

## Report

# Identification and Functional Analysis of a Defect in the Human *ALG9* Gene: Definition of Congenital Disorder of Glycosylation Type *IL*

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Defects of lipid-linked oligosaccharide assembly lead to alterations of N-linked glycosylation known as “type I congenital disorders of glycosylation” (CDG). Dysfunctions along this stepwise assembly pathway are characterized by intracellular accumulation of intermediate lipid-linked oligosaccharides, the detection of which contributes to the identification of underlying enzymatic defects. Using this approach, we have found, in a patient with CDG, a deficiency of the *ALG9*  $\alpha$ 1,2 mannosyltransferase enzyme, which causes an accumulation of lipid-linked-GlcNAc<sub>2</sub>Man<sub>6</sub> and -GlcNAc<sub>2</sub>Man<sub>8</sub> structures, which was paralleled by the transfer of incomplete oligosaccharides precursors to protein. A homozygous point-mutation 1567G→A (amino acid substitution E523K) was detected in the *ALG9* gene. The functional homology between the human *ALG9* and *Saccharomyces cerevisiae* *ALG9*, as well as the deleterious effect of the E523K mutation detected in the patient with CDG, were confirmed by a yeast complementation assay lacking the *ALG9* gene. The *ALG9* defect found in the patient with CDG—who presented with developmental delay, hypotonia, seizures, and hepatomegaly—shows that efficient lipid-linked oligosaccharide synthesis is required for proper human development and physiology. The *ALG9* defect presented here defines a novel form of CDG named “CDG-IL.”

Glycosylation is a widespread posttranslational modification that has a considerable impact on numerous biological processes (Varki 1993). The N-linked glycosylation pathway bears the hallmark of first assembling the GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> oligosaccharide core on the lipid carrier dolichylpyrophosphate (*DoIPP*) and then of transferring it *en bloc* to nascent polypeptides. In recent years, several defects along the N-linked glycosylation pathway have been characterized (Jaeken 2003; Thiel et al. 2003; Wu et al. 2003; Grubenmann et al. 2004; Kranz et al. 2004; Schwarz et al. 2004) and have been defined as types of congenital disorders of glycosylation (CDG). The majority of CDG known to date represent defects of dolichol-linked oligosaccharide assembly, classified as “CDG-I.”

Clinically, CDG are associated with a broad range of symptoms, such as the failure to thrive, axial hypotonia, cerebellar dysfunction, seizures, mental retardation, and coagulopathy (Krasnewich and Gahl 1997). These rather nonspecific features together with a variability in severity of these manifestations render it difficult to make a clear diagnosis and necessitate a genetic characterization to establish the causality of glycosylation disorders.

The study of yeast glycosylation mutants has provided a wealth of knowledge about the mechanisms of endoplasmic reticulum (ER)-associated N-glycosylation (Burda and Aebi 1999), where >20 enzymatic activities and an even greater number of genes are involved (Freeze 2001). In addition, these yeast glycosylation mutants have been instrumental in the characterization of several disorders of glycosylation (Aebi and Hennet 2001). Besides establishing the genetic basis of novel human diseases, the identification of additional defects along the assembly of dolichol-linked oligosaccharides allows an assessment of the biological importance, for humans, of each glycosylation step.

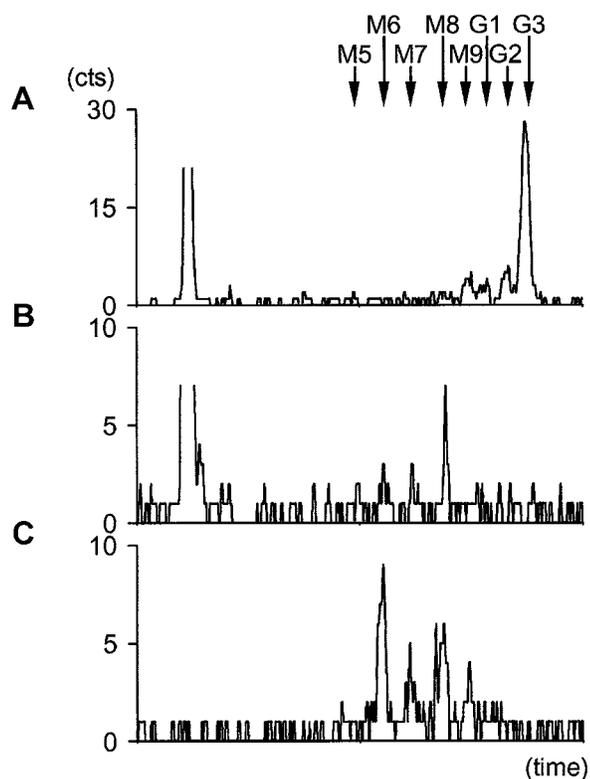
In the present study, we report a novel type of CDG-I identified in a female patient born at term as a twin with

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**Figure 1**  $[^3\text{H}]$ Mannose-labeled lipid- and N-linked oligosaccharide profiles from human primary fibroblasts. *A*, Separation of LLOs from normal control fibroblasts. *B*, Separation of LLOs from patient fibroblasts. *C*, Separation of NLOs from patient fibroblasts. Arrows mark the elution times of standard oligosaccharides from yeast (*M5*–*M9*,  $\text{GlcNAc}_2\text{Man}_{5-9}$ ; *G1*–*G3*,  $\text{GlcNAc}_2\text{Man}_9\text{Glc}_{1-3}$ ).

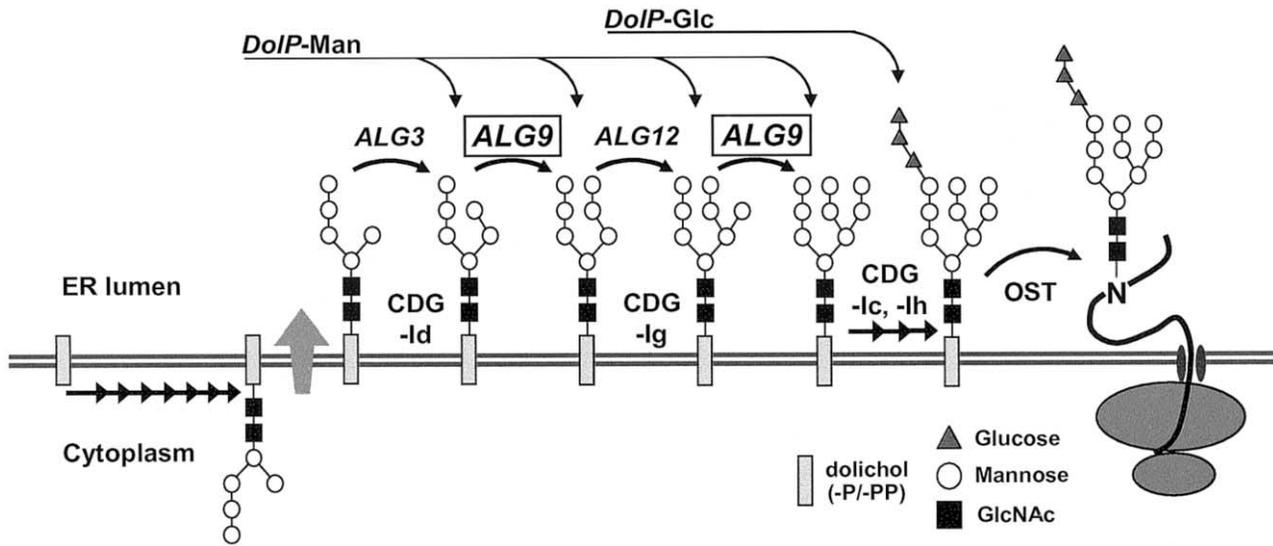
a birth weight of 2.75 kg, length of 44 cm, and head circumference of 31.5 cm. As she grew older, her clinical features included severe microcephaly, central hypotonia, seizures, hepatomegaly, developmental delay, and bronchial asthma. This pattern of clinical presentations was compatible with CDG, a diagnosis confirmed by isoelectric focusing analysis of serum transferrin. The patient sample showed significant increase of disialo- and asialo-transferrin, indicative of CDG-I (data not shown). Normal values of phosphomannomutase activity measured in patient fibroblast extracts excluded the most frequent CDG type, CDG-Ia (MIM 212065). To detect a possible defect of lipid-linked oligosaccharide (LLO) assembly, fibroblasts from the patient were labeled with  $[^3\text{H}]$ mannose, and the glycans released from the lipid carrier or cleaved from proteins were analyzed by high-performance liquid chromatography, as described elsewhere (Grubermann et al. 2002). The LLO profile revealed an accumulation of incomplete precursor structures corresponding to *DolPP*- $\text{GlcNAc}_2\text{Man}_6$  and *DolPP*- $\text{GlcNAc}_2\text{Man}_8$  (fig. 1*B*). In comparison, only the complete *DolPP*-

$\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$  LLO is found in healthy control fibroblasts (fig. 1*A*).

The analysis of protein N-linked oligosaccharides (NLO) confirmed the glycosylation alteration detected in the patient's fibroblasts. The normal NLO profile of human fibroblasts corresponds to three peaks representing the structures  $\text{GlcNAc}_2\text{Man}_8$ ,  $\text{GlcNAc}_2\text{Man}_9$ , and  $\text{GlcNAc}_2\text{Man}_9\text{Glc}_1$  (data not shown).  $\text{GlcNAc}_2\text{Man}_9$  and  $\text{GlcNAc}_2\text{Man}_8$  arise from the immediate trimming of NLO by ER resident glucosidase and  $\alpha$ -mannosidase enzymes (Kilker et al. 1981; Camirand et al. 1991). In the patient with CDG, an additional peak possibly corresponding to the incomplete  $\text{GlcNAc}_2\text{Man}_6$  structure was visible (fig. 1*C*), suggesting that truncated oligosaccharides were transferred to proteins in this patient.

The dual accumulation of *DolPP*- $\text{GlcNAc}_2\text{Man}_6$  and *DolPP*- $\text{GlcNAc}_2\text{Man}_8$  suggested a possible defect at the level of the *ALG9*  $\alpha$ 1,2-mannosyltransferase, which, in yeast, catalyzes the addition of the seventh (Burda et al. 1996) and ninth (C. G. Frank and M. Aebi, unpublished data) mannose residues on growing LLOs (fig. 2). A human homolog to the yeast *ALG9* gene (MIM 606941) has been described recently (Entrez accession number AF395532) (Baysal et al. 2002). The cDNA encodes a predicted multispanning membrane protein of 611 amino acids, with 43% similarity to the *Saccharomyces cerevisiae* Alg9 protein. A sequence alignment of several Alg9 proteins showed highly conserved domains, possibly representing regions responsible for the active site of this enzyme (fig. 3). The human *ALG9* cDNA was prepared from 4  $\mu\text{g}$  of total fibroblast RNA by use of the specific reverse-transcription primer 5'-TGTTACAGGCGATG-ACTTGC-3'. The protein-coding region of the *ALG9* cDNA was amplified by PCR with 3  $\mu\text{l}$  of reverse transcription product and the primers 5'-CGCGCTCACC-GACTTCATA-3' and 5'-GGAGTCAGGTCAGTGG-ATCA-3' through 30 cycles of amplification at 94°C for 45 s, at 53°C for 30 s, and at 72°C for 90 s. The PCR fragments were sequenced directly. In the patient *ALG9* cDNA, we detected the point mutation 1567G→A, which introduced the amino acid change E523K in the *ALG9* protein (fig. 3). This mutation was not found in the control cDNA samples that were sequenced nor in 108 expressed sequence tags retrieved from GenBank, excluding the possibility of a frequently occurring polymorphism. As a confirmation, the 1567G→A mutation was also detected at the genomic level by sequencing exon 14 of the patient *ALG9* gene (data not shown).

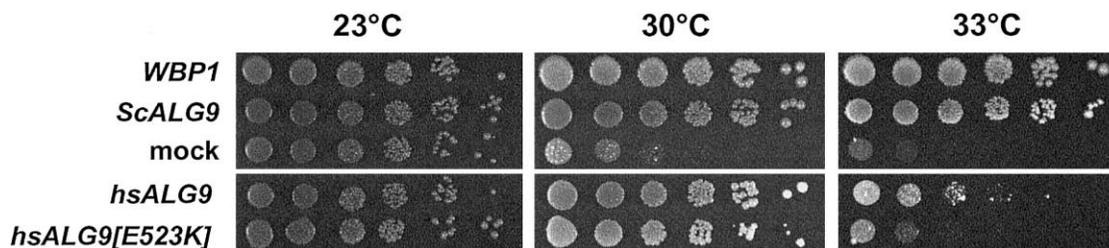
To verify that the E523K mutation impaired *ALG9* function, we analyzed the mutant human cDNA in a yeast complementation assay. Deletion of *ALG9* alone in yeast does not result in any obvious growth phenotype. We therefore used the synthetic phenotype of a  $\Delta\text{alg9 wbp1-2}$  double mutant. The combination of the deficiency in LLO biosynthesis ( $\Delta\text{alg9}$ ) and a reduced



**Figure 2** LLO biosynthesis pathway in the ER lumen. Arrows mark the reactions in which dolichol-phosphate (*DoIP*)–Man or *DoIP*–Glc are used as substrates and where the *DoIP*–Man-dependent transferases *ALG3*, *ALG9*, and *ALG12* are involved. The complete oligosaccharide  $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$  is transferred from its lipid carrier to nascent polypeptides by the oligosaccharyltransferase complex (OST). Known types of CDG are marked with the respective names CDG-Ic, -Id, -Ig, and -Ih.

Hs	MASRGARQLKKGSGASSGDTAPADKRELLG-----SREAGGAEHRTELSGNK-----AG-----QVWAPEGSTAKCLLSARICALLSNISDCDEIFNYWEPTHYLIVGEGEOTWEY	105
Ce	-----MVTHRRKGGSGPPQKPPRIVDRSSFDADKKIKVEKLYHKANNPDND-----WPPSFGSVSKMLLSIRISGATGTHNDCDEVYNYWEPLHLFVGEGETWEY	100
Dm	MAPPAARARYIANKADNQILSKKPKRPLNGNKKTEATPAGKKDKDAKRNQPTSGGQEKGLPNPIMPVQTAKTFSARICSAIAYIADCCEIFNYWEPLHYLIVGEGEOTWEY	120
Sp	-----LVSFVWTSILAVLRITSSSRVYDCCDEVYNYWEPLHYLIVGEGEOTWEY	61
Sc	-----MNCKAVIISLLELFRVYIQPTSLISDCDEIFNYWEPTHYLIVGEGEOTWEY	55
Hs	SPAYIRSRVYALHAWFAAHARILQTNKILVFPFRCLLAFVSCICCLYFYKAVCKKCCIHHSRMMADLVLSLGMFCSSAPLPSSECMYTTLLIMTCWYMDK-----TSLIAVLG	218
Ce	SPVYIRSRVYFYIHYTHASLFAANLPGDTKLVVFTLRLTIGLECLGCEYYAFDAICCKINNHATGRFFILRSHSSMFLASNAFVPSSECMATIFYIHCAYLNEN-----WTAGHFC	213
Dm	SPFCCLRSYFYLQGVFYGYQKLFNPSPLIFVYRMLGFCGAMRMYMKSHCOEFCCHRCMLHLGLESVEMFVSSHALLPSSESNYFGCAALAAWQON-----YCFAPHL	233
Sp	SPFYIRSRVYALHAWRG-FLARGLGLSRHLPYFRCLVACFSAPCDNLLIAVARNENRALHLTSLVLFVNSGMSHSSSFLPSSEANNVTLAISQLSPPST-----KRTVKVWSF	177
Sc	SPFYIRSRVAFLLPFYCYLIPVVMFTDLESHWNFTTRACLGFESFIMFKLHRRFAGSLALQANWITLIGLQENPFWHSAVELLPSAVALLLYVGCATRHSRYLSTGTSTSNFTSLAY	175
Hs	VAAGAILGWPFSAALGSLTAPDLLVMKH----RWKSEFHWSLMAALLFLVPVWVWDSVYVYKVIAPLNIIMLYNVF---PHGPDLYGEEFVWFYIKGELNFNVAVALALLVLELSTSM	331
Ce	VAFSTVVGWPFSAVLSLPIVADMLLKLG-----LRIRFILTSLVIGLCTGGVQVVDSDHYFGKTVLAPDNIPIYNVVS---GGPFLYGEELSFYKKNFNWNIIVFAAPFGPFLSAY	326
Dm	TATSAALLGWPFPAALGIPVILEMLRQR-----DWKTFVQVWTHSGATVAIPMADTSYFGKTFAPENIIMYVNVF---SHGPNIFGEEELSYVYIENFNENIWLWALQLPIMLVTD	346
Sp	ITTCGVIGWPFSAALSIFPILLLELDLGRFRHLFCRWFKALFVALLTIGICITVDSLFYERIQFVAMNIVRYNVLAK-DGRGPDYGEEMWYVFAVLSLQHNIVLWFAACCPBLVHA	296
Sc	NFLASILGWPFVLLSLPCLHYLFNHR--IISTIRTAFCDCILFSLTAFAVIVDSDHYFKIAPVSWNIIPIYVNIINASEESGPNIFGVEFVYVPELLENS-----HLPVIV	282
Hs	EYLORFHVQ-----NLGHPYWTIAFPMIIFLFIQHHKEERFLFPVYPLIICCGAVASALQCYH---FVQRYRLEHYTVTSMVLAGTIFLFGLSFSRSVALFRVHGELDL	442
Ce	FTKQWMSQDRNVALYQRFAPILLVAVTTAAWDLIFGSCAHKEERFLFPYVPEFAFFAALADATNRICLCK-----KLGMDNLSLFLILCFATFSASRTYSHNNYVGHVETL	433
Dm	YLLVPAKSKS-----TLNFPHYSLAPLWDLVFFAQQHKEERFLFPYVPEHSCGATIVDVYQVIFFRMKSVVFKIKAGVHYLDHSMFAHLVAVTSTLGLSRVFLYRNVHAKMDL	461
Sp	AFTNWINLDS-----FLDLSVVISFPIIIFLFIQHHKEERFMYPIYEVLCVAAATIGDMSLQMIQ---ILSSINETVRSKFPVRFVLCVVAIGCLSTARLQTN-VNARMII	405
Sc	LAIIGIHLR-----LWPVWAS-LETVAVFTQHHKEERFLYPIYGLTITSSASHAFYKVLNENR-----KPIKKCKKLSVLLIVAGQAMSERVAVLVNMYTAVIAV	379
Hs	VPEFYRATDPTTIHTVPEGRFVNVGVKEMVFPFSSLLFDN-----WQLQPTFSEFRGOLFKEPAGCP-----LAFRIIVFTDNDQNLBEPSPVYIDISKCHNVVDVDMTR--EPTPR	547
Ce	FRSLNAELNTRNFKN-FHDDIRVGVKMTFRFPSSFLPQTVSDGKKVEMRFLISEFRGLERKPLKSD-----KLVEVTRHIEEANNLAQBEISRNVVLDSDCYVVDMDMP--QSDR	545
Dm	MLELNQFKATPOYDDP-----VLYNVGVKMDRYEGSSEFBAKN-----FRLRFLSSEFRGLRAYVDEGQ-----NALLVQVYFENLQCEHMHFDYDRQDFVVEFDEGK--YHAL	564
Sp	VPAISPLETENNVTIN-----VGVKEMVFPFSSLLFDN-----RLRFVRSERDGLGEEVSNSTWV-NREGYVCEBEHNEFNNEPFRITS-ESQDFHLDLDFDHSKATVN	511
Sc	BEQFSSLNQGGVKAPV-----VN-VCTGRVWHPFSSLLFDN-----HRLRFVRSERDGLGEEVSNSTWV-IPFKITLLEKGMNRYNYDTGCREWPTTRQDFHLDLIVAPIN-LAKD	483
Hs	EKKVSSNKEWISLAYRFFLDAS-----RSKLLRAFYVPELSDQYTVVNYTILKPKKAKQIRKKSQG-----	611
Ce	EFDGRKMRQYKRRKTKKMKRIENAILQFVWLSLFEERIKFLNSNFSPFVFVDNFGLEQANSHYSFLKITGNQWIRPHSSMSNQDSDMGFFVHFHMFHSLPNTMS	653
Dm	EMVSKRSKDSVSKSLPFIPE-----KSHKLLRAFYVPELTDNHQVGDENLKKRKTNRNGR-----	623
Sp	BEIKSK-SDGMIPVMVYFIDTR-----QVPPVGRAPVPEETPKWGRMEILVKKPVKIDFSNLRASKQQA-----	577
Sc	VFNPLHMDNKNKACAAFDGE-----NSKILGRAFYVPEPINRIMQVLPKQNVQVYVGRVIDYCLFEKPTETT-----	555

**Figure 3** ALG9 protein sequence comparison. Amino acid sequences of ALG9 proteins from *Homo sapiens* (Hs) (Entrez accession number AAL25798), *Caenorhabditis elegans* (Ce) (Entrez accession number CAA90107), *Drosophila melanogaster* (Dm) (Entrez accession number NP\_651353), *Schizosaccharomyces pombe* (Sp) (Entrez accession number CAB57773), and *S. cerevisiae* (Sc) (Entrez accession number CAA96122) were aligned using the ClustalW program (Thompson et al. 1994). The amino acid change E523K is indicated for the human ALG9 protein.



**Figure 4** Complementation of  $\Delta alg9 wbp1-2$  yeasts with human *ALG9* cDNAs. Transformants were spotted in sixfold dilutions on YPD plates and were incubated at 23°C, at 30°C, or at 33°C for 120 h. Transformants carried either the vector alone (“mock”), a plasmid complementing the *wbp1-2* mutation (“*WBP1*”), the p*ALG9* plasmid expressing the *S. cerevisiae ALG9* gene (“*ScALG9*”), or plasmids expressing the normal human *ALG9* cDNA or the CDG patient *ALG9*[E523K] cDNA.

oligosaccharyltransferase activity (*wbp1-2*) results in a temperature-sensitive phenotype at 30°C (Burda et al. 1996). The  $\Delta alg9 wbp1-2$  strain was transformed with control plasmids and multicopy plasmids expressing the normal human *ALG9* or the *ALG9*[E523K] cDNAs, under control of the strong *TDH3* promoter. The human *ALG9* cDNA included the common polymorphism 865G→A described elsewhere (Baysal et al. 2002). In contrast to the human wild-type *ALG9* cDNA, transformation of the mutant *ALG9*[E523K] allele showed only a minor effect on growth of  $\Delta alg9 wbp1-2$  yeasts at 33°C (fig. 4). Both versions restored growth at 30°C, indicating a residual activity of *ALG9*[E523K]. This demonstrates that human *ALG9* is functionally homologous to yeast *ALG9* and that the E523K mutation has a detrimental effect on *ALG9* function. When the biochemical phenotype of the CDG fibroblasts is considered, this study demonstrates that the human *ALG9* protein catalyzes, like its yeast counterpart, the transfer of mannose onto the two acceptor substrates *DolPP*-GlcNAc<sub>2</sub>Man<sub>6</sub> and *DolPP*-GlcNAc<sub>2</sub>Man<sub>8</sub>.

Clinically, the *ALG9* deficiency was accompanied by symptoms that are common for most types of CDG-I; that is, developmental delay, hypotonia, and epilepsy. The fact that a significant residual *ALG9* activity was detected, as shown by the presence of normal NLOs in the cells of the patient with CDG and by the yeast complementation assay, underlines the importance of the reactions catalyzed by *ALG9*, since even a leaky mutation accounts for a severe disease phenotype. As for all types of CDG-I, the disease is caused by protein underglycosylation; that is, the nonoccupancy of critical N-glycosylation sites, which probably accounts for most of the clinical manifestations observed. However, transfer of truncated oligosaccharides to protein may also affect intracellular processes of glycoproteins, such as the chaperone-assisted protein folding, degradation of misfolded proteins in the ER (ERAD), the ER-Golgi trafficking, and the transport to lysosomes (Plempner and Wolf 1999; Parodi 2000; Helenius and Aebi 2001). The monogluco-

sylation of N-glycans, reciprocally controlled by glucosidase II and the UDP-Glc:glycoprotein glucosyltransferase (GT), regulates the association of glycoproteins with chaperone lectins in the so-called calnexin/calreticulin cycle (Parodi 2000; Helenius and Aebi 2001). In vitro studies showed that truncated N-glycan structures with <9 mannoses are suboptimal substrates for GT (Sousa et al. 1992) or for the lectin chaperones calreticulin and calnexin (Ware et al. 1995; Spiro et al. 1996). Sugar structures also control glycoprotein degradation. For example, in *ALG9*-null yeast mutants, ERAD of a malfolded glycoprotein was impaired (Jakob et al. 1998). Therefore, part of the phenotype for patients with CDG could result from transfer of shorter-than-normal glycans to protein, which might interfere with cellular protein quality-control mechanisms.

The neurological symptoms found in most types of CDG demonstrate the importance of N-glycosylation for development and function of the nervous system (Krasnewich and Gahl 1997; Jaeken and Matthijs 2001). Recently, Baysal et al. (2002) found carriers of a chromosome translocation interrupting the *ALG9* gene in a family showing a high frequency of bipolar affective disorder (BPAD [MIM 125480]). It could be speculated that *ALG9* activity, required at two steps along the pathway, becomes rate limiting in cells if only one productive copy of the gene is present. Similarly, mild neurological alterations and behavioral disorders have been described among mild cases of CDG-Ia (Grünwald et al. 2001; Briones et al. 2002). Although involvement of the *ALG9* mannosyltransferase activity in BPAD requires further study, it may be relevant to consider testing for alterations of N-glycosylation in patients with BPAD or related psychiatric diseases.

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## Electronic-Database Information

The accession numbers and URLs for data presented herein are as follows:

Entrez, <http://www.ncbi.nlm.nih.gov/Entrez/> (for *ALG9/DIBD1* cDNA for *H. sapiens* [accession number AF395532], *C. elegans* [accession number CAA90107], *D. melanogaster* [accession number NP\_651353], *S. pombe* [accession number CAB75773], and *S. cerevisiae* [accession number CAA96122])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for CDG-Ia, *ALG9/DIBD1*, and BPAD)

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