Protein glycosylation is an essential post-translational modification of various proteins, affecting their folding, sorting, and function. Inborn defects in the assembly and processing of glycans on glycoproteins are known as congenital disorders of glycosylation (CDG) and can affect both N- and O-glycosylation (for reviews see Marquardt and Denecke, Jaeken, and Grunewald et al.). N-glycosylation defects and especially defects in the assembly of the dolichol linked N-glycan precursor in the endoplasmic reticulum (ER) (CDG-I) result in hypoglycosylation of many kinds of (serum) glycoproteins. CDG-I is therefore a group of multisystemic disorders.

The assembly of the N-glycan precursor in the ER is a highly ordered process involving at least 30 known gene products. Mutations in 11 of these (PMM2, MPI, ALG6, DPM1, ALG3, MPDU1, ALG12, ALG8, ALG2, DPAT1, and ALG1) have been shown to cause CDG type 1 (CDG-Ia to CDG-Ik). Although it remains difficult to define a characteristic clinical phenotype for each type of CDG, mainly because only a limited number of patients have been assigned to most types, they generally share hypotonia and different degrees of mental retardation. Central nervous system defects are absent in CDG-Ib patients (with a deficiency of phosphomannose isomerase, MPI) and in the recently published CDG-Ih patient (with a deficiency of dolichyl-P-Glc:Glc 1Man 9GlcNAc 2-PP-dolichyl 1,3-glucosyltransferase (ALG8)). Thus far, the only patient has been described with a relatively mild clinical picture.

We describe the molecular and clinical features of three patients from two families with an ALG8 deficiency. All three patients presented with severe, life-threatening multi organ failure and died within their first months of life.

Diagnosis of CDG-Ih was based on the accumulation of the lipid-linked oligosaccharide intermediates dol-PP-GlcNAc 2Man 0 and dol-PP-GlcNAc 2Man 2Glc 1 in patients' fibroblasts. Mutation analysis of the ALG8 gene identified in both families a compound heterozygosity for a splice site mutation and a nonsense mutation (family 1: c.96-2A>G and p.T47P; family 2: c.672+4A>G and p.G275D). The functional effect of the ALG8 splice mutations was analysed on mRNA and the effect of the nonsense mutations was assayed in ALG8 deficient yeast strains.

CDG-Ih patients present with severe hepato-intestinal problems, and the clinical spectrum ranges from mild to very severe, including neonatal death. Central nervous system defects have not been observed in the four known patients.

METHODS

Cell culture

Primary fibroblasts obtained from skin biopsies were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM/F12, Gibco) with 4.5 g/l glucose and 10% fetal calf serum (FCS). To block α-glucosidase-I activity, cells were cultured in the presence of 250 μM castanospermine (CST) (Calbiochem) for 2 h, prior to labelling. The effect of the splice site mutations was investigated on cells grown for 16 h in the presence of 200 μg/ml puromycin (Sigma) to block nonsense-mediated mRNA decay.

Lipid-linked and protein N-linked oligosaccharide (LLO, NLO) analysis

LLO and NLO analyses were performed on extracts from fibroblasts as described in Grubemann et al. In brief, fibroblasts (about 450 cm 2) were grown to 90% confluence and labelled with 150 μCi [ 3 H]mannose for 1 h. LLOs were recovered from the cell suspension with 95% ethanol after acidification. LLOs were recovered from the cell suspension with 95% ethanol after acidification.

Abbreviations: CDG, congenital disorders of glycosylation; CPY, carboxypeptidase Y; CST, castanospermine; ER, endoplasmic reticulum; LLO, lipid-linked oligosaccharide; MPI, phosphomannose isomerase; NLO, N-linked oligosaccharide; NMD, nonsense-mediated mRNA decay.

Key points

- Congenital disorder of glycosylation type Ih (CDG-Ih) is caused by a defect in the dolichyl-P-Glc:Glc 1Man 9GlcNAc 2-PP-dolichyl 1,3-glucosyltransferase (ALG8). Thus far, the only patient has been described with a relatively mild clinical picture.
- We describe the molecular and clinical features of three patients from two families with an ALG8 deficiency. All three patients presented with severe, life-threatening multi organ failure and died within their first months of life.
- Diagnosis of CDG-Ih was based on the accumulation of the lipid-linked oligosaccharide intermediates dol-PP-GlcNAc 2Man 0 and dol-PP-GlcNAc 2Man 2Glc 1 in patients' fibroblasts. Mutation analysis of the ALG8 gene identified in both families a compound heterozygosity for a splice site mutation and a nonsense mutation (family 1: c.96-2A>G and p.T47P; family 2: c.672+4A>G and p.G275D). The functional effect of the ALG8 splice mutations was analysed on mRNA and the effect of the nonsense mutations was assayed in ALG8 deficient yeast strains.
- CDG-Ih patients present with severe hepato-intestinal problems, and the clinical spectrum ranges from mild to very severe, including neonatal death. Central nervous system defects have not been observed in the four known patients.
chloroform:methanol:H₂O extraction. Oligosaccharides released by mild acid hydrolysis from LLOs or by PNGaseF treatment of the residual pellet (NLOs) were then analysed by HPLC.

**Mutation analysis of ALG8**

Primer sets were designed to amplify the 3 exons of ALG8, including at least 50 bp of the flanking intronic regions, based on the genomic sequence of ALG8 (NT_039927.5 region 8011022:8049713). Primer sequences are available on request. The exons were amplified using standard PCR conditions, subsequently sequenced with Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied) and analysed on an ABI3100 Avant (Applied).

**Frequency of the polymorphism c.665A>G (p.N222S)**

The polymorphism c.665A>G creates an Hpy8I restriction site. Exon 6 was amplified from genomic DNA of a panel of 50 healthy individuals (Caucasians). A 10-μl sample of PCR product was subsequently digested with Hpy8I (Fermentas) and analysed on a 2% agarose gel.

**RT-PCR**

Total RNA was isolated from patients' fibroblasts and a normal control using Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was prepared with oligo-dT priming and Superscript II RNase-H reverse transcriptase (Invitrogen) using 20 μM total RNA in a total volume of 80 μl.

To analyse the effect of the splice mutations, the ALG8 sequence was amplified from cDNA with primers ALG8-1F (5'-TGG ATC ATC AGG AGA TGG GTG GGA AG) and ALG8-2R (5'-TTA CAG CAC TCA CGG ACA GC) for GB0243 and primers ALG8-2F (5'-TTT TCC AGA GAT TTG CCG TCA) and ALG8-4R (5'-CAA AAG GCT CAT TGG GGA AG) for NL0097. A 2-μl cDNA sample was used in a total volume of 50 μl with Taq DNA polymerase (Roche). Amplification conditions were 2 min at 94°C, 10 cycles of 20 s at 94°C, 30 s at 65°C (−1°C each cycle) and 1 min at 72°C followed by 25 cycles of 20 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

**Cloning of ALG8 in expression vectors**

For cloning of the ALG8 coding sequence, a yeast expression vector, ALG8 was amplified with primers ALG8-1F and ALG8-1R (5'-GCA GTT GTG GGA AGC AG) and ALG8-2R (5'-TGA CAG CTC CGA ACA GC) for GB0243 and primers ALG8-2F (5'-TTT TCC AGA GAT TTG CCG TCA) and ALG8-4R (5'-CAA AAG GCT CAT TGG GGA AG) for NL0097. A 2-μl cDNA sample was used in a total volume of 50 μl with Pwo-polymerase (Roche). The cycling conditions were 2 min at 94°C, 10 cycles of 15 s at 94°C, 30 s at 60°C, and 90 s at 68°C, followed by 20 cycles using the same conditions but increasing the extension time every cycle by 5 s. After amplification, 1 μl Taq polymerase (Roche) was added and the mixture was incubated at 72°C for 7 min to allow subsequent TA cloning.

A 2-μl sample of the ALG8 amplicon from GB0243, NL0097, and a normal control were cloned in pCR2.1 using the TA cloning kit (Invitrogen). Constructs were selected for the right orientation and inserts were sequenced in both directions. The wild type and the T47P ALG8 constructs both encode a serine (S) at position 222 while the G275D construct encodes an asparagine (N). The ALG8 inserts were then subcloned as BamHI/XhoI fragments in the BamHI/XhoI sites of p426GPD, placing them under the control of the strong glyceraldehyde-3-phosphate dehydrogenase promoter.

**Yeast strains and media**

*S. cerevisiae* strains used in this study were derivatives of YG98-1d and YG127; strain YG98-1d and its transfectants were propagated at 23°C and strain YG127 and its transfectants at 30°C.

**Complementation assays**

Western blot analysis of the carboxypeptidase Y glycoprotein (CPY) was performed as described previously. Temperature sensitivity of yeast strains was assessed on full medium (YPD) plates. For the dilution series, 5 μl of serial 10-fold dilutions of YG98-1d transfectants, grown in selective medium for 48 h, were spotted starting at 5×10³ cells/μl. Plates were incubated at 23°C, 29°C and 30°C for 46 h.

**RESULTS**

**Case reports**

The clinical and standard laboratory data of patient GB0243 were previously described in detail by Charlwood and colleagues. In brief, GB0243, the first child of healthy, unrelated Caucasian parents, showed intrauterine growth retardation, oligohydramnios, and reduced fetal movements. For the first 4 weeks of life he was sleepy, and difficult to feed with failure to thrive. He then developed diarrhoea, vomiting, and abdominal distension due to massive ascites. Routine investigations indicated anaemia and a marked tubulopathy. No arguments for CNS involvement were found based on clinical examination and cranial imaging. Despite intensive treatment with antibiotics, oxygen, diuretics, paracentesis, transfusions, electrolyte supplements, and thyroxine, he showed no real improvement and died at the age of 3 months. His sister (GB0244), conceived 4 years later, also showed poor growth in utero and reduced fetal movements. She became oedematous with electrolyte disturbance within hours of birth and died 3 days later.

Patient NL0097 was born prematurely (35 weeks) after an uncomplicated pregnancy, immediately presenting poor cardio-respiratory conditions possibly due to lung hypoplasia. Clinical examination revealed several dysmorphic features: asymmetric skull, wide open fontanel, hypertelorism, low-set, abnormally positioned ears, long philtrum, short neck, cryptorchism, camptodactyly of the third and fourth fingers of both hands, and club feet. Multiple haematomas and petechiae were present. Biochemical investigations showed low haemoglobin level of 6.1 mM/L, severe thrombocytopenia (18×10⁹/μl; nL 150–400), and primary hypothyroidism. Cardiological investigation revealed a perimembranous and trabecular ventricular septum defect as well as a patent ductus. At 16 days the patient had not yet regained his birth weight. There was no significant vomiting or diarrhoea. From 8 weeks on, a progressive ascites occurred leading to secondary complications including tachypnoea and dyspnoea. Despite all attempts, the disease course worsened and the patient died at the age of 3 months. Pathological studies revealed thorax and bilateral lung hypoplasia, multiple cystic dilated intra- and extrhepatic bile ducts, cholestasis, bilateral microcysts in all parts of the kidney, and absence of extramedular haematopoeisis. No central nervous system abnormalities were observed either on functional or anatomical basis.

An overview of the clinical presentation of the three patients described in this paper in comparison with patient MP (described by Chantret *et al.*) and the typical presentations of CDG-Ia and CDG-Ib are given in table 3.

**Diagnosis of CDG**

Both patients showed an abnormal glycosylation of serum transferrin on isoelectric focusing with increased asialo and disialotransferrin, indicative for CDG type I. Phosphomannomutase (PMM) and phosphomannomutase isomerase (MPI) activities measured in cultured fibroblasts were used. Strain YG98-1d and its transfectants were propagated at 23°C and strain YG127 and its transfectants at 30°C.
normal, thus excluding CDG-Ia and CDG-Ib. Analysis of \(^{1}H\)mannose labelled lipid-linked oligosaccharides (LLOs) from patients’ fibroblasts revealed accumulation of dolPP-GlcNac\(_{2}\)Man\(_{9}\) and dol-PP-GlcNac\(_{2}\)Man\(_{9}\)Glc\(_{1}\) in variable ratios (fig 1A). Blocking the glucosyltransferase-glucosidase shuttle\(^{1,2}\) with the ER glucosidase inhibitor castanospermine (CST) prior to LLO analysis resulted in a single peak of dolPP-GlcNac\(_{2}\)Man\(_{9}\)Glc\(_{1}\) on LLO analysis of fibroblasts from patient NL0097 (fig 1B). For patient GB0243 the effect of CST is less clear: the dol-PP-GlcNac\(_{2}\)Man\(_{9}\)Glc\(_{1}\) peak, which is consistent with an ALG8 defect (data not shown). This confirms the polymorphic nature of this variation. The father of patient GB0243 is homozygous for p.N222S (fig 2).

**Table 1** Clinical picture of four CDG-Ih patients compared with the typical CDG-Ia and Ib presentation

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>GB0243(^{*})</th>
<th>GB0244 (sib of GB0243)</th>
<th>NL0097</th>
<th>MP(^{a})</th>
<th>CDG-Ia</th>
<th>CDG-Ib</th>
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<tr>
<td>Abnormal electroretinogram</td>
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<table>
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<tr>
<th>Outcome</th>
<th>Died at 3 months</th>
<th>Died at 3 days</th>
<th>Died at 3 months</th>
<th>Required parenteral nutrition, albumin infusions. Alive at 3 years. Normal development</th>
<th>Mild to severe</th>
<th>Mild to severe</th>
</tr>
</thead>
</table>

**Functional analysis of the splice mutations**

The effect of the splice mutations on the mRNA processing was analysed by comparing ALG8 cDNA amplified from RNA isolated from patients’ fibroblasts cultured in the presence or absence of the translation inhibitor puromycin. For GB0243, the p.T47P allele represents at least 90% of the ALG8 transcripts in the absence of puromycin (fig 3A). Using RNA isolated from fibroblasts incubated with puromycin, both alleles could be amplified in nearly equal amounts (data not shown). The base variation c.96-2A\(\rightarrow\)G abolishes the regular splice acceptor of exon 2. Instead, a cryptic splice site 11 bp downstream is used, resulting in an 11-bp deletion and a premature stop at codon p.38 (fig 3B). Similarly, only the p.G275D allele could be amplified from RNA of fibroblasts from patient NL0097 unless puromycin was added to the cultures. Inhibition of nonsense-mediated mRNA decay (NMD) with puromycin stabilised the abnormal transcript which was lacking exon 6 due to the splice mutation c.672+4A\(\rightarrow\)G.

**Functional analysis of the missense mutations**

Due to the high evolutionary conservation of the LLO-biosynthesis pathway among eukaryotes, *S. cerevisiae* mutants have been very useful for functional analysis of mutant forms of human ALG proteins.\(^{20,22,23}\) To assess the effect of the missense mutations p.T47P and p.G275D on the function of ALG8, complementation assays of yeast strains (*S. cerevisiae*) deficient for ALG8 were performed. The coding region of *ALG8* cDNA was amplified from normal control and patient fibroblasts and cloned into a yeast expression vector under control of the strong GPD promoter. After transformation of yeast strains YG127 (*Alg8*) and YG98-1d (*Alg8wbp1–1*),\(^{20}\) two independent clones were picked for each plasmid and assayed.
The double mutant \( \Delta alg8wbp1-1 \) has a more severe underglycosylation than both single mutants, resulting in a reduced growth rate at restrictive temperatures. This synthetic phenotype was used to assay the functional effect of p.T47P and p.G275D on ALG8 activity. Incubation of dilution series of YG98-1d transformants at 29 \( ^\circ \)C showed a clear difference in complementation efficiency between the yeast and human ALG8 and the two mutant ALG8 constructs. At permissive temperature (23 \( ^\circ \)C) all transformants showed comparable growth, demonstrating that the dilution series was started with approximately equal amounts of viable cells. With this assay, no difference was observed between complementation with the yeast or human ALG8. In contrast, cells transformed with the mutant ALG8 constructs were more temperature sensitive and grew less well at 29\( ^\circ \)C, confirming the deleterious effect of the mutations T47P and G275D (fig 4A).

The underglycosylation of the \( \Delta alg8 \) single mutant yeast strain is less severe and does not result in a growth defect. Therefore, the effect on the degree of glycosylation of the marker protein carboxypeptidase Y (CPY) in the different YG127 transformants was analysed by SDS-PAGE followed by Western blot. The CPY hypoglycosylation pattern with the T47P or G275D mutant constructs was, within experimental variations, comparable to the mock control, while the wild type human ALG8 cDNA conferred a marked restoration of the glycosylation (fig 4B).

**DISCUSSION**

We describe three patients from two families with a very severe form of CDG caused by a deficiency of ALG8 (CDG-Ih). The principal pathology in the CDG-Ih patient described so far (this paper and Chantret et al\(^4\)) is a hepato-intestinal presentation, characterised by life-threatening oedema and ascites. The patients described in this paper have a more severe presentation compared to the previously described patient MP\(^4\) also presenting mild (GB0243) to severe (NL0097) dysmorphic features and haematopoietic deficiency. Two of the three patients even presented with antenatal symptoms. Although the three patients described here were too young for a definitive conclusion, central nervous system (CNS) abnormalities, a hallmark for all types of CDG-I except CDG-Ib, were not observed. These presentations—predominance of gastro-intestinal problems and the absence of CNS involvement—are reminiscent of CDG-Ib (table 1), but CDG-Ih is, at least in these patients, much more severe and not treatable by mannose therapy.

At present, the variable presentation and severity of the different defects along the ER N-glycosylation pathway is unexplained at the functional level.

The ALG8 defect is characterised by an accumulation of dol-PP-GlcNAc\(_2\)Man\(_9\) and dol-PP-GlcNAc\(_2\)Man\(_9\)Glc\(_1\). The ratio of the two LLO intermediates is variable. This might reflect the differences in glucosidase-glucosyltransferase activities depending on energy and stress during cell culture.\(^{21}\) Blocking this shuttle by CST indeed resulted in the accumulation of only dolPP-GlcNAc\(_2\)Man\(_9\)Glc\(_1\) in patient NL0097. It is unclear why fibroblasts from GB0243 after...
incubation with CST still accumulate both intermediates, in addition to the fully mature PP-GlcNAc2Man9Glc3. Mutation analysis confirmed the ALG8 deficiency. As expected, both splice mutations c.96-2A>G and c.672+4A>G represent a virtual null-allele. The adenine at position –2 in the 3′ splice site of exon 2 is strictly conserved,25 so the transition c.96-2A>G must affect splicing. Adenine at position +4 of the splice donor is less conserved (50%),25 but mRNA analysis clearly indicated mRNA instability for the c.672+4A>G allele of NL0097. Due to aberrant splicing and the creation of a premature stop codon, nonsense-mediated mRNA decay is activated in both cases as demonstrated by the stabilisation of these transcripts by puromycin treatment in culture.

The two missense mutations p.T47P and p.G275D affect conserved positions. The tyrosine at position 47 is highly conserved between the α1,3-glucosyltransferases, ALG6 and ALG8, from different species (fig 5). According to topology predictions, T47 is located in a long peptide motif in the first luminal loop of ALG8, a region which shows conservation

Figure 3  (A) Sequence analysis of the region around position p.T47 on genomic DNA (left panel) and cDNA (right panel) extracted from a normal individual (control) or patient GB0243. (B) The splice mutation c.96-2A>G abolishes the normal splice site, which leads to the use of a cryptic splice site 11 bp downstream of the normal splice site, which results in an 11-bp deletion at the level of the transcript (c.96_106del).

Figure 4  Functional analysis of the missense mutations p.T47P and p.G275D. (A) Dilution series of the S. cerevisiae strain YG98-1d (Δalg8 wbp1–1) transformed with the p426GPD vector alone (mock), the plasmid pALG8-1 which contains yeast ALG8 (yALG8), human ALG8 (ALG8), mutant human ALG8 (p.T47P and p.G275D) or the YEp352 vector expressing the WBP1 gene.24 A liquid culture was diluted and spotted to obtain approximately 50,000 (1), 5,000 (2), 500 (3), 50 (4), and five (5) cells. Plates were incubated for 72 h at 23°C and 29°C. (B) Western blot analysis of CPY from YG127 (Δalg8) transformed with the p426GPD vector (mock), the p426GPD vector expressing yeast ALG8 (yALG8), human ALG8, or mutant human ALG8 (p.T47P and p.G275D) and the parental yeast strain SS328. The bands corresponding to the fully glycosylated mature CPY (CPY) and CPY lacking 1 [CPY[−1]] or 2 [CPY[−2]] glycan chains are marked.
among the dolP-Man and dolP-Glc dependent mannosyl- and glucosyltransferases. Position 275 (glycine) is located in the cytoplasmic loop between the transmembrane domains 6 and 7 and is also conserved between ALG6 and ALG8 of different species but not in other mannosyl- or glucosyltransferases. Despite these differences in position and conservation, no significant difference has been observed in the effect of the mutations on the ALG8 function. Both in the ALG8-dependent growth assay and the analysis of CPY-glycosylation on Western blot, the mutant ALG8 constructs complement less efficiently compared to ALG8 but are comparable with each other. Although these assays are not quantitative, they have been used before to estimate the residual activity of mutant proteins. Hence, we can conclude that the p.T47P and p.G275D mutant proteins have a similar, low residual activity.

Based on these residual ALG8 activities and the instability of the aberrantly spliced transcripts, the genotypes of the patients GB0243 and NL0097 are comparable. They result in a severe hepato-intestinal problems with or without dysmorphic features depending on the genotype, but no obvious CNS defects.

In summary, the CDG-Ih clinical spectrum includes mild to very severe clinical presentations. Patient NL0097 presented with more dysmorphic features, but the general disease course was similar and resulted in early infant death. This is in contrast with the milder presentation of the previously described patient MP who has two truncating mutations that normally would be considered to be "null" mutations. Although the single base deletions, c.413delC and c.396insA, observed in patient MP normally result in unstable transcripts due to NMD, the authors identified 10–20% residual ALG8 mRNA transcript on Northern blot, which might code for functional proteins. We believe that a minimal residual ALG8 activity is essential for viability and that the clinical outcome reflects the sum of the residual activities of the respective proteins.

Figure 5  Alignment of the sequence region around mutations p.T47P and p.G275D from ALG6 and ALG8 from human (Hs), C. elegans (Ce), D. melanogaster (Dm), S. cerevisiae (Sc) and S. pombe (Sp) (ClustalX scheme in Jalview, modified from Pfam3155, http://www.sanger.ac.uk/Software/Pfam/index.shtml).

REFERENCE


