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Reduced Heparan Sulfate Accumulation in Enterocytes Contributes to Protein-Losing Enteropathy in a Congenital Disorder of Glycosylation

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Intestinal biopsy in a boy with gastroenteritis-induced protein-losing enteropathy (PLE) showed loss of heparan sulfate (HS) and syndecan-1 core protein from the basolateral surface of the enterocytes, which improved after PLE subsided. Isoelectric focusing analysis of serum transferrin indicated a congenital disorder of glycosylation (CDG) and subsequent analysis showed three point mutations in the *ALG6* gene encoding an α1,3-glucosyltransferase needed for the addition of the first glucose to the dolichol-linked oligosaccharide. The maternal mutation, C998T, causing an A333V substitution, has been shown to cause CDG-Ic, whereas the two paternal mutations, T391C (Y131H) and C924A (S308R) have not previously been reported. The mutations were tested for their ability to rescue faulty *N*-linked glycosylation of carboxypeptidase Y in an *ALG6*-deficient *Saccharomyces cerevisiae* strain. Normal human *ALG6* rescues glycosylation and A333V partially rescues, whereas the combined paternal mutations (Y131H and S308R) are ineffective. Underglycosylation resulting from each of these mutations is much more severe in rapidly dividing yeast. Similarly, incomplete protein glycosylation in the patient is most severe in rapidly dividing enterocytes during gastroenteritis-induced stress. Incomplete *N*-linked glycosylation of an HS core protein and/or other biosynthetic enzymes may explain the selective localized loss of HS and PLE. *(Am J Pathol 2000, 157:1917–1925)*

Congenital disorders of glycosylation (CDG) are caused by defective synthesis of precursors in the glycosylation pathway.[1–4] These defects reduce the amount or change the structure of the lipid-linked oligosaccharide (LLO) precursor for *N*-linked glycosylation, leading to underglycosylation of many proteins,[3] including antithrombin-III, factor XI, or protein C. This reduces their levels, leaving patients at risk for coagulopathy. Mutations in phosphomannose isomerase (*MPI*, causing CDG-Ib) and phosphomannomutase (*PMM2*, causing CDG-Ia) reduce the amount of GDP-Man, the immediate precursor of LLO,[5] thus reducing the amount of *N*-linked glycosylation. Mutations in *ALG6*, which encodes an α1,3-glucosyltransferase, cause CDG-Ic.[6–8] This enzyme is required for the addition of the first of three glucose residues to LLO, and without the first glucose, further glucosylation is prevented. The nonglucosylated precursor oligosaccharide is a poor substrate for the oligosaccharyltransferase complex and is inefficiently transferred to proteins.[9,10] Furthermore, the absence of glucose on the oligosaccharides will affect the quality control and folding of the proteins.[11]

PMM-deficient patients have severe psychomotor and mental retardation, peripheral neuropathy, cerebellar hypoplasia, and pericardial effusions among other symptoms.[1,12] They often have feeding problems and failure to thrive.[13] On the other hand, PMI-deficient patients have normal intelligence, achieve developmental milestones, and do not show neuropathy, cerebellar hypoplasia, or pericardial effusions. Instead, they present with hypoglycemia, liver fibrosis, and protein-losing enteropathy (PLE).[14–16] The α1,3-glucosyltransferase-deficient patients (CDG-Ic) have a milder CDG-Ia-like appearance.[6,8] The deficiency has previously been shown to result from a homozygous point mutation in the *ALG6* gene leading to an A333V change in the protein.[6,7] Here we describe a patient with this and two other *ALG6* mutations that severely compromise the transferase function. The patient was initially seen for PLE, and an intestinal biopsy showed a complete absence of the normal heparan sul-

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fate (HS) on the basolateral surface of enterocytes.[17,18] However, it improved after PLE subsided. The *ALG6* mutations seem to compromise *N*-linked glycosylation most severely in rapidly dividing cells.

Materials and Methods

Media and Materials

S. cerevisiae cells were grown in standard yeast peptone dextrose and synthetic complete media.[19] *E. coli* was grown in Luria-Bertani medium.[20] Restriction enzymes and T4 DNA ligase were from Promega (Madison, WI). Oligonucleotides were from Genbase (San Diego, CA). Sequencing was performed using BigDye sequencing kit on an ABI 377 DNA sequencer, both from Applied Biosystems (Foster City, CA).

Enzyme Assays

The lysate from fibroblasts were centrifuged at 10,000 × *g* × 5 minutes and the supernatant used to assay activities of PMM (EC 5.4.28) and PMI (EC 5.3.1.8) as described[21] and modified.[22,23] The Dol-P-Glc synthase (EC 2.4.1.177) assay was previously described.[24] GDP-mannose pyrophosphorylase (GDPMP, EC 2.7.7.13) assay has been described.[25]

Analysis of Serum Transferrin

Isoelectric focusing (IEF) analysis of transferrin was done using sera from the patient, defined cases of CDG-1a, CDG-Ib, and normal controls were as described previously.[26]

Analysis of the Genes

Total RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA). First-strand cDNA of the human *ALG6* was synthesized at 45°C for 40 minutes using Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) and primer oVW118: 5′ AAT GGT AAT TTC ATT TAT ACA TAG C 3′. The cDNA was used as template in a polymerase chain reaction with primers oVW117: 5′ AAG AAG TGA TTG ACC ACG TTT 3′ and oVW118 using the following cycle: 94°C for 2 minutes; 20× (94°C for 20 seconds, 48°C for 30 seconds, 70°C for 1 minute; 20× (94°C 20 for seconds, 48°C for 30 seconds, 70°C for 2 minutes); 70°C for 10 minutes. The 1700-bp fragment was used as template for the sequencing reaction and for further amplification with primers oVW132: 5′ TTG CGC ACA GAA TTC CCC TCC CTA AAT 3′ and oVW133: 5′ GAC TGG AAC CTC GAG GAA ACA ATT TGT TTA GG 3′ that introduced the restriction sites *Eco*RI and *Xho*I at each end of the *ALG6* gene. Both the amplified cDNA and the recloned *ALG6* fragments were sequenced essentially as described previously[26] using primers oVW117 or 132, oVW 118 or 133, oVW126: 5′ GCT GAT CTG CTG ATT 3′, oVW105: 5′ CCT AGG GTC ACT GGC, oVW106: 5′ GTG TCA CTA CCA GTC 3′, oVW108: 5′ GAC TGG TAG TGA CAC, oVW109: 5′ GCC AGT GAC CCT AGG 3′, and oVW143: 5′ GGG CAT TAG GAG TTC 3′.

Strains and Plasmids

The *ALG6*-deficient *S. cerevisiae* strain YG227 (*Matα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg6::HIS3*)[27] was used to express the human α1,3-glucosyltransferase gene *ALG6*. Subcloning and transformation of *E. coli* and yeast were performed using standard procedures.[20] The expression vector pWE85 was constructed by subcloning a *Not*I-*Eco*RI fragment containing the strong constitutive *NOP1* promoter (a gift from S. Emr, University of California, San Diego, CA) into the same sites of pRS426.[28] pWE137 and pWE126 encoding the maternal A333V and the paternal Y131H, S308R α1,3-glucosyltransferase, respectively, were constructed by digesting the polymerase chain reaction products obtained from above with *Eco*RI and *Xho*I and cloning the fragment into the same sites of pWE85. To separate the two paternal mutations, pWE126 was digested with *Avr*II and *Xho*I, and the fragment containing the DNA encoding the S308R mutation was recloned into the same sites of pWE187 carrying the wild-type human *ALG6* gene. This resulted in pWE355. The Y131H-encoding plasmid was constructed by digesting pWE126 with *Eco*RI and *Avr*II and cloning of the fragment into the same sites of pWE187, resulting in plasmid pWE215. All plasmids were resequenced before transformation into the *ALG6*-deficient *S. cerevisiae* strain YG227.[27]

In Vivo *Glycosylation of Carboxypeptidase Y (CPY)*

S. cerevisiae strain YG227 containing one of the following plasmids: pWE85 (without the *ALG6* gene), pWE187 (containing wild-type human *ALG6* cDNA), pWE134 (encoding A333V α1,3-glucosyltransferase), pWE126 (encoding Y131H, S308R α1,3-glucosyltransferase), pWE215 (encoding Y131H α1,3-glucosyltransferase), and pWE355 (encoding S308R α1,3-glucosyltransferase), was used to investigate the effects of the mutations in *ALG6* on the *in vivo* glycosylation of CPY. The transformed yeast cells were grown in SC medium without uracil[19] overnight at 30°C. The next day the cells were diluted to an optical density (OD) at 600 nm of 0.05 and followed spectrophotometrically. Cells were harvested at either exponential phase (OD_{600} = 0.3 to 0.5) or early stationary phase ($OD_{600} \geq 2.0$) by centrifugation. One OD_{600} unit of cells was suspended in 10 μl of phosphate-buffered saline (PBS) with protease inhibitors (Complete, Mini Ethylenediaminetetraacetic acid-free; Boehringer Mannheim, Mannheim, Germany) and 10 μl of 2× sodium dodecyl sulfate-loading buffer.[20] After heating for 10 minutes at 100°C, the samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels followed by Western blot using rabbit antiserum against CPY (a generous gift from Jakob Winther, Carlsberg Laboratory, Copenhagen, Denmark).

Immunohistochemical and Histochemical Analysis

Endoscopic biopsies of the small intestine were processed in formalin or snap-frozen in liquid nitrogen. Mucosal inflammation was assessed on frozen sections using monoclonal antibodies (mAbs) (DAKO, Cambridgeshire, UK) against CD3, CD4, CD8, CD19, CD25, and HLA-DR. Epithelial and basement membrane composition analysis used mAbs against laminin and collagen IV, (DAKO) and tenascin (Sigma, Gillingham, UK) using dilutions previously determined on sections of tonsil, spleen, small intestine, and colon. Bound antibody was localized by peroxidase or alkaline-phosphatase immunohistochemistry. Epithelial glycosaminoglycans (GAG) distribution was done on formalin-fixed tissue using antibody mAb 10E4 (Seikagaku, Abingdon, UK) that recognizes HS chains, and visualized with avidin-biotin (Vectastain Elite, Vector, Peterborough, UK). Histochemical detection of sulfated GAGs using cationic colloidal gold in PBS at pH 1.2 (polylysine gold, 1/100; Biocell Int., Cardiff, UK) together with a silver enhancer, as previously reported.[29–31] Specific enzymatic digestions confirm that this technique detects HS in epithelial and endothelial cells, and chondroitin and dermatan sulfates within the mucosal tissues.[29,31] Immunostaining of syndecan-1, the major HS core protein, was done as previously described,[17] using mAb MCA681H (Serotec, Kidlington, UK) specific for human syndecan-1.[32,33]

Results

Clinical History

The patient was seen at age 3 months for profound PLE after gastroenteritis. The usual features seen in PLE (such as lymphangiectasia) were not found, and endoscopic small bowel biopsy showed only minimal abnormality with slight crypt elongation. The colonoscopy was normal. Proteinuria was absent. The postenteritis PLE normalized within a month, but during the next year, he had several episodes of life-threatening PLE after acute gastroenteritis, requiring large amounts of intravenous-infused albumin. He was cortisol-deficient (<50 nmol/L) and the episodes settled with cortisol replacement. At 14 months he had the first of several tonic-clonic fits, with a normal electroencephalogram.

At 9 months he showed prolonged blood coagulation time, Factor XI deficiency, and marked delay in motor development including peripheral hypotonia, but maintained normal reflexes and muscle bulk. Bayley scales showed global developmental delay to 5 months. He had little visual attention, no nystagmus, and visual evoked potentials were normal bilaterally, whereas magnetic resonance imaging scan showed delayed myelination.

The combination of periodic PLE and Factor XI deficiency prompted transferrin IEF analysis (Figure 1), which showed an abnormal pattern typical of CDG-I.[34] Attempted therapy with oral mannose gave no clinical improvement, and no significant increase in Factor XI activity. He continues to show marked developmental delay, hypotonia, and remains hypocortisolic, with severe Factor XI, antithrombin III, heparin cofactor II, Protein C, and Protein S deficiencies. Occasional convulsions occur, but repeated electroencephalogram has been normal.

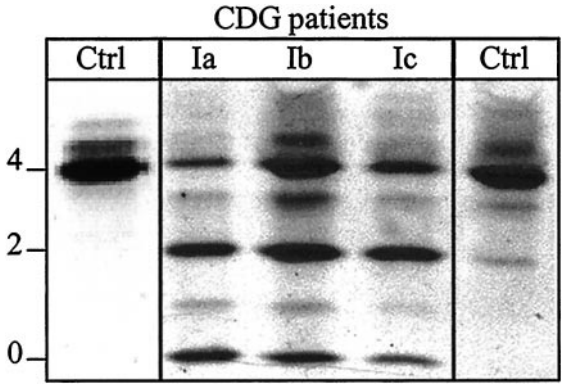

Figure 1. Altered IEF pattern of serum transferrin indicates CDG. Serum transferrin IEF analyses were done on: age-matched controls (ctrl), patients with verified CDG-Ia, CDG-Ib, and the patient described in this article with CDG-Ic. The oligosaccharides of normal transferrin contain four sialic acids (4), whereas the abnormal bands correspond to molecules of di-sialo- (2) and asialo (0)-transferrin.

Histological, Histochemical, and Immunohistochemical Analysis of Mucosal Biopsies

Small bowel biopsies were performed on two occasions, the first during PLE and the second while the patient was well. Histological assessment was mostly normal showing only slight villous blunting with no crypt hyperplasia or inflammatory enteropathy. Lamina propria and intraepithelial lymphocytes, CD3, CD4, CD9, CD19, and CD25 populations were within normal limits on both samples (data not shown). The epithelium during PLE was HLA-DR-negative (Figure 2A). Basement membrane laminin, collagen IV, and tenascin distributions were normal (data not shown), but the distribution of HS was abnormal, especially during acute PLE.

The basolateral epithelial membrane usually has abundant heparanase-sensitive, chondroitinase-insensitive sulfated GAGs,[31] but these were completely absent during PLE (Figure 2, B and C). A small amount of punctate, intraepithelial staining was found, mostly above the nucleus (arrow in Figure 2B). This abnormal pattern and punctate staining (arrows in Figure 2, D and F) is seen using a mAb (10E4) specific for HS chains (Figure 2, D–G) and another that recognizes the major HS core protein in enterocytes, syndecan-1[17,18] (Figure 2, I and J). Sulfated GAGs and immunoreactive HS were normal within the lamina propria and on vascular endothelium in both biopsies. The subepithelial basement membrane seemed intact. The second biopsy, taken while the patient was well (Figure 2H), showed similar findings in only 11 of 16 identifiable villi, whereas 5 of 16 villi showed a

more normal, but slightly fainter basolateral-staining pattern. This extended throughout the villus in 2 of 5 and was patchy in 3 of 5. Villi with more normal basolateral staining had considerably fewer HS-positive intracellular aggregates. In contrast, both biopsies showed normal basolateral HS staining in the patient's colon (Figure 2K), stomach, or esophagus (data not shown), suggesting that the pathology was restricted to the small intestine.

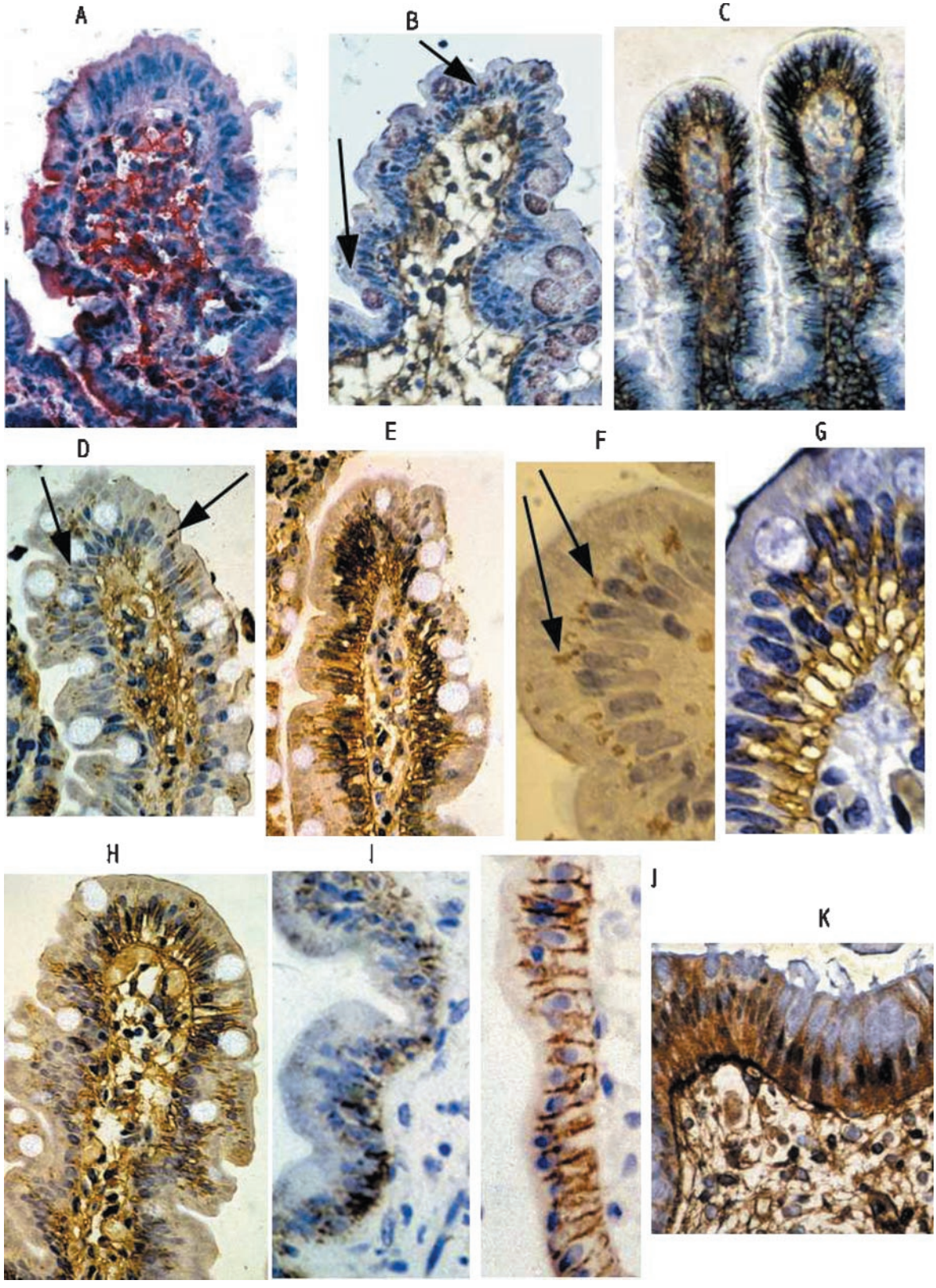

Table 1. Enzymatic Activities Expressed as nmol · min^{-1} · mg $protein^{-1}$ in Fibroblasts from Healthy Controls and the Patient (Mean Value ± SD)

	Enzymatic activity		
	PMM	PMI	GDP-M-P
Control	2.7 ± 0.7 (n ± 9)	23 ± 9 (n ± 8)	610 ± 120 (n ± 7)
Patient	3.0	24	840

The activities were measured in a number (n) of different controls as described in the table and in Materials and Methods. The values for the patient are the average of at least two independent determinations. These varied less than 10% for PMM and PMI, and less than 20% for GDP-M-P.

Analysis of Fibroblasts

Fibroblasts from the patient were analyzed to identify the specific CDG defect. PMM, PMI, and GDP-Man pyrophosphorylase activities were normal (Table 1). LLOs were isolated from the patient's and the control's fibroblasts labeled with [2-^{3}H]mannose, and the size of the sugar chains was analyzed by amine adsorption high pressure liquid chromatography (Figure 3). The oligosaccharide from control LLOs gave the expected pattern with the major peak co-eluting with $Glc_3Man_9GlcNAc_2$. Sugar chains from the patient were smaller and co-eluted with a nonglucosylated sugar chain derived from LLO glycan made by a *S. cerevisiae* strain with a nonfunctional *ALG6* gene.[27] This gene codes for an α1,3-glucosyltransferase which is required for the addition of the first glucose residue during LLO biosynthesis. The patient's oligosaccharide was completely sensitive to α-mannosidase digestion, whereas digestion of the corresponding oligosaccharide from control cells produced free mannose and another product, $Glc_3Man_4GlcNAc_2$. The smaller size of the patient's oligosaccharide and its sensitivity to α-mannosidase digestion suggested that the first glucose residue was not added during LLO biosynthesis. This could result from deficiencies in either Dol-P-glucose synthesis or α1,3-glucosyltransferase, the defective enzyme in CDG-Ic.[6–8] Direct assay of Dol-P-glucose synthase activity in the patient's fibroblasts showed normal levels compared to controls and to other CDG patients with known defects in other genes[26] (data not shown), suggesting a defective α1,3-glucosyltransferase.

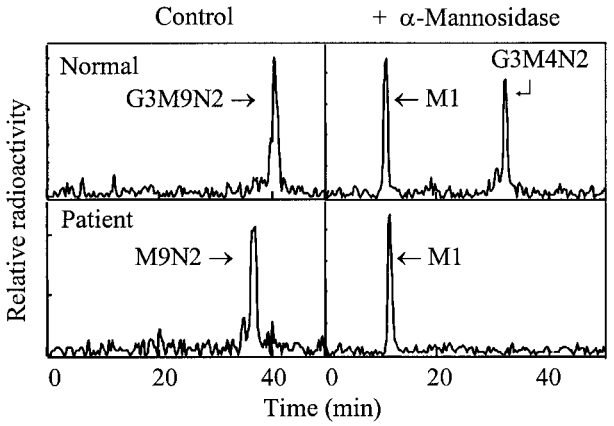

Figure 3. HPLC analysis of LLO precursors. [2-^{3}H]Mannose-labeled LLO was isolated from fibroblasts and analyzed by amine adsorption HPLC either before or after α-mannosidase digestion. Identification of peaks was based on known [2-^{3}H]mannose-labeled standards from CHO cells ($Glc_3Man_9GlcNAc_2$, G3M9N2) or from *ALG6*-deficient yeast ($Man_9GlcNAc_2$, M9N2). M1 is free mannose.

Analysis of ALG6 in the Patient

Using reverse transcriptase-polymerase chain reaction we analyzed the sequence of the *ALG6* cDNA from the patient, his asymptomatic parents, and several controls. The controls had the expected published sequence for human *ALG6*, but the patient had three base substitutions in the coding region of the gene. A C → T at position 998 on the cDNA level (C998T) changes A → V at position 333 of the Alg6 protein (A333V), the T391C mutation results in Y131H, and the C924A mutation results in S308R (Figure 4). The first mutation is present in the maternal allele (Figure 4, top) and the second two in the paternal allele (Figure 4, middle). Previously, a homozygous C998T substitution was reported in CDG-Ic patients[6,7] indicating that this homozygous substitution alone can be responsible for the phenotypes seen in CDG-Ic.

Rescue of Altered CPY Glycosylation in a Yeast Strain Defective in ALG6

ALG6-deficient *S. cerevisiae* strains inefficiently glycosylate their glycoproteins.[27] For example, the vacuolar

Figure 2. Small intestine enterocytes are deficient in basolateral HS accumulation during PLE. **A:** Mucosal expression of HLA-DR is normal during acute PLE. The infant was grossly edematous with ascites, and had required pre-endoscopy albumin infusion (plasma albumin, 14 g/L: normal, >40 g/L). Immunohistochemistry shows normal numbers of DR^+ subepithelial macrophages with no expression on epithelium (endogenous alkaline phosphatase is seen in the brush border). Mucosal $CD3^+$ and $CD25^+$ cells were also normal (not shown). Thus, there was no histological or immunohistochemical evidence of inflammatory enteropathy or epithelial activation in the patient. Original magnification, ×100. **B:** Patient's abnormal distribution of sulfated GAGs, stained by specific cationic probe, shows absence of basolateral staining and abnormal punctate staining above nuclei (**arrows**). Basement membrane and lamina propria GAGs are preserved. Original magnification, ×100. **C:** Sulfated GAG expression, stained as in **B**, in histologically normal pediatric small intestinal biopsy, showing striking localization on the basolateral enterocyte surface, as previously reported.[30] Original magnification, ×100. **D:** Patient's HS immunohistochemistry (mAb10E4) shows a similar appearance to the sulfated GAG staining shown in **B**, with no expression in lateral intercellular spaces and punctate supranuclear staining (**arrows**). Original magnification, ×100. **E:** HS immunohistochemistry in histologically normal biopsy from control infant shows staining of basolateral membrane, similar to sulfated GAG staining in **C**, but none of the intracellular aggregates seen in the patient. Original magnification, ×100. **F:** High-power view (×400) of HS distribution in patient's epithelium, showing supranuclear localization of intracellular inclusions (**arrows**). **G:** High-power view (×400) of HS distribution in normal epithelium, showing contrasting basolateral localization. **H:** Biopsy taken from patient when not protein-losing (original magnification, ×100) shows partial normalization of HS distribution compared to **D**, especially in slower growing mature cells near the villus tip. **I:** Syndecan-1 immunohistochemistry in the patient during acute PLE. Original magnification, ×200. Some patchy basolateral localization, with supranuclear aggregates, is also seen. However substantially less syndecan-1 has reached the basolateral epithelial surface than in normal pediatric small intestine. **J:** Syndecan-1 distribution in normal small intestine (original magnification, ×200) resembles the normal sulfated GAG histochemistry and HS immunohistochemistry above, although the basement membrane is unstained. **K:** Normal sulfated GAG distribution in patient's colon during PLE, contrast to GAG loss in small intestine shown in **B**. Original magnification, ×100.

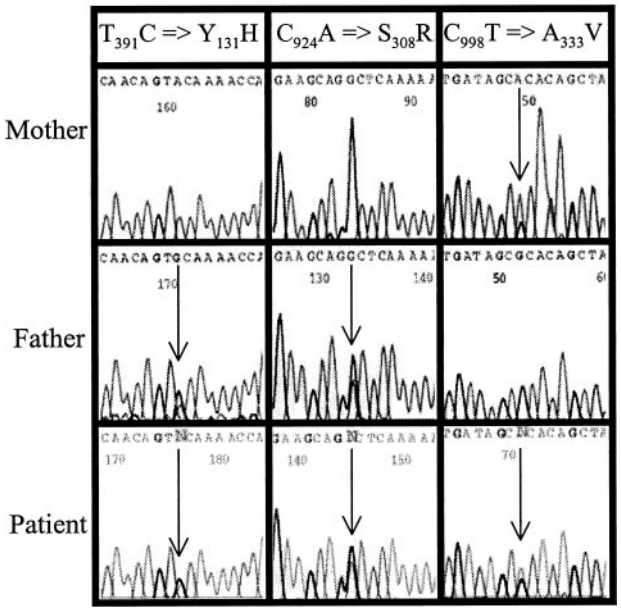

Figure 4. Mutations in *ALG6*. The panels show the electropherograms of the *ALG6* sequence obtained from the mother, father, and CDG-Ic patient. Sequencing was performed using primer oVW109 (for the Y131H change) and oVW108 for S308R and A333V, both read in the 3′ to 5′ direction, but only the region surrounding the transitions is shown. **Top:** The maternal sequence with the C/T change at position 998. **Middle:** The paternal sequence with the T/C and C/A change at positions 391 and 924, respectively. **Bottom:** The sequence from the CDG-Ic patient, where all three changes are seen.

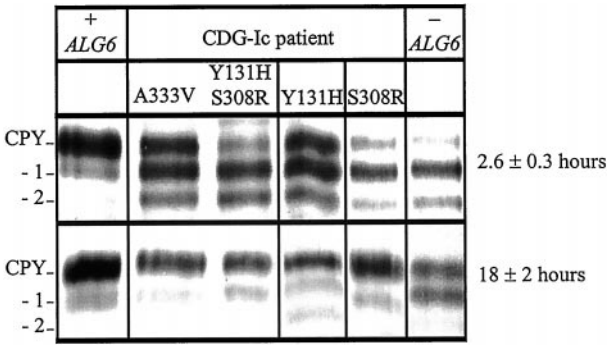

Figure 5. Complementation of faulty CPY glycosylation in an *ALG6*-deficient yeast strain. **Top:** Fast growing cells, generation time = 2.6 ± 0.3 hours. An Δ*alg6* strain of *S. cerevisiae* was harvested at exponential growth phase. The strain is unable to fully glycosylate CPY and predominantly makes molecules lacking one or two *N*-linked chains (−*ALG6*). Transformation with control *ALG6* restores glycosylation to normal (+*ALG6*), whereas transformation with the maternal allele encoding the A333V protein only has slight ability to rescue glycosylation. The paternal allele encoding the Y131H, S308R protein cannot rescue glycosylation of CPY. When each paternal mutation is expressed separately, the S308R mutation seems more severe than Y131H. **Bottom:** Slow growing cells, generation time = 18 ± 2 hours. The severity of the same mutations is assessed during early stationary phase. The effects of the mutations are considerably less severe at this slower growth rate.

carboxypeptidase CPY has four *N*-linked chains, but in Δ*alg6* strains, it lacks one or two of these chains. Full glycosylation can be restored by transformation with a plasmid carrying either the yeast or human *ALG6*.[6,7] We examined the functional consequences of each mutation by testing its ability to restore normal glycosylation to CPY. Expressing the various alleles from a multicopy plasmid under a strong constitutive promoter showed that normal human *ALG6* completely restores CPY glycosylation in the Δ*alg6* strain in the exponential growth phase (generation time = 2.6 ± 0.3 hours) (Figure 5, +*ALG6*). In contrast, the maternal allele, encoding the A333V Alg6 protein, only partially restores normal glycosylation because CPY glycoforms lacking both 1 and 2 oligosaccharide chains are seen. The paternal *ALG6* allele containing two substitutions resulting in Y131H and S308R cannot correct CPY glycosylation, and the glycosylation pattern is similar to yeast without Alg6p function (Figure 5, −*ALG6*) where the majority of CPY lacks oligosaccharide chains. To determine which of these paternal mutations was more critical for activity, each was tested separately for CPY glycosylation rescue. Expression of *ALG6* with only the Y131H substitution seems to have a similar severity to A333V (Figure 5, top). Some fully glycosylated CPY is seen, but the majority of CPY still lacks 1 and 2 oligosaccharides. S308R Alg6p alone weakly restores CPY glycosylation (Figure 5, top). Endoglycosidase H digestion to release all of the sugar chains, produces only one band indicating that the size differences in immunoprecipitated CPY are based on the number of oligosaccharide chains (data not shown). These results suggest that this patient has a more substantial loss of *ALG6* activity than previously described patients homozygous for A333V[6] and for the recently described S478P,[7] which could explain the severe clinical presentation.

Interestingly, when investigating cells in early stationary growth phase (generation time = 18 ± 2 hours), it became clear that the *ALG6* mutations have much more severe consequences in rapidly dividing cells compared to slowly growing cells (Figure 5, top and bottom panels, respectively). We interpret this to mean that the impaired glycosylation capacity is overwhelmed in fast-growing cells, but tolerated in slow growing yeast cells, where the glycosylation-demands are less.

Discussion

The patient presented in this article is deficient in several coagulation factors (Factor XI, antithrombin III, heparin cofactor II, Protein C, and Protein S) similar to CDG-Ia-c patients.[3,8] In addition, this is the first reported CDG-Ic patient who is also cortisol-deficient. This is likely because of the absence of critical *N*-linked oligosaccharides on transcortin.[35] The deficiencies in the patient could occur because most secretory and membrane proteins require glycosylation for correct folding and thereby for the further transport through the secretory pathway. In mammalian cells, calnexin and calreticulin seem to be important for quality control. They are lectins that interact specifically with partially trimmed, monoglucosylated *N*-linked oligosaccharides.[36–38] Thus, the absence of glucose on the *N*-linked chains can alter their association with the quality control apparatus, which can delay their movement through the secretory pathway or lead to their degradation in the endoplasmic reticulum. Furthermore, the oligosaccharyl transferase complex, responsible for the transfer of the sugar chains to the polypeptide, has a

decreased affinity for the incomplete substrate.[9,27] Thus, glucosylation is crucial for efficient transfer of the LLO to the protein and thereafter for proper quality control. However, a mutant form of the yeast vacuolar protease, CPY, lacking all glycosylation sites is still transported to the vacuole, but at a slower rate than fully glycosylated CPY.[39]

Patients with several forms of CDG that result in protein underglycosylation often have gastrointestinal pathologies. For instance, PMI-deficient CDG-Ib patients have severe diarrhea, vomiting, and PLE.[14–16] Small intestine biopsies show partial villus atrophy and the enterocyte endoplasmic reticulum is distended and engorged with insoluble precipitated proteins associated with the abundant chaperone BiP.[14] Oral mannose therapy effectively relieves nearly all of the symptoms of these patients.[14,40] PMM-deficient CDG-Ia patients also show a failure to thrive and many have gastrointestinal abnormalities. Duodenal biopsies show shortened villi, increased inflammatory cells in the stroma, and dilated smooth endoplasmic reticulum.[13] Patients with CDG-Ie, caused by a defect in the synthesis of dolichol-P-mannose, have persistent reflux and poor weight gain despite caloric supplementation.[26] It is likely that the high synthetic and growth demands placed on intestinal epithelial cells make them especially vulnerable to glycosylation-based pathologies.

We are uncertain about the basis of localized HS loss in this patient, but it is seen only in the small intestine, not in the more slowly turning over epithelial cells of the stomach, colon, and esophagus.[41,42] Altered glycosylation may directly affect the synthesis and stability of the core protein, syndecan-1, or it may be indirect because many of the HS biosynthetic enzymes in the Golgi are most likely *N*-linked glycosylated. Intracellular accumulation of the small amount of residual HS-immunoreactive material in the nuclear region, perhaps the Golgi, suggests that the sulfated chains do not reach the surface. The syndecan-1 protein has two potential *N*-linked glycosylation sites (NFS at position 43 and NQS at position 231), and both are conserved between rat and human. The first potential *N*-linked glycosylation site is strongly conserved between mouse, hamster, rat, and human. All of the syndecan-1 proteins examined so far contain at least one potential *N*-linked glycosylation site, indicating that *N*-linked glycosylation is important. Underglycosylation may cause misfolding leading to degradation of the majority of core protein in the endoplasmic reticulum, whereas the remainder is inefficiently targeted. Thus, underglycosylation could directly or indirectly affect the interaction of HS with extracellular ligands involved in receptor-signaling complexes and other cell surface interactions.[43]

Severe PLE in children usually results from structural lesions where epithelial HS is preserved (eg, lymphangiectasia)[44] or inflammatory enteropathy (eg, celiac disease, Crohn's disease). The molecular basis is unknown, although albumin loss from inflammatory-based degradation of epithelial HS has been proposed as a pathogenetic mechanism in Crohn's disease.[30] The involvement of HS in PLE was also seen in a study of three infants with normal small bowel biopsies, who had massive PLE from birth and loss of HS from the basolateral enterocyte membrane.[31] HS loss from the glomerular basement membrane leads to albumin leakage across the vascular endothelium in kidney[45–48] and thus enterocyte HS deficiency may be analogous to congenital nephrotic syndrome.[47] The findings here suggest that CDG-1c causes a similar loss of HS from the enterocyte basolateral membrane. Loss of syndecan-1 is also seen in reparative cells from patients with inflammatory bowel disease, and this is thought to compromise their ability to bind basic fibroblast growth factor.[49] The beneficial effects of exogenous heparin in promoting healing may result from its ability to substitute for the missing HS chains and restore high-affinity growth factor binding and proliferation. In addition, heparin has also been reported to reduce PLE related to Fontan cardiac surgery in children.[50] Therapy aimed at increasing epithelial HS expression may be clinically useful to prevent chronic intestinal protein loss.

The first biopsy of the patient taken during PLE showed a complete absence of basolateral HS staining, whereas the subsequent biopsy taken while well showed substantial basolateral HS in ~1 of 3 villi. Intracellular HS only occurred in those villi where basolateral staining was absent. It is striking that this child's several episodes of severe and prolonged life-threatening PLE all followed acute gastroenteritis, where crypt cell proliferation increases to repair damaged epithelium. We speculate that the already inefficient glycosylation is overwhelmed by the increased epithelial turnover in gastroenteritis, effectively leading to complete loss of HS in the small intestinal epithelium. Failure to detect any HS abnormalities in the patient's colon, stomach, and esophagus because of slower cellular turnover is supported by a twofold to threefold slower turnover in these organs in rats.[41,42] It is also consistent with our finding that the *ALG6* mutations impact CPY glycosylation most severely in rapidly dividing yeast. This pattern of episodic severe PLE, often associated with recurrent infections, rather than a consistent gradual protein-loss, has been seen in other children with CDG-I[14] (and our unpublished observations). Unexplained PLE is a life-threatening complication of many multisystemic disorders. It is possible that enteric protein loss seen in some individuals results from environmental stresses that exceed their glycosylation capacity.

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