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Abstract

The medical significance of N-glycosylation is underlined by a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). One key step in the biosynthesis of the Glc(3)Man(9)GlcNAc(2)-PP-dolichol precursor, essential for N-glycosylation, is the translocation of Man(5)GlcNAc(2)-PP-dolichol across the endoplasmic reticulum membrane. This step is facilitated by the RFT1 protein. Recently, the first RFT1-deficient CDG (RFT1-CDG) patient was identified and presented a severe N-glycosylation disorder. In the present study, we describe three novel CDG patients with an RFT1 deficiency. The first patient was homozygous for the earlier reported RFT1 missense mutation (c.199C>T; p.R67C), whereas the two other patients were homozygous for the missense mutation c.454A>G (p.K152E) and c.892G>A (p.E298K), respectively. The pathogenic character of the novel mutations was illustrated by the accumulation of Man(5)GlcNAc(2)-PP-dolichol and by reduced recombinant DNase 1 secretion. Both the glycosylation pattern and recombinant DNase 1 secretion could be normalized by expression of normal RFT1 cDNA in the patients' fibroblasts. The clinical phenotype of these patients comprised typical CDG symptoms in addition to sensorineural deafness, rarely reported in CDG patients. The identification of additional RFT1-deficient patients allowed to delineate the main clinical picture of RFT1-CDG and confirmed the crucial role of RFT1 in Man(5)GlcNAc(2)-PP-dolichol translocation.
RFT1 deficiency in three novel CDG patients

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ABSTRACT

N-glycosylation is an essential form of protein modification, whose medical significance is underlined by the group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). A key step in the N-glycosylation pathway, the translocation of Man₅GlcNAc₂-PP-dolichol across the endoplasmic reticulum membrane, is catalyzed by the RFT1 protein and the first RFT1-deficient CDG patient was recently identified by Haeuptle and co-workers.

In the present study, we describe three novel CDG patients with an RFT1 deficiency. The first patient was found to be homozygous for the earlier reported missense mutation c.199C>T (p.R67C), while two new point mutations were identified in the other two patients. One was homozygous for the missense mutation c.454A>G (p.E152K), while the other was homozygous for a G to A transition at nucleotide position 892 (c. 892G>A; p.K298E). In the patients’ cells, the pathogenic character of these new mutations was illustrated by the typical accumulation of the LLO intermediate Man₅GlcNAc₂-PP-dolichol and by reduced recombinant DNase 1 secretion. Both the LLO profile and recombinant DNase 1 secretion could be normalized by expression of normal RFT1 cDNA in the patients’ fibroblasts. Due to the identification of these three additional patients, the clinical phenotype of RFT1 deficiency could be further delineated and comprises severe mental retardation, failure to thrive, hypotonia, epilepsy, myoclonic jerks, decreased visual acuity, sensorineural deafness and feeding problems.
Key Words

Glycosylation

Congenital Disorders of Glycosylation

RFT1

Dolichol cycle
**Introduction**

N-glycosylation is a post-translational modification of proteins found in eukaryotic and prokaryotic organisms (Weerapana and Imperiali, 2006). In eukaryotes, protein N-linked glycans are involved in many essential biological processes including the immune response, intracellular targeting, cell-cell recognition, protein folding and protein stability (Varki, 1993).

The eukaryotic N-glycosylation pathway starts with the assembly of the Glc₃Man₉GlcNAc₂ oligosaccharide precursor on a dolichol-PP carrier in an enzymatic multistep and well-ordered process known as the dolichol cycle (Burda and Aebi, 1999). This dolichol cycle takes place in two compartments: the cytosol and the endoplasmic reticulum (ER). It starts with the elongation of dolichol-P to Man₅GlcNAc₂-PP-dolichol on the cytosolic face of the ER membrane, while the elongation of Man₅GlcNAc₂-PP-dolichol to the complete Glc₃Man₉GlcNAc₂–PP-dolichol occurs in the ER lumen. For this reason, the Man₅GlcNAc₂-PP-dolichol intermediate has to be translocated across the ER membrane. In yeast, the ER membrane protein Rft1 was shown to facilitate the translocation of Man₅GlcNAc₂-PP-dolichol to the ER lumen in a bidirectional and ATP-independent manner (Helenius et al., 2002). Once the fully assembled oligosaccharide precursor is synthesized, Glc₃Man₉GlcNAc₂ is transferred onto selected asparagine residues of polypeptide chains by the oligosaccharyltransferase (OST) complex (Burda and Aebi, 1999).

Congenital Disorders of Glycosylation (CDG) are a group of inherited human disorders characterized by deficient protein glycosylation. Up to now, 14 different CDG types
deficient in protein N-glycosylation site occupancy have been identified. Based on the serum sialotransferrin pattern, these N-glycosylation disorders can be classified into two subgroups: defects of oligosaccharide precursor assembly and transfer to proteins (formerly known as CDG-I) and defects of N-glycoprotein processing (formerly known as CDG-II) (Eklund and Freeze, 2006; Freeze, 2007; Jaeken and Matthijs, 2007; Leroy, 2006).

Recently, the first RFT1-deficient CDG patient was identified (Haeuptle et al., 2008). Haeuptle and co-workers described a young girl presenting with marked psychomotor retardation, hypotonia, seizures, hepatomegaly and coagulopathy. This patient was homozygous for the missense mutation c.199C>T (p.R67C) in the RFT1 protein and accumulated the Man₅GlcNAc₂-PP-dolichol intermediate on lipid-linked oligosaccharide (LLO) analysis. However, no Man₅GlcNAc₂ was transferred onto glycoproteins, which pointed to a deficient translocation of the accumulated LLO across the ER membrane.

In the present study, we describe three additional RFT1-deficient patients including two novel pathogenic point mutations. While only one RFT1-deficient patient was known so far, the identification of these three additional patients allowed us to refine the clinical phenotype characteristic for RFT1 deficiency, designated as RFT1-CDG according to the novel nomenclature (Jaeken et al., 2008).
Materials and methods

Cell culture
Primary skin fibroblasts from healthy controls and patients were cultured at 37 °C under 5% CO₂ in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum (Clone III, HyClones).

Mutation analysis
Total RNA was isolated from 2 x 10⁷ fibroblasts using the TRIzol LS reagent (Invitrogen) according to the manufacturer’s instructions. The human RFT1 cDNA was prepared and the protein coding region was amplified by PCR as described before (Haeuptle et al., 2008). The PCR products were sequenced (Synergene Biotech) after removal of the unincorporated nucleotides with QIAquick columns (QIAGEN). Carrier analysis in the parents and healthy siblings was performed on DNA extracted from blood. Primers were designed to amplify exons 3, 4 and 9 of the RFT1 gene, based on the genomic sequence (NM_052859). Primer sequences are available on request. These exons were amplified using standard PCR conditions, subsequently sequenced with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applera) and analyzed on an ABI3100 Avant (Applera).

Metabolic radiolabelling
Fibroblasts (8 x 10⁶ per labelling) were grown overnight in a 175 cm² tissue culture flask. After 24 h, cells were pre-incubated in 0.5 mM glucose for 45 min and then pulse radiolabelled for 1 h with 150 μCi of 2-[³H]-mannose (16 Ci/mmol, Amersham)
Biosciences). After metabolic labelling, the cells were scraped with 1.1 ml MeOH/H₂O (8:3) followed by the addition of 1.2 ml CHCl₃. Sequential extraction of oligosaccharide material was performed as previously described (Cacan and Verbert, 1997).

**Analysis of oligosaccharide material**

Glycoprotein fractions obtained at the end of the sequential extraction were digested overnight at room temperature with trypsin (1 mg/ml; Sigma) in 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were treated with 0.5 U PNGase F (Roche) in 50 mM phosphate buffer, pH 7.2 for 4 h to release the oligosaccharides from the peptides. The oligosaccharides were desalted on Bio-Gel P2 columns and eluted with 5% acetic acid. LLO fractions obtained after sequential extraction were subjected to mild acid treatment (0.1 M HCl in THF) for 2 h at 50 °C. Purification of the released oligosaccharides was performed as described above.

The oligosaccharides were separated by HPLC on an amino derivated Asahipak NH₂P-50 column (250 mm x 4.6 mm; Asahi) applying a gradient of acetonitrile/H₂O ranging from 70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier et al., 2002). Elution of the radiolabelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard).

**Complementation of the LLO profile**

Lentiviruses containing *RFT1* and *EGFP* cDNAs were prepared as previously described (Haeuptle et al., 2008). Briefly, 3 x 10⁶ HEK293T cells were transfected with 20 µg
pLenti6-hRFT1 or pLenti6-EGFP and 36 µg of the packing plasmid mix (Invitrogen) by calcium-phosphate precipitation. Two days after transfection, the supernatants were collected and used to transduce the patient fibroblasts. After selection with 5 µg/ml blasticidin (Invitrogen), the cells were metabolically labelled with 2-[³H]-mannose and the extracted LLO were analysed by HPLC (Zufferey et al., 1995).

**Complementation of DNase I secretion**

Bovine DNase I cDNA was subcloned into the pSVK3 vector as previously reported (Nishikawa et al., 1997). Patients’ fibroblasts were transduced with an adenovirus expressing bovine DNase I (Eklund et al., 2005; Fujita et al., 2008). Two days post transduction, the cells were washed twice with PBS and incubated at 37 °C with 1 ml DMEM without methionine/cysteine, containing 10% dialysed fetal bovine serum, 10 mM NH₄Cl and 0.2 mCi [³⁵S]-Met/Cys labelling mixture. After a labelling time of 4 h, the culture medium was harvested and DNase I was immunoprecipitated as previously described (Nishikawa and Mizuno, 2001). The immunoprecipitated samples were subjected to SDS-PAGE using a 13% acrylamide gel. After electrophoresis, the gels were rinsed with a mixture of 7% acetic acid and 10% methanol for 15 min and soaked in Amplify solution (Amersham Pharmacia Biotech) for 15 min. The gel was dried, autoradiography was carried out and the intensity of the bands corresponding to DNase I was quantified by scanning densitometry (using Quantity One software PDI). **Band intensity was normalized against protein levels.**
Results

Biochemical and molecular diagnosis

Three patients with a type 1 pattern of serum sialotransferrins were further investigated. Subsequently, phosphomannomutase (CDG-Ia) and phosphomannose isomerase (CDG-Ib) deficiencies were excluded on the basis of enzymatic activity measurements in the patients’ fibroblasts (data not shown).

In order to identify a defect in the assembly of the oligosaccharide precursor within the dolichol cycle, structural analysis of the LLO was performed by HPLC after metabolic labelling. In healthy control fibroblasts, the LLO profile is characterized by the fully assembled oligosaccharide precursor Glc₃Man₉GlcNAc₂-PP-dolichol. In contrast, an accumulation of Man₅GlcNAc₂-PP-dolichol in combination with only a minor amount of complete LLO was detected in all three patients (Figure 1, panels A, B, C and D). Additionally, protein-linked glycan structures were analyzed. In both, healthy control and patients, Man₈GlcNAc₂, Man₉GlcNAc₂ and Glc₁Man₉GlcNAc₂ structures were obtained (Figure 1, panels E, F, G and H). Notably, no Man₅GlcNAc₂ structure could be detected on the patients’ glycoproteins.

The accumulation of Man₅GlcNAc₂-PP-dolichol on LLO combined with normal protein-linked glycans is typical for a defect in the translocation of Man₅GlcNAc₂-PP-dolichol to the ER lumen and was recently reported in a RFT1-deficient patient (Haeuptle et al., 2008). To this end, mutation analysis of the RFT1 cDNA was performed in the present patients and all three carried RFT1 mutations. The first patient was homozygous for the earlier reported missense mutation (c.199C>T; p.R67C), while the parents were found to be heterozygous carriers of this mutation (Figure 2A) (Haeuptle et al., 2008). Two novel
point mutations were identified in two other patients. Sequencing of the second patient’s
*RFT1* cDNA revealed an A to G transition at nucleotide position 454, leading to the
conversion of a lysine into a glutamic acid at position 152 on the protein level (Figure
2B). Both parents as well as a healthy sibling were heterozygous for this mutation. In
model eukaryotic organisms, the lysine at position 152 was mostly conserved and a
conversion of a lysine into a glutamic acid was not observed (data not shown). The third
patient was homozygous for a G to A transition at nucleotide position 892, causing a
glutamic acid to lysine change at position 298 (Figure 2C). The mutated glutamic residue
is strictly conserved among eukaryotes (data not shown). No material of the parents and
siblings was available for carrier analysis.

In addition, all converted amino acids were found in hydrophilic domains of the RFT1
protein predicted to be oriented to the ER lumen (*TMpred* (Hofmann and Stoffel, 1993);
*TMHMM* (Krogh et al., 2001)) (Figure 3). As previously reported, the p.R67C mutation
is located in the first luminal loop of the RFT1 protein (Haeuptle et al., 2008). The
p.K152E mutation was found in the second luminally oriented 25 amino acid long
hydrophilic stretch and the p.E298K mutation was positioned in the largest luminal loop,
bearing a potential N-glycosylation site at position p.N227 and ranging over 130 amino
acids (Figure 3).

*Complementation of the LLO profile*

To demonstrate the pathogenicity of the two new missense mutations, wild-type *RFT1*
cDNA was transduced into patients’ fibroblasts using a lentiviral construct to
complement the Man$_5$GlcNAc$_2$-PP-dolichol accumulation. Healthy control and patients’
fibroblasts were thus infected with recombinant lentiviruses expressing either the wild-type \textit{RFT1} cDNA or \textit{EGFP} as a negative control. Compared to \textit{EGFP} expression in the patients’ fibroblasts, analysis after \textit{RFT1} expression revealed a normalization of the LLO profile characterized by decreased levels of Man\textsubscript{5}GlcNAc\textsubscript{2}-PP-dolichol and increased levels of the complete Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}-PP-dolichol (Figure 4). \textit{EGFP} expression in the patients’ fibroblasts did not affect the LLO profile, as expected.

\textit{Complementation of DNase 1 secretion}

In another, alternative method to prove the pathogenicity of the two new mutations, secretion of recombinant, bovine DNase 1 was investigated in all three RFT1-deficient patients. A modified version of bovine DNase 1 has a single potential N-glycosylation site (p.N106) and is secreted when expressed in human fibroblasts (Nishikawa and Mizuno, 2001). However, upon expression in the fibroblasts of CDG patients, DNase 1 secretion was strongly reduced (Eklund et al., 2005; Fujita et al., 2008). As shown in Figure 5 (lanes 3, 5 and 7), DNase 1 secretion was significantly reduced in all three RFT1-deficient patients. Transduction of the patient’s fibroblasts with lentiviruses coding for wild-type \textit{RFT1} restored the levels of DNase 1 secretion (Figure 5, lanes 4, 6 and 8), thus demonstrating the pathogenicity of the new mutations.

\textit{Clinical phenotype}

To address the main clinical characteristics of RFT1 deficiency, the clinical phenotype of the three different patients was compared. Patient 1 was the first child of healthy, unrelated North Americans of Scottish-English origin and presented with respiratory
insufficiency, severe generalized epilepsy with intractable seizures, infantile spasms, microcephaly, failure to thrive, hypotonia, sensorineural deafness and decreased visual acuity. In addition, this patient showed severe mental retardation, with virtually no development. Dysmorphic features included micrognathia, short neck, small nose, drooping eyelids, valgus feet and adducted thumbs. This patient also had severe feeding problems requiring gastrostomy and died at the age of eight months. Brain MRI at early age did not reveal cerebral nor cerebellar atrophy. However, at autopsy, weight and gyral/sulcal pattern consistent with a degree of cerebral atrophy was reported (Table 1).

The second patient was the second son of healthy, consanguineous Italian parents. During the first year of life, he presented with severe developmental delay, microcephaly, nystagmus, sensorineural deafness, relapsing aspiration pneumonia, a generalized hypotonia and inverted nipples. Additionally, this patient was frequently hospitalized because of drug-resistant epilepsy and feeding problems requiring gastrostomy. At the age of 4 years, CT scan of the brain revealed a stroke-like episode affecting the left frontal lobe, and the following year he started to suffer from recurrent deep venous thrombosis of the left leg. Clinical examination at the age of five years showed a severe mentally retarded child with microcephaly, spastic tetraparesis and kyphoscoliosis. Serial brain MRI examinations showed progressive cortical and subcortical atrophy with no cerebellar involvement and liver function has always been normal (Table 1).

The third patient was the ninth child of healthy, consanguineous parents from Algerian origin. This family has two children presenting with the autosomal recessive disorder Hemophagocytic Lymphohistiocytosis. One child died at the age of three months, while the other is still alive due to bone marrow transplantation at the age of four months. In
addition, one son died at the age of four days and presented with similar characteristics as his brother. Patient 3 presented with respiratory insufficiency, hypotonia, body spasm, failure to thrive, epilepsy, bilateral glaucoma and sensorineural deafness. Dysmorphic features included slightly inverted nipples, infiltrated ears, short neck, retrognathism, glossoptosis, adducted thumbs and valgus feet. Brain MRI did not show cerebral or cerebellar atrophy. This patient also had feeding problems and chronic infections of the respiratory tract. In addition, he showed severe mental retardation with the absence of visual contact and liver function was normal (Table 1).

**Discussion**

Based on the analysis of LLO and protein-linked glycan profiles in fibroblasts, three potential RFT1-deficient patients were identified. All three patients showed an accumulation of the LLO Man₅GlcNAc₂-PP-dolichol, while no Man₅GlcNAc₂ was detected on glycoproteins. Mutation analysis of the *RFT1* gene revealed that one patient was homozygous for the earlier reported missense mutation (c.199C>T, p.R67C). This is the second report of the p.R67C mutation in a patient of British origin (Imtiaz et al., 2000), which could point to a founder effect of the p.R67C mutation. The other patients were homozygous for the new missense mutations c.454A>G (p.E152K) and c.892G>A (p.K298E). The pathogenic character of these novel mutations was demonstrated by the complementation of the abnormal LLO profile and reduced DNase 1 secretion upon expression of wild-type *RFT1* in the patients’ fibroblasts.

All three *RFT1* mutations identified so far are located in one of the hydrophilic loops predicted to be within the ER lumen. It can be assumed that these regions are of major
importance for the translocation of Man$_5$GlcNAc$_2$-PP-dolichol in the ER lumen or for the maintenance of Man$_5$GlcNAc$_2$-PP-dolichol on the luminal side. Further structural analysis will be required to confirm the predicted orientation of the RFT1 protein. Determination of the occupancy of the putative N-glycosylation site at position p.N227 (Figure 3) would certainly contribute to establish the topology of the RFT1 protein.

In yeast, the Rft1 protein was genetically identified as a protein mediating the translocation of LLO across the ER membrane (Helenius et al., 2002). However, recent evidence suggests that the RFT1 protein would not be the flippase enzyme itself, but would play a critical accessory role in translocating Man$_5$GlcNAc$_2$-PP-dolichol to the ER lumen (Frank et al., 2008; Sanyal et al., 2008). Anyhow, the identification of three additional RFT1-deficient patients clearly underscores the major importance of RFT1 in this translocation event.

Finally, the identification of three additional patients allowed us to refine the clinical phenotype characteristic for RFT1 deficiency. All four known RFT1-deficient patients showed very similar characteristics including severe mental retardation, hypotonia, epilepsy, myoclonic jerks, decreased visual acuity, sensorineural deafness and feeding problems (Table 1). In comparison to other CDG defects, RFT1 deficiency is thus mainly a neurological disorder. A striking clinical feature is the presence of sensorineural deafness in all four RFT1-deficient patients (Imtiaz et al., 2000). Deafness has been reported in only a few other CDG patients (Imtiaz et al., 2000; Kranz et al., 2007; Hutchesson et al., 1995). More RFT1-deficient patients have to be studied in order to know whether this is a consistent feature of RFT1-CDG.
Acknowledgements

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References


### Tables

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**Brain MRI:**

- cerebral atrophy reported at autopsy.

**Table 1:** Clinical phenotype of the three new RFT1-deficient patients compared to the phenotype of the originally reported case (KS) (Clayton and Grunewald, 2009; Hauptle et al., 2008; Imtiaz et al., 2000). Symbols: na: not available; *: cerebral atrophy reported at autopsy.
Figure Legends

Figure 1: (A-D) HPLC analysis of the LLO in control and patients’ fibroblasts revealing the accumulation of Man$_5$GlcNAc$_2$-PP-dolichol in the case of CDG. (E-H) Protein N-linked oligosaccharides of control and patients were separated by HPLC, demonstrating that no aberrant glycan structures were detected in the patients. The retention times of the standard oligosaccharides Glc$_3$Man$_9$GlcNAc$_2$-PP-dolichol (G$_3$M$_9$) and Man$_{1-9}$GlcNAc$_2$-PP-dolichol (M$_{1-9}$) are marked above the HPLC profiles.

Figure 2: Sequence alignment of the RFT1 cDNA fragments in control and patients. (A) Patient 1 is homozygous for the earlier reported missense mutation c.199C>T (p.R67C). (B) In patient 2, the homozygous A to G transition at nucleotide position c.454 was detected, leading to a conversion of a lysine into a glutamic acid at position p.152. (C) Analysis of the RFT1 cDNA in patient 3 revealed a homozygous G to A transition at nucleotide position c.892, causing a glutamic acid to lysine change at protein position p.298.

Figure 3: Schematic representation of the identified missense mutations in the predicted topology of the RFT1 protein. All three mutations were found in the predicted luminal loops of the protein. The orientation of the model was supported by the favoured position of the 11 transmembrane domains and the localization of a potential N-glycosylation site (p.N227) (TMpred (Hofmann and Stoffel, 1993); TMHMM (Krogh et al., 2001)).
**Figure 4:** Complementation of the Man$_5$GlcNAc$_2$-PP-dolichol accumulation. Fibroblasts of patient 2 (A) and patient 3 (C) express wild-type *RFT1* cDNA, thereby leading to a decreased accumulation of Man$_5$GlcNAc$_2$-PP-dolichol. Lentiviral mediated expression of *EGFP* in the fibroblasts of patient 2 (B) and patient 3 (D) showed no effect. The retention times of the standard oligosaccharides Glc$_3$Man$_9$GlcNAc$_2$-PP-dolichol (G$_3$M$_9$) and Man$_1$-$\text{GlcNAc}_2$-PP-dolichol (M$_{1,9}$) are marked above the HPLC profiles.

**Figure 5:** Rescue of DNase 1 secretion in the patients’ fibroblasts. Secretion of DNase 1 is reduced in the fibroblasts of CDG patients (lanes 3, 5 and 7). Expression of wild-type *RFT1* cDNA (lanes 4, 6 and 8) restored the secretion of DNase 1 to a level equal to controls (lanes 1 and 2). Symbols: o = non-glycosylated DNase 1; 1 = singly glycosylated DNase 1.
Patient 1

**A**

Control

- c.199 C>T
- p.R67C

Patient 2

**B**

Control

- c.454 A>G
- p.K152E

Patient 3

**C**

Control

- c.892 G>A
- p.E298K