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ALG12 mannosyltransferase defect in congenital disorder of glycosylation type Ig

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In the endoplasmic reticulum (ER) of eukaryotes, N-linked glycans are first assembled on the lipid carrier dolichyl pyrophosphate. The $GlcNAc_2Man_9Glc_3$ oligosaccharide is transferred to selected asparagine residues of nascent polypeptides. Defects along the biosynthetic pathway of N-glycans are associated with severe multisystemic syndromes called congenital disorders of glycosylation. Here, we describe a deficiency in the ALG12 ER α1,6-mannosyltransferase resulting in a novel type of glycosylation disorder. The severe disease was identified in a child presenting with psychomotor retardation, hypotonia, growth retardation, dysmorphic features and anorexia. In the patient's fibroblasts, the biosynthetic intermediate $GlcNAc_2Man_7$ oligosaccharide was detected both on the lipid carrier dolichyl pyrophosphate and on newly synthesized glycoproteins, thus pointing to a defect in the dolichyl pyrophosphate–$GlcNAc_2Man_7$-dependent ALG12 α1,6 mannosyltransferase. Analysis of the ALG12 cDNA in the CDG patient revealed compound heterozygosity for two point mutations that resulted in the amino acid substitutions T67M and R146Q, respectively. The impact of these mutations on ALG12 protein function was investigated in the *Saccharomyces cerevisiae alg12* glycosylation mutant by showing that the yeast ALG12 gene bearing the homologous mutations T61M and R161Q and the human mutant ALG12 cDNA alleles failed to normalize the growth defect phenotype of the *alg12* yeast model, whereas expression of the normal ALG12 cDNA complemented the yeast mutation. The ALG12 mannosyltransferase defect defines a new type of congenital disorder of glycosylation, designated CDG-Ig.

INTRODUCTION

Glycosylation is a widespread post-translational modification affecting properties and functions of proteins, and thus having a considerable impact on numerous biological processes (1). Accordingly, defects of glycosylation often result in developmental alterations, and, when not lethal, lead to various clinical manifestations. Within the last few years, the range of clinical phenotypes related to glycosylation defects has been extended to hereditary multiple exostoses (2), progeroid syndromes (3) and muscular dystrophies (4). Hundreds of genes are involved in the shaping of several classes of glycoconjugates (5) suggesting a large number of potential defects along these biosynthetic pathways leading to pathological conditions.

Alterations of N-linked glycan biosynthesis have been grouped under the general designation *congenital disorders of glycosylation* (CDG), where 10 distinct defects have been described to date (6,7). Defects affecting the assembly of the dolichyl pyrophosphate (*DolPP*)-linked $GlcNAc_2Man_9Glc_3$ oligosaccharide and its subsequent transfer to nascent glycoproteins are classified as CDG-I, whereas alterations of N-glycan processing represent types of CDG-II (8). Clinically, most CDG patients present with psychomotor retardation, hypotonia, cerebellar hypoplasia, hormonal disorders and stroke-like episodes (9,10). However, the constellation of symptoms often differs from case to case, hence rendering a CDG diagnosis solely based on clinical examination a difficult task. The detection of underglycosylated glycoproteins, such as serum transferrin, by isoelectric focusing (11) represents a simple diagnostic tool, although this test does not discriminate between the different causes of CDG.

Genetic models, such as yeast and Chinese hamster ovary cell glycosylation mutants, have been instrumental in elucidating the molecular basis of CDG (12). Because of the conservation in glycosylation pathways among eukaryotes, these models provide essential clues to relate a glycosylation phenotype to its

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underlying genetic defect. In the present study, we report the identification of a novel type of N-glycosylation disorder characterized by a deficiency of the endoplasmic reticulum (ER)-resident ALG12 α1,6 mannosyltransferase.

RESULTS

The patient NJ is the first child of unrelated Danish parents. He was delivered at 34 weeks of gestation by Caesarean section due to deterioration of the pregnancy as reflected by hemolysis, elevated liver enzymes and a low platelet count (HELLP syndrome). Birth weight was 1742 g, and the neonatal course was complicated by respiratory distress, hypoglycemia, lethargy and feeding difficulties. At age 13 months, he was referred because of developmental delay. A computed tomography at age 16 months showed slight widening of the frontal subarachnoidal space. Presently, at age $2\frac{1}{2}$ years, psychomotor retardation and hypotonia are prominent, and he is not able to sit without support. Dysmorphic features include triangular face, epicanthus, inverted nipples, micropenis, undescended testes, club foot and sandal gaps. Eye examination is normal except for a convergent squint. He has feeding problems, and his weight and length are far below the 3% percentile.

Insulin-like growth factor (IGF)-1 and IGF-binding protein 3 are undetectable in the patient, but growth hormone stimulation test as well as thyroid hormones are normal. Antithrombin III (ATIII) is low (0.24 U; normal range 0.82–1.18). Alanine aminotransferase (ALT) is normal. Notably, immunoglobulins are repeatedly low: IgA 0.09 g/l (normal range 0.70–3.65), IgG 1.1 g/l (normal range 6.1–14.9) and IgM 0.14 g/l (normal range 0.39–2.08). He is receiving infusions of immunoglobulins at regular intervals. He has had pneumonia three times, but no severe infections. The karyotype is normal. Urine metabolic screening for amino and organic acids is unremarkable.

The combination of psychomotor retardation and low ATIII levels led to the suspicion of CDG. Isoelectric focusing of serum transferrin showed decreased amounts of tetrasialotransferrin and increased amounts of disialo- and asialotransferrin, thus confirming the occurrence of a glycosylation disorder (data not shown). Phosphomannomutase and phosphomannose isomerase activities measured in cultured fibroblasts were normal, thus excluding CDG-Ia and -Ib (data not shown). Analysis of lipid-linked oligosaccharides (LLO) in the patient's fibroblasts revealed an accumulation of the incomplete oligosaccharide *DolPP*–$GlcNAc_2Man_7$ and the absence of the mature core *DolPP*–$GlcNAc_2Man_9Glc_3$ that is normally detected in cells from healthy subjects (Fig. 1). The LLO *DolPP*–$GlcNAc_2Man_9Glc_3$ is the preferred substrate of the oligosaccharyltransferase (OTase) complex (13), although truncated LLO may still be transferred to proteins at low efficiency, as shown in yeast (14).

Once on proteins, oligosaccharides are trimmed to $GlcNAc_2Man_9$ by glucosidase-I and -II (15,16). The $GlcNAc_2Man_9$ core usually undergoes cycles of reglucosylation and deglucosylation during the process of protein folding (17), thereby yielding $GlcNAc_2Man_9Glc_1$ besides $GlcNAc_2Man_9$. Before leaving the ER, the oligosaccharide core is further trimmed by α-mannosidase to $GlcNAc_2Man_8$ (18). Therefore, the normal N-linked oligosaccharide (NLO) profile of newly synthesized proteins mainly consists of the species $GlcNAc_2Man_8$, $GlcNAc_2Man_9$ and $GlcNAc_2Man_9Glc_1$ (Fig. 2A). In contrast, the NLO profile of patient NJ fibroblasts predominantly showed the $GlcNAc_2Man_7$ structure (Fig. 2C).

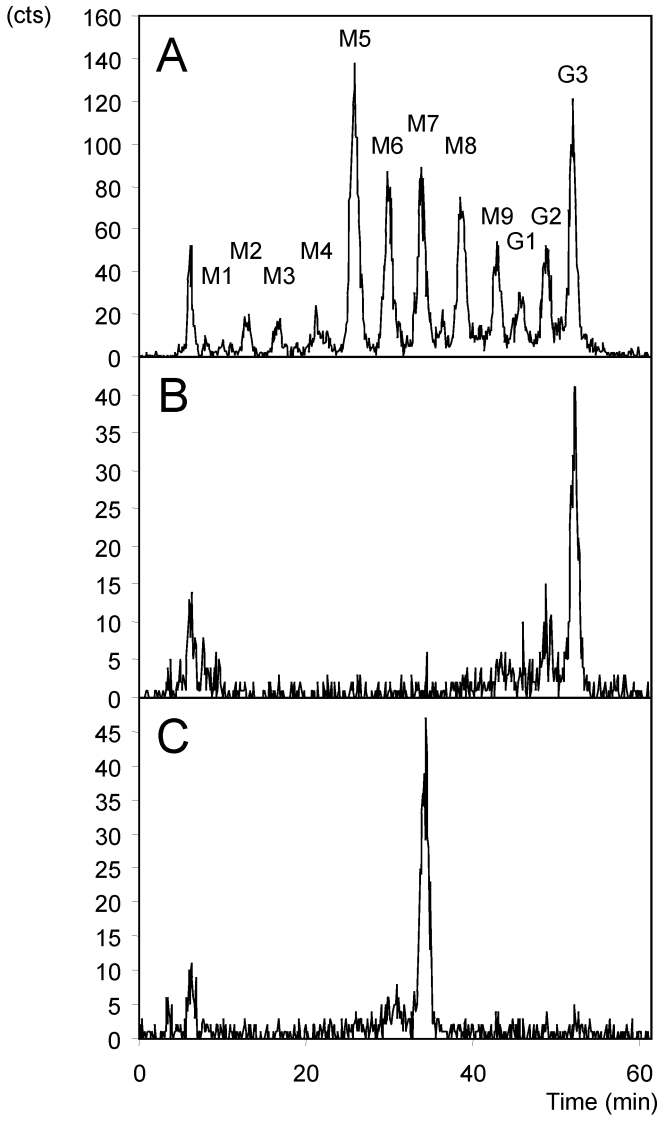

Figure 1. Lipid-linked oligosaccharide profiles. HPLC separation of [^{3}H]mannose-labeled lipid-linked oligosaccharides from yeasts (**A**), control human fibroblasts (**B**) and patient NJ fibroblasts (**C**). The identity of the oligosaccharide peaks is marked on (A) ranging from $GlcNAc_2Man_1$ (M1) to $GlcNAc_2Man_9Glc_3$ (G3). (C) shows the $GlcNAc_2Man_7$ peak accumulating in patient NJ cells, whereas $GlcNAc_2Man_9Glc_3$ is normally found in control cells, as shown in (B).

The detection of this truncated NLO in patient NJ fibroblasts suggested that an oligosaccharide species that lacks two mannose residues is transferred to glycoproteins in the patient's cells. To address the question whether *DolPP*–$GlcNAc_2Man_7$ or *DolPP*–$GlcNAc_2Man_7Glc_3$ served as a substrate for the OTase in this patient's cells, we analyzed the NLO profile of control and patient NJ fibroblasts in the presence of the glucosidase inhibitor castanospermine. Whereas in control cells castanospermine enables the detection of untrimmed

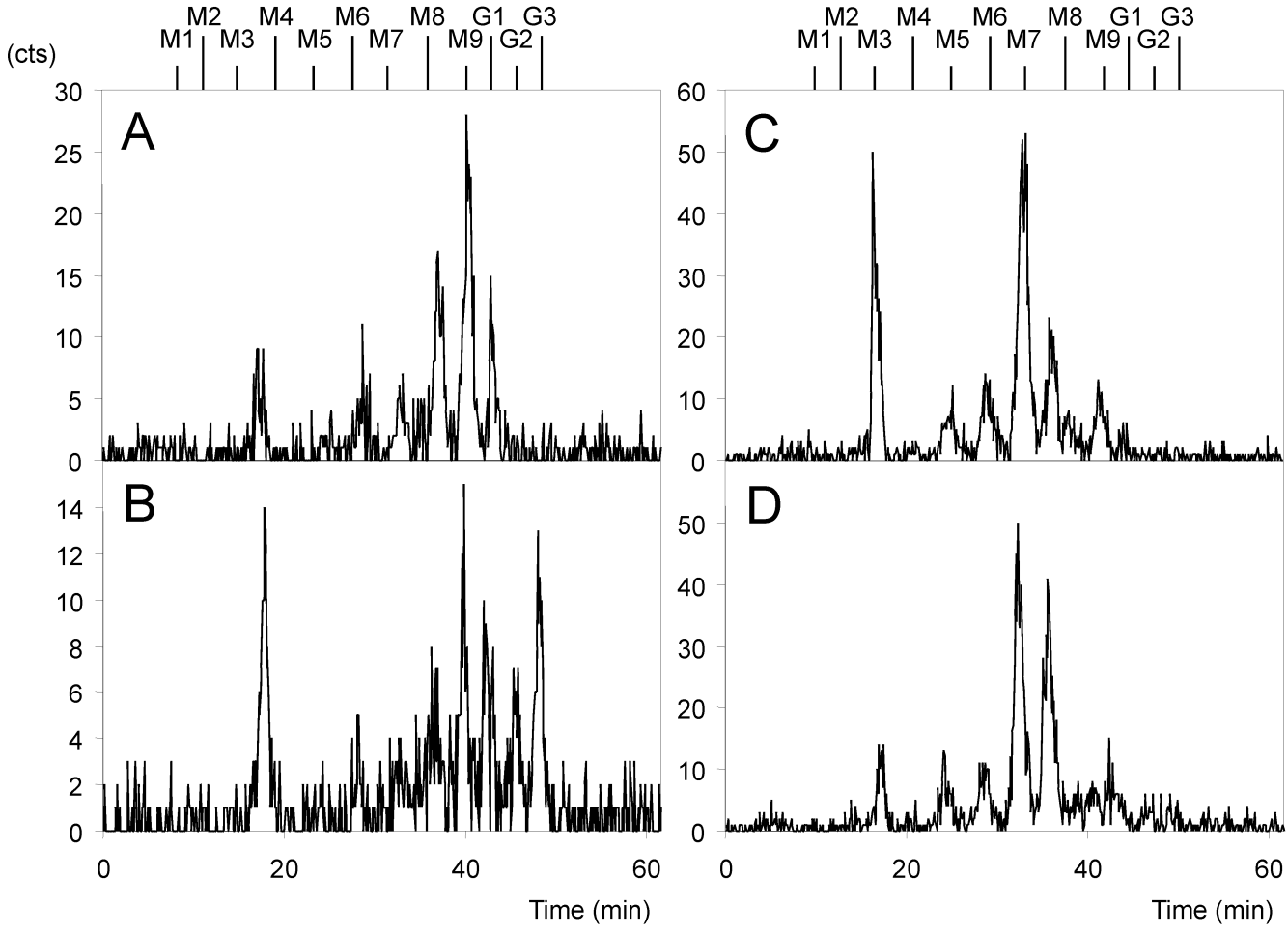

Figure 2. N-linked oligosaccharide profiles. HPLC analysis of [^{3}H]mannose-labeled N-linked oligosaccharides from control (**A**, **B**) and patient NJ (**C**, **D**) fibroblasts after cleavage from proteins with N-glycosidase F. The elution positions of standard oligosaccharides from $GlcNAc_2Man_1$ (M1) to $GlcNAc_2Man_9Glc_3$ (G3) are marked at the tops of the panels. In (A) and (C), cells were metabolically labeled for 1 h at 37°C. In (B) and (D), cells were treated with the glucosidase inhibitor castanospermine (250 μM) for 2 h prior to metabolic labeling and extraction of N-linked oligosaccharides.

$GlcNAc_2Man_9Glc_3$ (Fig. 2B), an untrimmed $GlcNAc_2Man_7Glc_3$ species was not visible in the NLO profile of patient NJ fibroblasts (Fig. 2D). This indicates that $GlcNAc_2Man_7$ acts as donor substrate for the OTase complex in patient NJ cells.

The accumulation of the LLO *DolPP*–$GlcNAc_2Man_7$ has been previously documented in the *Saccharomyces cerevisiae alg12* mutant strain. This strain is characterized by a defect in the ER-resident *DolPP*–$GlcNAc_2Man_7$-dependent α1,6 mannosyltransferase enzyme (19). The human ortholog to the yeast ALG12 gene has not been described so far, but a BLAST search in GenBank revealed the cDNA entry NM_024105, which included an open reading frame encoding a protein of 488 amino acids displaying 44% similarity to the yeast ALG12 protein. Genes encoding homologous proteins were also detected in the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes (Fig. 3). Primary sequence alignment of putative ALG12 proteins showed highly conserved motifs, thus pointing to regions possibly involved in the catalytic activity. The overall identity was 32–42% between the animal sequences and 24–26% between all the sequences.

The NM_024105 cDNA was amplified from control and patient NJ fibroblasts and directly sequenced. We detected compound heterozygosity for two point mutations, 200C>T and 437G>A, in the patient-derived cDNA. These two mutations introduced the amino acid substitutions T67M and R146Q, respectively, in the ALG12 protein sequence. These two mutations were not found in the control cDNAs sequenced or in the 25 expressed sequence tags retrieved from GenBank representing fragments of the NM_024105 cDNA. The amino acid changes introduced were at positions strictly conserved among the eukaryotic homologous proteins analyzed (Fig. 3). The human ALG12 gene was localized on chromosome 22 according to the working draft sequence of the clone CITF22-1A6 (GenBank accession no. AL671710). The ALG12 gene included 10 exons spanning 15 kb (Fig. 4A). The 200C>T and 437G>A mutations detected in patient NJ mapped to exons 3 and 4, respectively. Sequencing of these exons in parental DNA samples indicated that the T67M mutation was of maternal origin and the R146Q mutation was inherited from the father (Fig. 4B).

To determine whether the T67M and R146Q amino acid substitutions affected ALG12 activity, the corresponding

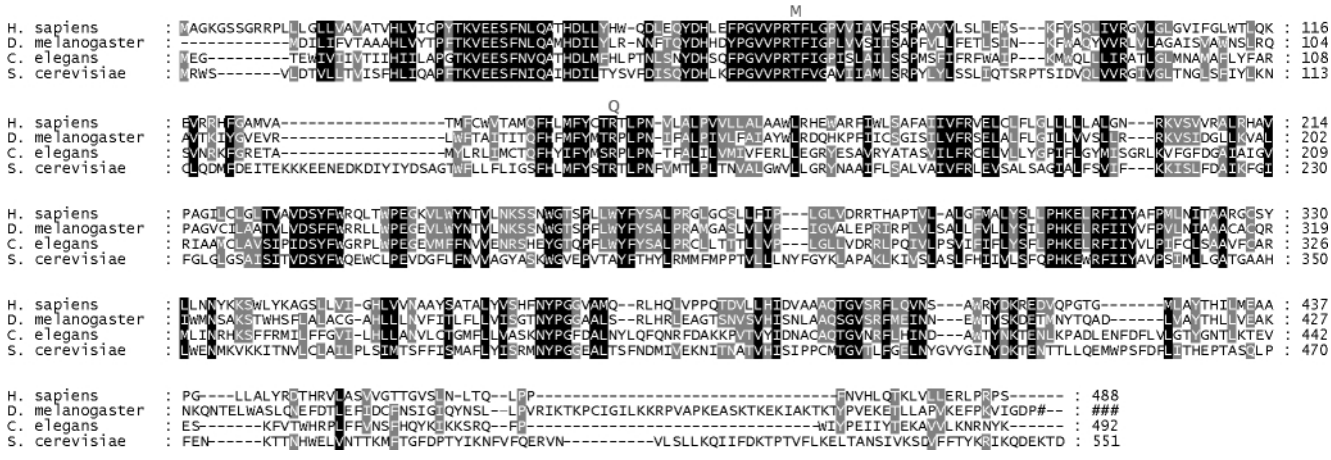

Figure 3. Comparison of ALG12 protein sequences. ClustalW alignment (38) of ALG12 proteins from *Homo sapiens* (GenBank accession no. NM_024105), *D. melanogaster* (RE17513), *C. elegans* (NM_072670) and *S. cerevisiae* (NP_014427). Positions conserved in the four proteins are shaded in black and residues conserved in three proteins are shaded in gray. The amino acid substitutions T67M and R146Q detected in patient NJ are indicated above the human sequence. The end of the *D. melanogaster* predicted ALG12 protein is truncated to fit this alignment.

changes (T61M and R161Q) were introduced in the *S. cerevisiae ALG12* locus. As *Δalg12* strains display no obvious growth phenotype, the effect of *alg12* mutant alleles was investigated in a *Δalg12wbp1–2* strain. This strain shows ALG12-dependent growth at 23°C on full medium (YPD) containing low amounts of hygromycin B (Fig. 5A). This aminoglycoside antibiotic is known to be more potent against many yeast glycosylation mutants (20).

As opposed to the wild-type gene, the mutant *ALG12[T61M]* gene failed to complement this growth defect. The R161Q mutation had a weaker effect on the integrity of the ALG12 protein than did the T61M mutation, since growth of *Δalg12wbp1–2* yeasts was restored by expression of *ALG12[R161Q]* (Fig. 5A). The double mutant T61M/R161Q had the same phenotype as the T61M mutant. We also assessed the glycosylation status of the vacuolar glycoprotein carboxypeptidase Y (CPY) in *Δalg12* yeasts transformed with *alg12* mutant alleles. Although CPY is only mildly hypoglycosylated in *Δalg12* mutant strains, its mobility is increased owing to truncated oligosaccharides transferred to protein (Fig. 5B) (19). Transformation with the wild-type yeast ALG12 gene restored a normal CPY glycosylation profile, but transformation with the mutant *ALG12[T61M]* DNA did not. As observed with the growth phenotype of *Δalg12wbp1–2* yeast, the mutation R161Q only slightly affected ALG12 function, since the glycosylation of CPY was nearly normal (Fig. 5B).

We expressed the NM_024105 human ALG12 cDNA under the control of the GPD promoter in *Δalg12wbp1–2* yeasts. As shown in Figure 6, growth of *Δalg12wbp1–2* yeasts was restored by expression of this cDNA, thus establishing the function of the corresponding protein. Importantly, the two mutant ALG12 alleles found in the CDG patient NJ, namely *ALG12[T67M]* and *ALG12[R146Q]*, did very weakly restore growth to the levels reached with the normal human ALG12 *cDNA.* This analysis confirmed the direct relation between these ALG12 mutations and the strongly reduced activity of the encoded mannosyltransferase. In conjunction with the identical phenotype observed both in patient NJ cells and *alg12* mutant yeast cells, namely the accumulation of incompletely assembled LLO, we postulate a novel type of CDG, specifically called CDG-Ig, that is due to a deficiency in the ALG12 locus.

DISCUSSION

The present study defines a novel genetic disease in humans by identifying a defect at the ALG12 mannosyltransferase locus as cause of a glycosylation disorder in a patient presenting with psychomotor retardation, hypotonia and various dysmorphic features. The ALG12 defect was characterized by an accumulation of the LLO *DolPP*–$GlcNAc_2Man_7$ oligosaccharide in the ER. Because this LLO is a poor substrate for the OTase complex, several N-glycosylation sites of glycoproteins remained unoccupied. It is noteworthy that the failure to detect the mature oligosaccharide $GlcNAc_2Man_9Glc_3$, either as an LLO or an NLO, indicates that the mutations detected in patient NJ inactivate ALG12 activity. Thus, this CDG case represents the first occurrence of a complete block along the LLO biosynthetic pathway identified in humans. In fact, in all cases of CDG-I analyzed so far, a significant portion of NLO have been shown to be derived from the mature LLO *DolPP*–$GlcNAc_2Man_9Glc_3$ (21–24) (C.E. Grubenmann and C.G. Frank, unpublished data).

The accumulation of *DolPP*–$GlcNAc_2Man_7$ observed in ALG12-deficient fibroblasts indicates that, as in yeast, this oligosaccharide is not a suitable substrate for the *ALG6* glucosyltransferase, which transfers Glc via an α1,3 linkage to the terminal mannose of the α1,2-mannose branch on *DolPP*–$GlcNAc_2Man_9$ (25). In consequence, the OTase complex, which recognizes the three terminal Glc residues on oligosaccharides, will only transfer the truncated $GlcNAc_2Man_7$ species at low efficiency to nascent glycoproteins. However, as indicated by the NLO profile of castanospermine-treated cells, it is evident that $GlcNAc_2Man_7$ is transferred to glycoproteins in patient NJ cells. The detection of the oligosaccharide

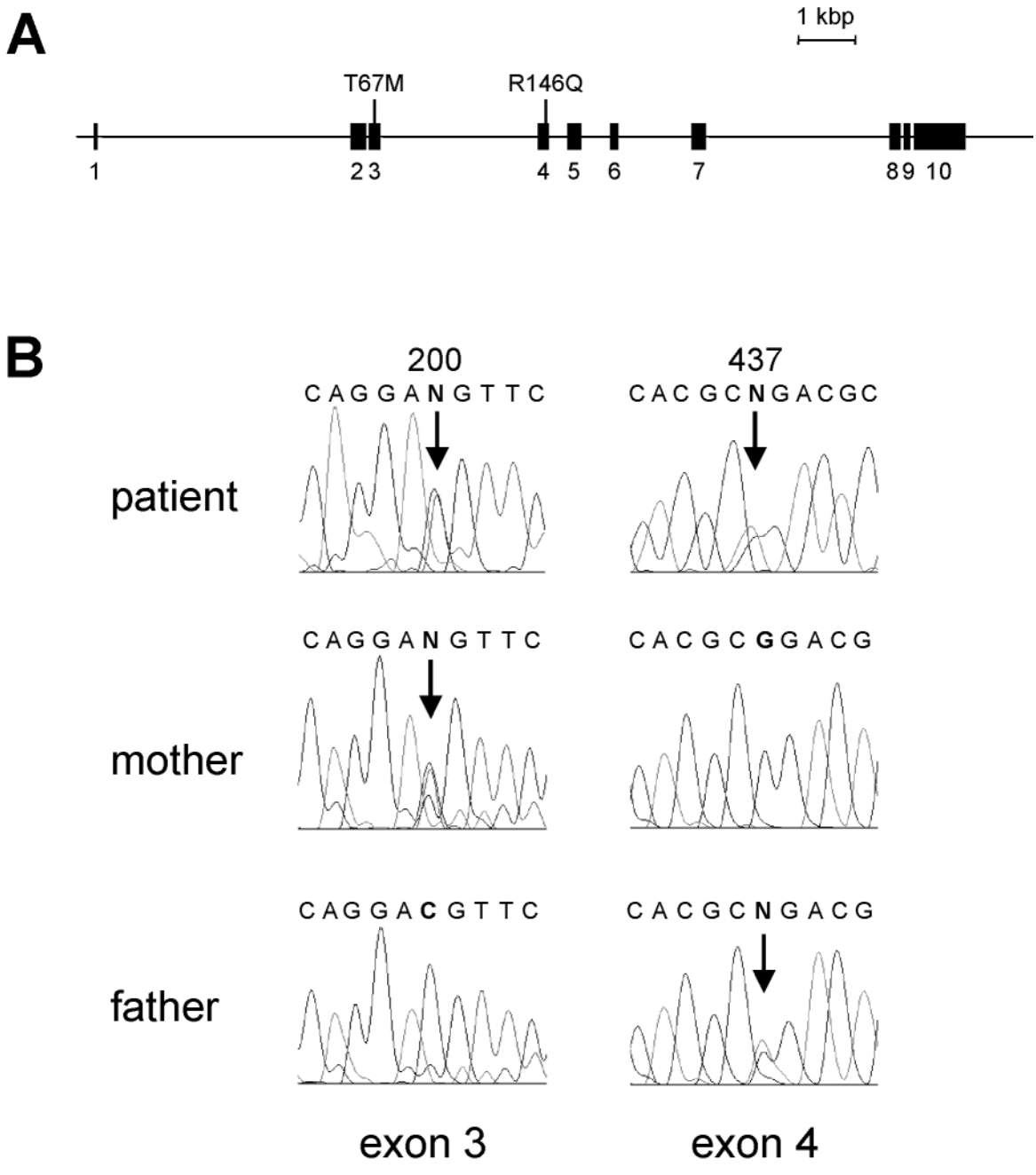

Figure 4. Human *ALG12* gene. (**A**) Genomic organization of the human *ALG12* gene, with exons represented as numbered boxes. Introns are indicated as solid lines. The positions of the T67M and R146Q mutations are marked on exons 3 and 4, respectively. (**B**) Electropherograms of *ALG12* exons 3 and 4 sequenced from patient NJ and his parents. The 200C>T and 437G>A mutations are indicated by arrows.

$GlcNAc_2Man_7Glc_1$ in the fibroblasts of patient NJ is certainly caused by the reglucosylation of $GlcNAc_2Man_7$ catalyzed by the ER-resident UDP–Glc glucosyltransferase enzyme involved in the quality control of protein folding (17).

The absence of mature oligosaccharides in patient NJ cells not only decreases the amount of N-glycan chains on proteins but also likely affects the functions assumed by this oligosaccharide along the secretion pathway of glycoproteins. After transfer to proteins, N-linked oligosaccharides function as signals in the process of protein folding (26) and for the export of misfolded glycoproteins to the cytosol for degradation (27,28). In the latter events, it has been shown in *S. cerevisiae* that the transition from $GlcNAc_2Man_9$ to $GlcNAc_2Man_8$ favors the targeting of misfolded proteins to the degradation pathway (29). The degradation of misfolded proteins was especially slowed down in *Δalg9* and *Δalg12* yeasts, indicating that $GlcNAc_2Man_6$ and $GlcNAc_2Man_7$ lacked the signaling ability for degradation of proteins.

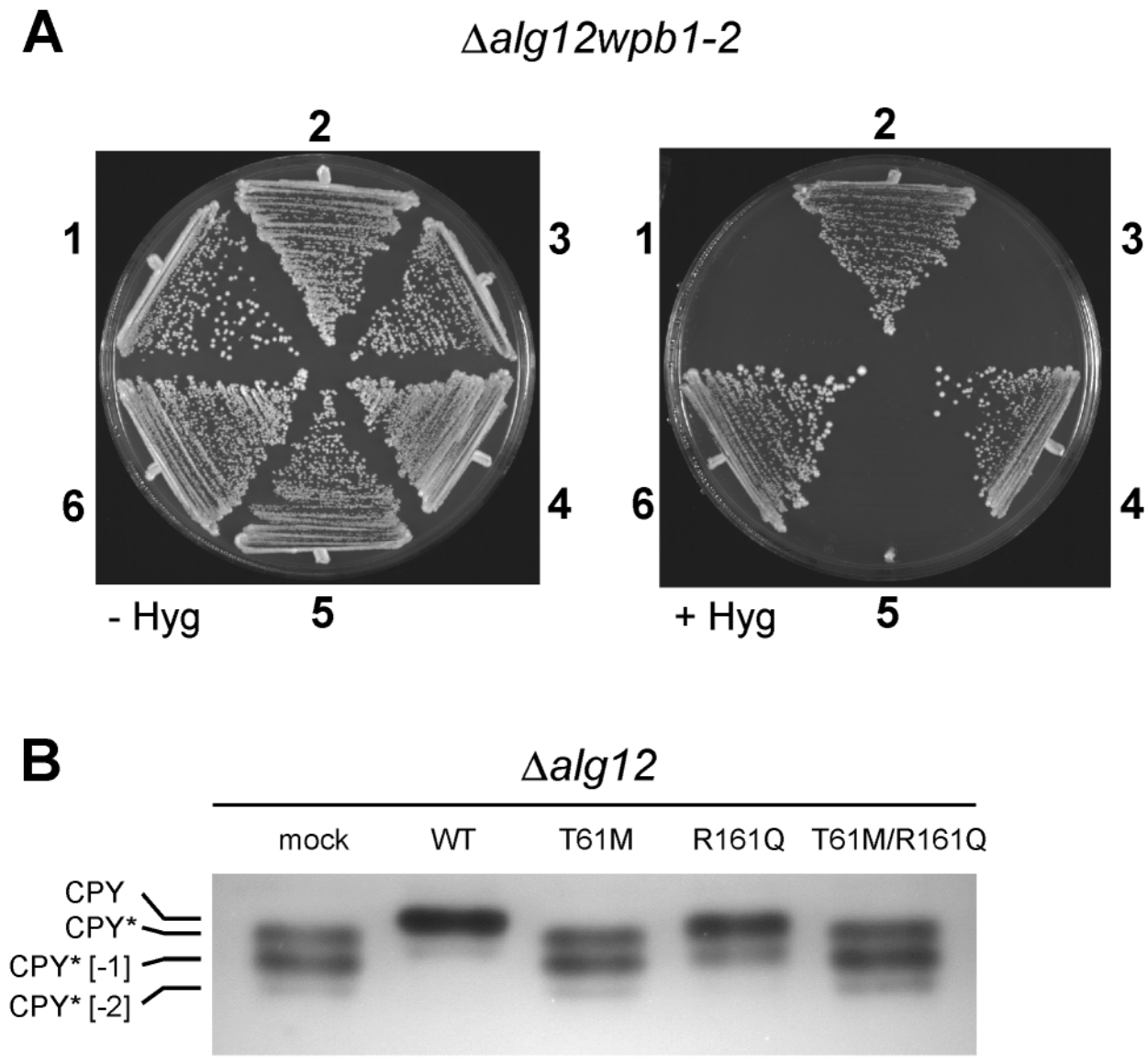

Figure 5. Phenotypes of *ALG12* yeast mutants. (**A**) Growth phenotype of Δ*alg12wpb1–2* yeasts transformed with (1) empty pRS416 vector or with pRS416 vector expressing (2) the normal *S. cerevisiae ALG12* gene, (3) the *S. cerevisiae ALG12[T61M]* gene, (4) the *S. cerevisiae ALG12[R161Q]* gene, (5) the *S. cerevisiae ALG12[T61M,R161Q]* gene or (6) the *S. cerevisiae* normal *WBP1* gene. Yeasts were grown on YPD medium at 23°C without hygromycin (left panel) and with hygromycin at 50 μg/ml (right panel). (**B**) Western blot analysis of carboxypeptidase Y (CPY) in Δ*alg12* yeasts transformed with an empty pRS416 expression vector (mock) or with a pRS416 vector expressing the normal *S. cerevisiae ALG12* gene (WT), the *S. cerevisiae ALG12[T61M]* gene (T61M), the *S. cerevisiae ALG12[R161Q]* gene (R161Q) or the *S. cerevisiae ALG12[T61M,R161Q]* gene (T61M,R161M). At the left side, the positions of normally glycosylated CPY and of those glycoforms with four, three and two truncated oligosaccharides (CPY*, CPY*[−1] and CPY*[−2], respectively) are marked.

Inhibition of mannose trimming by deoxymannojirimycin also slows down the degradation of misfolded proteins in mammalian cells (30), indicating that mannose trimming also functions as a degradation signal in higher eukaryotes.

Besides acting as signals in the quality control of glycoprotein folding and in the ER-associated protein degradation response (31), N-linked oligosaccharides also function as ligands for lectin proteins such as ERGIC53 and VIP36 (32) involved in the intracellular transport of cargo glycoproteins. It is reasonable to expect that the combination of under-occupancy of N-glycosylation sites and the occurrence of truncated $GlcNAc_2Man_7$ oligosaccharides may interfere with the folding, intracellular trafficking and ER-elimination of glycoproteins in patient NJ cells. Therefore, altered glycoprotein folding and intracellular transport may account for some of the features identified in patient NJ. For example, the absence or low levels of the serum glycoproteins IGF-binding protein 3, ATIII and immunoglobulins may be related to alterations along the secretory pathway. The availability of the ALG12-deficient cells from patient NJ now allows us to address this eventuality and to compare the intracellular maturation of proteins with other types of CDG.

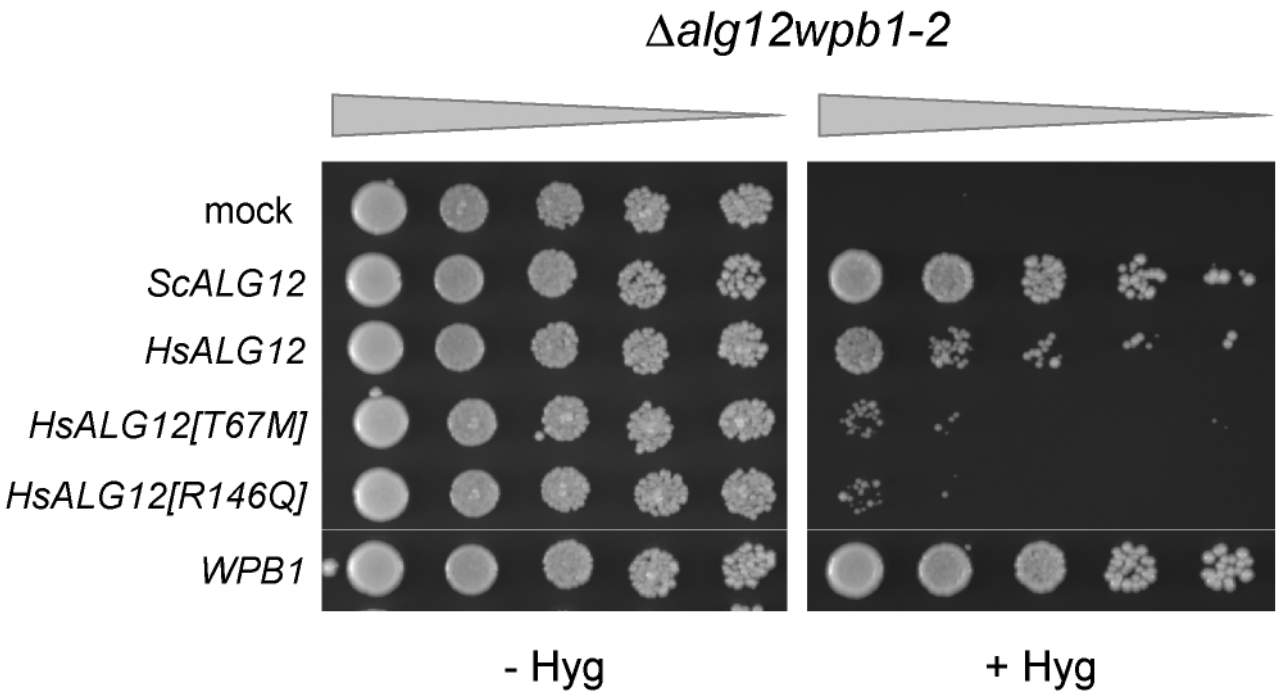

Figure 6. Complementation of Δ*alg12wbp1–2* yeasts with human *ALG12* cDNAs. YG843 transformants were spotted in 10-fold dilutions on YPD plates and incubated at 23°C for 6 days in the absence (left panel) or presence of 30 µg/ml hygromycin (right panel). Transformation was done with the pRS416 vector alone (mock), the pRS416 vector expressing the *S. cerevisiae ALG12* gene (*ScALG12*), the p426GPD vector expressing the human *ALG12* cDNA (*HsALG12*) or the mutant Hs*ALG12* cDNAs (*HsALG12[T67M]* and *HsALG12[R146QJ*), and with the YEp352 vector expressing the *WBP1* gene (39) (*WBP1*).

MATERIALS AND 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).

Mannose labeling

Fibroblasts were grown to 90% confluence on 450 cm^2. Before labeling, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 90 min at 37°C in 45 ml minimal Eagle's medium (Gibco) supplemented with 5% dialyzed FCS (Gibco). When specified, the glucosidase inhibitor castanospermine (Calbiochem) was added at 250 µM to the cells 2 h prior to labeling. For labeling, 150 µCi [^{3}H]mannose (54.0 Ci/mmol, Amersham Bioscience) was added to the cell medium and incubated for 1 h at 37°C. Cells were washed once with ice-cold PBS and scraped from the plates in 10 ml of methanol/0.1 mM Tris pH 7.4 (8 : 3 v/v).

Extraction of lipid- and protein-linked oligosaccharides

Chloroform (10.9 ml) was added to the cell suspension (10 ml) and the mixture was centrifuged at 5000*g* for 5 min after thoroughly mixing by vortexing. The upper and lower phases were removed and 3 ml chloroform/methanol/water (10 : 10 : 3 v/v/v) were added to the solid interphase. LLO were recovered from the supernatant after vortexing and centrifugation at 5000*g* for 5 min. The extraction was repeated three times, and the supernatants were pooled. The samples were dried under N_2 at 37°C, and the dried LLOs were hydrolyzed and prepared as described previously (33). NLO were recovered from the solid pellet of the LLO extraction after drying under N_2 at 37°C. Proteins were solubilized and denatured in 200 µl 0.5% SDS/2% β-mercaptoethanol at 100°C for 10 min. Oligosaccharides were cleaved from proteins by digestion with 1 unit of N-glycosidase F (Roche Diagnostics) in 300 µl of 50 mM sodium phosphate buffer pH 7.5, 1% Nonidet-P40, 0.33% SDS and 1.33% β-mercaptoethanol for 12 h at 37°C. Thereafter, 900 µl of cold ethanol was added, and the samples were centrifuged for 20 min at 2600g. The supernatants were then spun again for 30 min at 20 000*g* at 4°C. This supernatant was dried in a Speed-vac concentrator and resuspended in 400 µl of acetonitrile/water (7 : 3 v/v), passed through a 0.45 µm filter (Millipore) and subjected to HPLC.

HPLC analysis of oligosaccharides

[^{3}H]Mannose-labeled oligosaccharide samples were separated on a 250 mm × 4.6 mm LC-NH_2 aminopropyl column (Supelco), equipped with an LC-NH_2 guard column, as described previously (33).

RT–PCR

Total RNA was extracted from fibroblasts (2×10^7) using Tri-reagent (Sigma), following the instructions of the manufacturer. The human ALG12 cDNA was prepared from 4 µg of total

RNA using the primer 5′-TGCCCAGTCCTTTGACTTGCTTC-3′ and 4 units of Omniscript reverse transcriptase (Qiagen). The 20 µl reaction mixtures were incubated at 37°C for 2 h. The protein-coding region of the human ALG12 cDNA was amplified by PCR from 2 µl of RT product with the primers 5′-CAGTGCTAACGGCTGGTGTCTC-3′ and 5′-CTGGTAGTGATAACAGCTCCTGGA-3′. The cycling conditions were 35 cycles at 94°C for 45 s, 60°C for 30 s and 72°C for 2 min. Primers and unincorporated nucleotides were removed with QIAquick columns (Qiagen), and the PCR products were sequenced (Microsynth, Balgach, Switzerland).

Genomic DNA PCR

Genomic DNA from patient NJ and his parents was isolated from 10^7 fibroblasts and 5 ml of blood, respectively. The human ALG12 gene sequence was included in the chromosome 22 clone CITF22-1A6 (GenBank accession no. AL671710). ALG12 exons 3 and 4 were amplified with the primers 5′-AAGTGGAGGAGAGCTTCAAC-3′, 5′-GCAGGCAAGACTAACAGACA-3′ and 5′-GCTGGCATTGCAGCAGCATA-3′, 5′-GCTGTTGGCCAGGAAGTGTG-3′, respectively, for 35 cycles at 94°C for 45 s, 55°C for 30 s and 72°C for 1 min. The PCR products were sequenced (Microsynth, Balgach, Switzerland) after removal of primers and unincorporated nucleotides.

Plasmid construction

Human ALG12 cDNAs were amplified from normal and CDG-Ig patient cDNA with the primers 5′-CTAAAGAATTCTGTCTCGCACTGTTG-3′ and 5′-GCTTCTAGAAGACCTGTGGCTGCTGA-3′ containing *Eco*RI and *Xba*I restriction sites, respectively, and subcloned into the same sites of pBluescript-II KS+ (Stratagene). The human ALG12 cDNAs were subcloned as blunted *Hin*dIII-*Xba*I fragments into the *Sma*I site of p426GPD (34), placing them under control of the strong glyceraldehyde-3-phosphate dehydrogenase promoter. The homologous T61M and R161Q substitutions were introduced into the yeast ALG12 gene by quick-change mutagenesis (Stratagene) of pALG12 (19) using the oligonucleotides 5′-GGAGTAGTCCCTAGAATGTTCGTTGGTGCTGTGATTATTGC-3′ and 5′-GCAATAATCACAGCACCAACGAACATTCTAGGGACTACTCC-3′ for T61M and the oligonucleotides 5′-CCTCATGTTCTACAGCACTCAAACTCTGCCTAATTTTGTCATGAC-3′ and 5′-GTCATGACAAAATTAGGCAGAGTTTGAGTGCTGTAGAACATGAGG-3′ for R161Q. The 1.2 kb *Eco*81I–*Sac*I fragments from the resulting plasmids were subcloned into pCFZ14–416ALG12 (see below), replacing its *Eco*81I–*Sac*I fragment, to yield the mutated forms. A second round of mutagenesis following the same procedure was performed to combine the mutations. The regions between the *Eco*81I and *Sac*I sites of the mutated plasmids were verified by sequencing. To create pCFZ14–416ALG12, a single-copy plasmid with the yeast ALG12 gene, the 2.4 kb *Kpn*I–*Sac*I fragment of pALG12 was subcloned into the same sites of pRS416 (35).

Yeast strains and media

S. cerevisiae strains used in this study were derivatives of YG840 (*MAT*a *ade2-101 ura3–52 his3Δ200 lys2–801 Δalg12::kanMX4*) (19) or YG843 (*MAT*α *ade2–101 ade3 ura3–52 his3Δ200 leu2 Δalg12::kanMX4 wbp1–2*) (19). Standard yeast media and genetic techniques were used (36). Strain YG840 and its transformants were propagated at 30°C, and strain YG843 and its transformants at 23°C.

Complementation of *alg12* yeast mutants

Western blotting of the carboxypeptidase Y glycoprotein was performed as described previously (37). Hygromycin B sensitivity of *Δalg12 wbp1–2* transformants was assessed on full medium (YPD) plates containing 30 or 50 µg/ml hygromycin B (Roche Diagnostics). For the spot assay, 5 µl of serial 10-fold dilutions of YG843 transformants grown overnight in liquid medium were spotted, starting at 5×10^5 cells. Plates were incubated at a given temperature for 6 days.

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