GM are patients with CDG-Ik. [^{14}C]-labeled $GlcNAc_2$-PP-dolichol was incubated in the presence of detergent-solubilized membrane extracts from control and patient fibroblasts. Dolichol-linked oligosaccharides were analyzed by HPLC. In control extracts, nearly half of the substrate *A*). To demonstrate that the subsequent mannosylation reaction was unaffected, Man_1[^{14}C]$GlcNAc_2$-PP-dolichol was used as substrate (*B*). In both patients with CDG-Ik (*B.c, B.d*), elongation occurred equal to that in the control (*B.a, B.b*). GN = N-acetylglucosamine; M = mannose.

in the first mannosylation reaction and unaffected subsequent biosynthesis steps.

Analysis of the HMT-I *Gene*

In the first patient (NK), mutation analysis of the *HMT-1* gene revealed compound heterozygosity for mutations c.773C→T (Ser258Leu [paternal]) and c.1025A→C (Glu342Pro [maternal]). Mutation analysis of the *HMT-1* gene of the second patient (GM) revealed homozygosity for the mutation c.773C→T (Ser258Leu). These mutations, as well as the heterozygous carrier status of the parents of both patients, were confirmed at the genomic level by direct sequencing. Both amino acid substitutions occurred at semiconserved positions in the protein.

Growth Phenotype of Transformed ALG1 Yeast

It has been shown that ALG1 yeast cells can be complemented by transformation with the human *MT-I* coding sequence (Takahashi et al. 2000). Since the human *ALG1* homologue can complement the deficient yeast gene, yeast cells can serve as a model system to study the functional consequences of the mutations found in the two patients. *S. cerevisiae* PRY56 cells were transformed with an expression vector containing either the wild-type coding region of *HMT-1* or one of the mutated alleles. Mock transfection was done with the vector carrying no insert. Transformed uracil auxotroph cells were selected by growth on YNB plates containing no uracil. After the selection, cells were placed in sequential 10-fold dilutions on YPD plates. PRY56 cells have a temperature-dependent phenotype (Huffaker and Robbins 1982). At the permissive temperature of 26°C, cells transformed with the mutated alleles had growth comparable to wild-type or mock-transformed cells (fig. 4, *left panel*). At the restrictive temperature of 36°C, the mock-transformed *ALG1*-deficient cells showed decreased growth compared with wild-type transformed cells (fig. 4, *right panel*). Cells transformed with either the 1025A→C allele or the 773C→T allele showed a partial restoration of the wild-type phenotype but still had decreased growth when compared with the wild-type transformed cells. Neither of the two mutations was able to restore normal growth in PRY56 cells, demonstrating an impaired function of the corresponding alleles. The same phenotype was found by analysis of LLO (not shown) and of carboxypeptidase Y (CPY) glycosylation (see online-only appendix A).

Discussion

CDG-Ik is caused by a defect in the first mannosylation reaction of N-glycan biosynthesis. Compared with other CDG types, the phenotype is very severe, with rapid

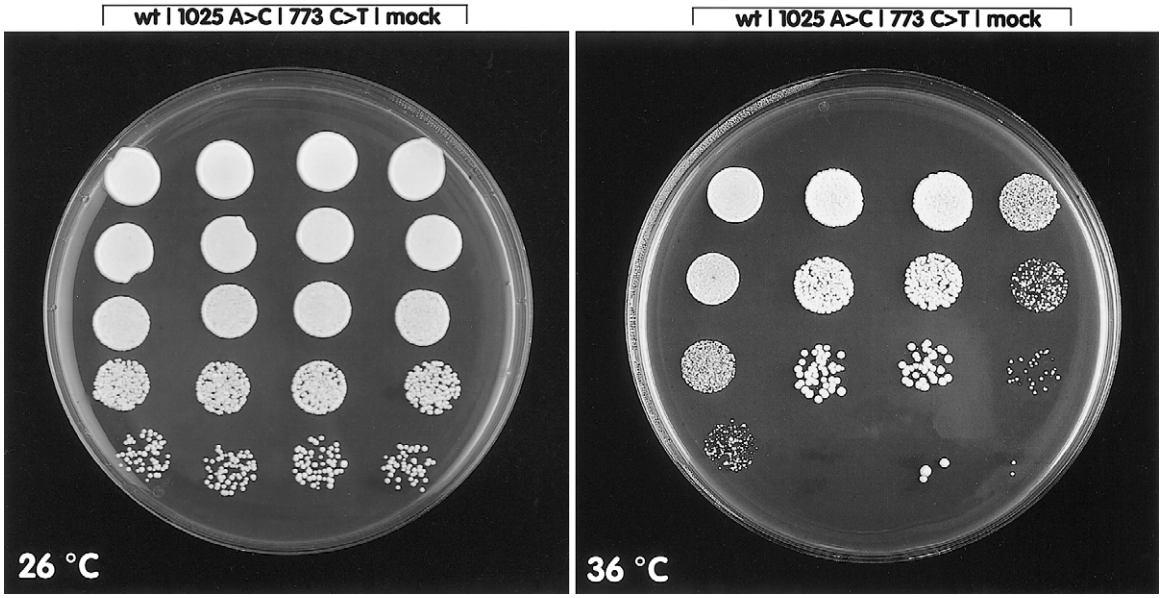

Figure 4 Growth characteristics of transformed PRY56 yeast cells showing 10-fold dilutions from top to bottom. Pictures were taken after 1 wk at the permissive temperature (26°C, *left*) or at the restrictive temperature (36°C, *right*). From left to right, wild-type transformed cells, transformed cells with the 1025A→C (NK) allele, transformed cells with the 773C→T allele (both patients), and mock-transformed cells.

development of microcephaly, seizures refractory to treatment, progressive stupor, and death in early infancy.

The hypoglycosylation of liver-derived serum glycoproteins was more profound than in CDG-Ia. Metabolic labeling of the cells with 2-[^{3}H]D-mannose, the standard procedure for the investigation of CDG fibroblasts, revealed a total amount of newly synthesized dolichol-linked oligosaccharides that was reduced to 30%–40% of that of control cells. HPLC analysis of LLO, as well as TLC of dolichol-linked oligosaccharides after radioactive labeling, demonstrated that the patients' fibroblasts were still able to synthesize full-length dolichol-linked glycans. However, the cells accumulated approximately three times more truncated LLO compared with control cell lines. The defect of the first mannosylation step was confirmed by an enzyme assay measuring the ability to elongate $GlcNAc_2$-PP-dolichol, the substrate of *MT-I,* that clearly demonstrated a profound reduction of the elongation ability. The subsequent mannosylation steps were unaffected.

Overexpression of the mutated alleles in ALG1 yeast demonstrated an accumulation of $GlcNAc_2$-PP-dolichol, whereas wild-type transformed cells were phenotypically corrected. A residual activity of about 10% in CDG-Ik fibroblasts, as well as the partial restoration of the LLO and glycosylation phenotypes in ALG1 yeast, imply a relevant residual activity of the mutated enzyme. The residual activity correlates very well with the missense mutations in semiconserved regions of the gene. It has to be assumed that a more profound deficiency of the enzyme will be lethal in humans, maybe even during intrauterine life, since, even in less complex cellular systems like yeast cells, a total *Alg1* disruption leads to inviability. For other CDG, it has been shown that a complete loss of enzymatic activity is incompatible with life (Matthijs et al. 1998).

CDG-Ik is the 15th metabolic defect in N-glycan biosynthesis discovered in the 8 years since the first disorder was described (van Schaftingen and Jaeken 1995). Because of the complexity of N- and O-glycan biosynthesis and processing, more CDG will be discovered in the years to come, making CDG one of the major groups of inherited metabolic disorders. CDG are a new challenge for basic researchers, as well as for physicians; it is more demanding than ever to use the understanding of the molecular bases of the diseases to develop effective treatments for them as has been done so far only for CDG-Ib and CDG-IIc (Niehues et al. 1998; Marquardt et al. 1999; Lühn et al. 2001).

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izinische Forschung (to J.D.). During the preparation of this manuscript, we learned that the group of C. Körner in Göttigen had independently identified and characterized a third CDG-Ik patient.

Electronic-Database Information

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

GenBank, http://www.ncbi.nih.gov/GenBank/ (for *HMAT-1* [accession number AB019038], genomic-level *HMT-1* [accession number NT_010552], and the coding region of *HMT-1* [accession numbers AB019038 and NM_019109])
Online Mendelian Inheritance of Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *MT-I* and ALG1)
Saccharomyces Genome Database, http://www.yeastgenome.org/ (for yeast genetics)

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