A new inborn error of glycosylation due to a Cog8 deficiency reveals a critical role for the Cog1–Cog8 interaction in COG complex formation

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The hetero-octameric conserved oligomeric Golgi (COG) complex is essential for the structure/function of the Golgi apparatus through regulation of membrane trafficking. Here, we describe a patient with a mild form of a congenital disorder of glycosylation type II (CDG-II), which is caused by a homozygous nonsense mutation in the hCOG8 gene. This leads to a premature stop codon resulting in a truncated Cog8 subunit lacking the 76 C-terminal amino acids. Mass spectrometric analysis of the N- and O-glycan structures identified a mild sialylation deficiency. We showed that the molecular basis of this defect in N- and O-glycosylation is caused by the disruption of the Cog1–Cog8 interaction due to truncation. As a result, Cog1 deficiency accompanies the Cog8 deficiency, preventing assembly of the intact, stable complex and resulting in the appearance of smaller subcomplexes. Moreover, levels of β1,4-galactosytransferase were significantly reduced. The defects in O-glycosylation could be fully restored by transfecting the patient's fibroblasts with full-length Cog8. The Cog8 defect described here represents a novel type of CDG-II, which we propose to name as CDG-IIh or CDG caused by Cog8 deficiency (CDG-II/Cog8).

INTRODUCTION

In eukaryotic cells, the Golgi apparatus is a central player in the post-translational modification, sorting and transport of newly synthesized glycolipids, proteoglycans and glycoproteins. It consists of stacks of flattened cisternae which are dynamically maintained through active recycling between the endoplasmic reticulum (ER) and Golgi. This active transport is required to preserve the steady-state distributions of Golgi glycosidases and glycosyltransferases through a constant flow of membranes and a vectorial flow of newly synthesized proteins. Several models have been proposed to explain intra-Golgi transport and the current view boils down to two general models: the anterograde vesicular transport model, where vesicles ferry synthesized proteins forward, and the cisternal maturation model, where the Golgi resident proteins are carried back by a retrograde vesicular traffic from trans- to cis-localized Golgi cisternae (1). The Golgi contains two distinct pools of proteins, the glycosylation enzymes and the matrix proteins, which contribute differently

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to maintaining Golgi structure. Multisubunit peripheral membrane protein complexes appear to play key roles in Golgi-associated membrane fusion/fission events. Of emerging interest is the conserved oligomeric Golgi (COG) complex consisting of eight subunits, Cog1 to Cog8 (reviewed in 2). Mutations in individual subunits have recently been linked to some cases of a rare genetic disease named congenital disorders of glycosylation type II or CDG-II (3–5). These patients suffer from defects in the processing/trimming of glycan chains once transferred onto glycoproteins and/or the biosynthesis of O-linked oligosaccharides. This is in contrast to type I (CDG-I), with defects in the initial synthesis or the transfer of the Glc3Man9GlcNAc2-PP-dolichol precursor to the nascent protein chain.

In general, these syndromes demonstrate clearly that either mutations in glycosylation enzymes or a different distribution of these enzymes within Golgi stacks leads to the appearance of glycoproteins displaying abnormal glycan structures. Although some CDG-II patients are deficient in Golgi glycosyltransferases or transporters, it now appears that more cases may be linked to a trafficking defect between the ER and Golgi or within the Golgi. Indeed, two cases of CDG-II were characterized with a deficiency in the Cog7 (6) and Cog1 (7) subunits of the octameric COG complex which is localized peripherally to Golgi membranes and apparently plays a role in the retrograde trafficking of intra-Golgi vesicles (2,8,9). The subunit architecture of COG has previously been studied and led to the suggestion that the COG complex is essentially formed by two subcomplexes, lobe A (Cog1–4) and lobe B (Cog5–8) (2,7,10–12). In this article, we identify a new CDG-II in a girl with psychomotor and mental retardation, which has a homozygous point mutation in the COG8 gene. The mutation generates a premature stop codon resulting in a truncated Cog8 protein. Our data reveal the importance of Cog8 in COG complex assembly or stability and emphasize the important role of analyzing CDG-II-linked COG mutations with respect to complex assembly and architecture.

RESULTS

Description of a CDG-II patient with an O-glycan deficiency

The patient reported here is an 8-year-old girl born at term of consanguineous parents of Spanish origin (Fig. 1A) (13). Although the neonatal period and early infancy were normal, at the age of 6 months, she presented with an acute encephalopathy and loss of psychomotor abilities, hypotonia, alternating esotropia, pseudo-ptosis and mental retardation. She later developed a cerebellar syndrome with prominent ataxia and action myoclonus. At 17 months, during gastroenteritis, she had a unilateral status epilepticus and lethargy lasting for 5 days. During infancy, she exhibited PFAPA syndrome (periodic fever, aphthous stomatitis, pharyngitis and adenitis); although these episodes occurred normally without worsening of the neurological problems, during one of them, she had encephalopathic symptoms and became lethargic with increased hypotonia for several days. During some other episodes, she presented spontaneous hematomas, coincident with alteration of the coagulation factors and a decrease in the prothrombin time, together with increased levels of transaminases and of creatine kinase. At the age of 6 years, brain magnetic resonance imaging (MRI) showed cerebellar atrophy and slight brainstem atrophy (Fig. 1B). Minor dysmorphic features were also seen (Fig. 1C).

From the age of 7, her cerebellar ataxia has worsened. Still, at the age of 8, brain MRI has not shown significant changes from the examination 2 years ago. She has been able to make some developmental and learning progress by developing simple and abstract language and understanding. A detailed clinical evaluation now revealed an oculomotor apraxia with dysnergia oculocephalica, in addition to the pseudo-ptosis and alternating esotropia; fundoscopy was normal. She has symptoms of neuropathy in the lower limbs because she walks with ataxia and foot drop; she has abolished achilles tendon reflexes. Nerve conduction velocities were not tested because the parents refused a neurophysiological exam. She has not suffered further epileptic seizures other than the status convulsivus at 17 months and she does not need antiepileptic drugs.

Because of her occasional coagulation problems, bleeding or thrombosis, and the fluctuation of the coagulation parameters throughout development (protein C and protein S deficiency, decreased prothrombin time and coagulation factors), a wide study for ‘increased risk of thrombosis’ was performed. Although homocysteine, vitamin B12 and folic acid levels were normal, a heterozygous C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene was found. The factor V Leiden and factor II mutations were absent.

Her father also has a very high level of plasma homocysteine, undetectable vitamin B12 levels, antibodies against intrinsic factor and incipient macrocitic anemia. He was diagnosed with a pernicious anemia. The mother is heterozygous...
Table 1. Summary of clinical features in the present case

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at the onset</td>
<td>6 months</td>
</tr>
<tr>
<td>Present age</td>
<td>8 years</td>
</tr>
<tr>
<td>Family history</td>
<td>Consanguinity, mental retardation in other consanguineous relatives. Pernicious anemia in the father. Heterozygous mutation C677T in MTHFR gene in the mother.</td>
</tr>
<tr>
<td>First symptoms</td>
<td>Neurological regression</td>
</tr>
<tr>
<td>Mental retardiation</td>
<td>+</td>
</tr>
<tr>
<td>Acquired microcephaly</td>
<td>+ (&lt;3 SD from 4 years old)</td>
</tr>
<tr>
<td>Seizures</td>
<td>Status convulsivus (only one)</td>
</tr>
<tr>
<td>Episodiose of acute encephalopathy</td>
<td>+</td>
</tr>
<tr>
<td>Increased infections</td>
<td>+</td>
</tr>
<tr>
<td>PFAFA syndrome</td>
<td>+ (height &lt;3 SD)</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>Epicanthus</td>
</tr>
<tr>
<td>Minor dysmorphic features</td>
<td>Small hands and feet. Hypoplasia of the first phalanx of some fingers and toes. Wide space between the first and second toes. Clinodactyly of the third and fourth toes.</td>
</tr>
<tr>
<td>Brain MRI</td>
<td>6 months: normal. 22 months, 6 and 7 years: cerebellar atrophy and slight branstem atrophy.</td>
</tr>
<tr>
<td>Coagulation studies</td>
<td>Normal</td>
</tr>
<tr>
<td>Transaminases</td>
<td>Increased inconstantly</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>Increased (180–273 U/l)</td>
</tr>
<tr>
<td>Thyroid hormones</td>
<td>Normal</td>
</tr>
<tr>
<td>Immunological studies</td>
<td>Protein S deficiency</td>
</tr>
<tr>
<td>Coagulation studies</td>
<td>Protein C deficiency inconstantly Antithrombin III inconstantly decreased Prothrombin time and coagulation factors decreased (ILV, VII, X, IX, XI) during infections and transitory phases.</td>
</tr>
</tbody>
</table>

for the C677T mutation in the MTHFR gene. Other basal laboratory analyses, coagulation studies, creatin kinase, lipids, thyroid hormones and levels of CDT in serum were normal in both parents. A summary of the clinical features is presented in Table 1.

The diagnosis of CDG in this patient was established by standard isoelectric focusing (IEF) of transferrin (Fig. 3A): the marked increase of asialo-, mono-, di- and trisialotransferrins when compared with control is reminiscent of type II CDG (7). Moreover, the abnormal IEF of ApoC-III (data not shown) indicates an O-glycosylation deficiency (13). This was confirmed by PNA lectin staining which detects galactose residues of O-glycans not protected by a terminal sialic acid residue. As shown in Figure 2B and C, significantly more staining is seen in the patient’s cells when compared with the control fibroblasts. This difference disappeared after neuraminidase treatment (Fig. 2D and E) consistent with the specificity of the PNA lectin for the terminal galactose residues and demonstrating a partial O-glycan sialylation defect in the patient. To confirm this O-glycan deficit, the O-glycans were permethylated, purified and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Fig. 2F and G representing O-glycans released from the patient and control serum, respectively). The most intense ions are at m/z 895 and 1256, which correspond to O-glycans with the compositions NeuAc1Hex1, HexNAcitol and NeuAc2Hex1HexNAcitol, respectively. Three other molecular ions were observed at m/z 534, 1344 and 1706 and correspond to compositions Hex1HexNAcitol (m/z 534), Neu5AcHex1HexNAcitol (m/z 1344) and Neu5Ac2HexNAcitol (m/z 1706). Comparison of the glycan profiles for the patient and control revealed differences in the relative amounts of glycans Hex1, HexNAcitol (m/z 534) and NeuAc2HexNAcitol (m/z 1256). The ion at m/z 1256 for this patient was decreased, whereas the ion at m/z 534 was increased. These results are consistent with the abnormal profile for plasma ApoC-III and confirmed an O-glycan sialylation defect in this patient.

Next, the N-linked glycans from serum glycoproteins were analyzed using mass spectrometry, as summarized in Table 2 (7,14). Despite the patient’s clinical phenotype and transferrin IEF, her N-linked glycans exhibited only subtle differences from controls in the relative amounts of individual oligosaccharide structures with few abnormal structures. First, as a trend, there seems to be a general decrease in the fully sialylated triantennary glycans (I and J in Table 2) as well as in the N-linked glycans lacking a single sialic acid residue (D, ion 2078). This is compensated by the appearance of an abnormal glycan structure corresponding to the mass of a monotruncated biantennary N-linked glycan lacking a terminal sialic acid and galactose residue (A, ion 1769). These results were confirmed by analyzing the N-glycan structures of transferrin (data not shown) and overall suggest a mild defect in sialylation. In summary, we have demonstrated and clearly documented an N- and O-glycosylation deficiency in this patient.

Identification of a homozygous nonsense mutation in the hCOG8 gene

Since combined deficiencies in N- and O-linked glycosylation were already observed in Cog1- and/or Cog2-deficient Chinese hamster ovary cells (15) and recently in CDG-II patients deficient in Cog7 and Cog 1 (6,7), we decided to sequence the cDNA encoding all eight COG subunits after RT–PCR amplification from the patient’s fibroblasts. We found a nonsense mutation (C to G) at position c.1611 in the COG8 cDNA, and this was confirmed by the genomic DNA sequence. This mutation, for which the patient is homozygous, changes the codon for Tyr at position 537 into a premature stop codon (Fig. 3A), resulting in a truncated COG8 lacking the 76 C-terminal amino acids. As shown by SDS–PAGE and western blotting, the mutant Cog8 subunit indeed displays a significantly increased mobility, confirming that the c.1611 C>G allele encodes a truncated Cog8 polypeptide. Moreover, compared with control, only 25% of the immunoreactivity was recovered, suggesting that the transcript or the truncated Cog8 protein is unstable (Fig. 3B).

To investigate whether the mutant Cog8 affects the stability of other COG subunits, we used semi-quantitative western blot analysis (7) to analyze their steady-state levels and compared those with a control and a Cog7-deficient patient (6), since...
Figure 2. Abnormalities in protein glycosylation. (A) IEF pattern of serum transferrin from two controls and the patient under investigation. The number of negative charges is indicated on the right. (B–E) Staining of control (B and D) and patient fibroblasts (C and E) before (B and C) and after (D and E) treatment with neuraminidase. (F and G) MALDI-TOF-MS spectra of the permethylated O-glycans from sera of control (F) and patient (G). The permethylated derivatives were analyzed in positive-ion reflective mode, as [M + Na]^+. Low levels of N-glycans were also observed. Symbols: open square, galactose; closed square, N-acetylglucosamine; crossed square, reduced N-acetylgalactosamine; open triangle, N-acetyl neuraminic acid.
both Cog7 (in a subcomplex with Cog5 and Cog6) and Cog8 are part of COG’s lobe B (2,10). Although the Cog7 deficiency affected the stability of all lobe B subunits, only a slight but significant decrease in Cog6 immunoreactivity was observed for the Cog8 patient (Fig. 4). Surprisingly, the truncated Cog8 dramatically affected the steady-state level of the lobe A component Cog1 (76% decrease) (Fig. 4). The previously proposed subunit map of COG, in which Cog8 associated directly with Cog1 (16), and our interaction studies (given subsequently) strongly suggest that the observed instability of Cog1 is a direct consequence of the Cog8 mutation.

Characterization of the Cog1–Cog8 interactions
The selective effect of the patient’s mutation in the hCOG8 gene on the stability of Cog1 suggests that the normal Cog1–Cog8 interaction may be altered in the Cog8-deficient patient. To investigate this hypothesis, we used an in vitro co-translation/co-immunoprecipitation assay (16) to measure directly the physical interactions between metabolically radiolabeled Cog1 and Cog8. In one series of co-immunoprecipitations, hemagglutinin (HA) epitope-tagged wild-type Cog1 was co-expressed with either wild-type Cog8 or its truncation mutant (Y537X Cog8, indicated as ‘8’/C3) defined in this study (Fig. 5A, right). Conversely, HA-tagged wild-type Cog8 was co-expressed with either wild-type Cog1 or a C-terminal 80-residue truncation mutant of Cog1 (2660iC Cog1, indicated as ‘1’/C3) previously identified in a different COG-deficient CDG-II patient (7) (Fig. 5A, left).

As expected, the wild-type Cog1 and Cog8 subunits reciprocally co-immunoprecipitated using HA antibodies, confirming that they directly interact. However, when a truncated

Table 2. Predicted identity and relative amounts of glycan structures released from total serum glycoproteins

<table>
<thead>
<tr>
<th>Structure</th>
<th>Observed mass (m/z)</th>
<th>CDG-II patient</th>
<th>Control 1b</th>
<th>Control 2b</th>
<th>Control 3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1769</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>1915</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>1932</td>
<td>31</td>
<td>15</td>
<td>28</td>
<td>37</td>
</tr>
<tr>
<td>D</td>
<td>2078</td>
<td>14</td>
<td>15</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>E</td>
<td>2223</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>F</td>
<td>2369</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>G</td>
<td>2588</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>H</td>
<td>2735</td>
<td>2</td>
<td>Nd</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>2880</td>
<td>12</td>
<td>25</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>J</td>
<td>3025</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

aSymbols identifying sugar residues are as follows: ■, N-acetylg glucosamine; ○, mannose; □, galactose; ♦, sialic acid; ◆, fucose. Linkages between sugar residues have been removed for simplicity.
bAmounts (%) of individual glycans relative to the most abundant ion (D, m/z 2223), which corresponds to the disialylated biantennary glycan D.
Cog8 (Y537X) or Cog1 (2660iC) was co-translated with the cognate HA-tagged wild-type Cog1 or Cog8, no co-immunoprecipitation was observed. Thus, the C-termini of both Cog8 and Cog1 are independently required for the formation of a stable intermolecular complex that can be detected using this method. If this is indeed the case, the Y537X Cog8 mutation (and hence the lack of 76 C-terminal amino acids) should be sufficient to disrupt the whole COG complex. We tested this by fractionating cytosol from the patient and control fibroblasts using glycerol velocity-gradient centrifugation. Western blot analysis of the gradient fractions from control cytosol clearly showed that Cog1, Cog3, Cog6 and Cog8 subunits co-distribute in the middle of the gradient, most likely reflecting the full octameric complex (Fig. 5B). However, in the case of cytosol derived from the mutant Cog8 patient cells, all subunits remained in the upper part of the gradient, indicating that they are either associated with complexes that are significantly smaller in size or individual proteins (Fig. 5B). The same pattern was observed for the other subunits (data not shown). These smaller structures may represent independent lobes A and B or subcomplexes (e.g. Cog5–7). Our results, therefore, demonstrate that the C-terminal part of the Cog8 subunit is required for its interaction with Cog1 and the assembly of the stable intact hetero-octameric COG complex.

The Y537X Cog8 mutant fails to localize to the golgi and causes mislocalization of Cog2 and Cog6

In normal cells, COG subunits co-localize with Golgi markers, reflecting the peripheral association of the COG complex with Golgi cisternae (6–9,17). We used confocal microscopy to study the effect of the mutant Cog8 on the association of COG subunits with the Golgi apparatus. In control cells (Fig. 6, upper left, top panels), Cog8 clearly co-localizes with the cis-Golgi marker GM130 and there was some apparently non-specific background staining of the nucleus. No Golgi localization of Cog8 was observed in the patient’s cells, although the background nuclear staining was still visible (Fig. 6, lower left). Instead, the truncated Cog8 appeared to be distributed throughout the cytoplasm, as indicated by the increased diffuse cytosolic immunostaining. Although weaker in immunofluorescence signal, as expected from the western blot analysis, the Cog1 subunit was associated with the Golgi in both the patient and control fibroblasts (Fig. 6, lower left), although as expected from immunoblotting (Figs. 4 and 5), the intensity of Cog1’s immunofluorescence signal was lower in the patient’s cells. These data suggest that Golgi association of Cog8 is dependent on its interaction with Cog1, whereas Cog1’s interaction with the Golgi may not require its interaction with Cog8. This is consistent with the COG model (2,7,11) and the proposal that Cog1 may mediate Golgi binding of lobe A (T. Oka, E. Vasile, D.U., F.M. Hughson and M.K., unpublished data). Indeed, consistent with this model, there was virtually no Golgi localization of Cog6 nor was there intense localized staining (Fig. 6, lower right, compare bottom with top (control) panels), presumably because of Cog6’s dispersal throughout the cytoplasm. Distribution of Cog2 in the patient’s cells (Fig. 6, upper right, compare bottom with top (control) panels) was also consistent with the model. There was also a relative reduction in Golgi-localized staining of Cog2, with a compensatory increase in more diffuse cytoplasmic Cog2 staining in the patient’s cells, as one might expect from the overall reduction in abundance of Cog1. Surprisingly, whereas the model would predict that Cog3 and Cog4 would behave similar to Cog2 and Cog5 and Cog7 would mimic Cog6, this was not the case. In the
patient and control cells, the Golgi localization and immunos-amping intensity for Cog3, Cog4 and Cog5 were indistinguish-able (data not shown). Only for Cog7, a slight mislocalization and decrease in immunoreactivity were observed in the patient (data not shown). These data indicate that the model of the structure of COG and its interaction with the Golgi requires further refinement.

To determine whether the truncation in Cog8 was directly responsible for the abnormalities in the other COG subunits and in the glycoconjugate synthesis in the patient’s cells, we...
attempted to complement the mutant phenotypes in these cells by overexpressing full-length (HA-tagged) Cog8 (HA-Cog8). In transfected patient cells, HA-Cog8 exhibited a characteristic perinuclear Golgi distribution (Fig. 7A and B, left panels). This was accompanied by, first, an increase in the intensity of Cog1 staining that co-localized with HA-Cog8 in the Golgi (Fig. 7A, top, compare transfected cells expressing HA-Cog8 with their untransfected neighbors) and, secondly, a restoration of Cog6’s Golgi localization (Fig. 7A, bottom). As can be seen in Figure 7B, a reduction of PNA staining was also observed when using a lentiviral system coding for wild-type Cog8 to complement the patient’s cells, whereas in the non-transduced cells, the PNA staining remains higher. Thus, the truncated Cog8 mutant appears to be causative for the abnormalities in COG integrity and Golgi-associated glycosylation and thus in the CDG-II phenotype of the patient.

Influence of the truncated Cog8 mutant on the abundance, localization and trafficking of Golgi glycosylation enzymes

Current models of COG function suggest that it affects the resident localization of Golgi proteins through its influence on retrograde transport within the Golgi (2,18). If so, Cog8 subunit deficiencies may affect the distribution of glycosylation enzymes or even their stability, as has been demonstrated for other COG subunits (7,18–20). To test whether Cog8 Y537X could influence the localization and stability of glycosylation enzymes, we compared the abundance (western blotting) and intracellular localization (confocal microscopy) in the patient and control fibroblasts of two Golgi-glycosylation enzymes, mannosidase II and β1,4-galactosyltransferase, which have previously been shown to depend on COG and thus are members of the family of COG-dependent Golgi proteins called ‘GEARs’ (7,19,20). Both enzymes appeared to exhibit a normal perinuclear Golgi distribution in the patient’s cells (Fig. 8A). There were no differences between patient and control cells in the intensity of staining (Fig. 8A, left) or abundance of mannosidase II (Fig. 8B, left). However, both were reduced for β1,4-galactosyltransferase (Fig. 8A and B, center and right) (65% compared with control, n = 3) (Fig. 8C).

To assess whether there is also a trafficking defect associated with the Cog8 mutant, we treated the patient and control fibroblasts with Brefeldin A (BFA), which is a selective inhibitor of the GTP-exchange factor for ADP-ribosylation factor 1 (Arf1) (21) and causes the redistribution of Golgi proteins to the ER via retrograde transport.
Figure 7. Rescue of Cog1 and Cog6 levels and localization in patient fibroblasts by transient transfection with HA-COG8 cDNA. (A) Indirect immunofluorescent staining of Cog6 and Cog1 in patient fibroblasts transiently transfected with an expression vector encoding Cog8 with an HA tag at the C-terminus. Cells overexpressing HA-Cog8 are marked with an arrow (scale bar, 20 μm). (B) PNA–Alexa fluor 488 and Cog8 stainings of patient fibroblasts transduced and non-transduced with a lentiviral vector coding for the wild-type COG8. Scale bar represent 20 μm. The images were processed with ADOBE PHOTOSHOP 7.0.
Figure 8. Effects of Cog8 truncation on glycosylation enzymes and BFA-induced dissociation for the Golgi. (A) Indirect immunofluorescence staining of the patient and control fibroblasts using anti-Golgi mannose II (left), anti-β1,4GalT (center) and the merged images (right). All the images were collected using the same laser power and settings. (B) Total cell extracts were analyzed by immunoblotting using anti-Golgi mannosidase II and anti-β1,4GalT. Anti-Vti1a antibodies were used here as a loading control. About 20 μg of the total cell extracts, respectively, were loaded for the control and the patient, except in lanes 1, 2 and 3 where 20, 26 and 32 μg were loaded for the patient. Abundance of the β1,4GalT was quantified and the signals obtained were integrated using the Image J software. β1,4GalT levels are shown in the graph after normalization to a loading control. (C) Percentage of cells with Golgi remnants quantified from Golgi mannosidase II immunofluorescent staining as a function of time after BFA treatment. The values represented are the average of three independent experiments. Error bars indicate standard deviations. The symbols used are follows: open squares, control; closed squares, Cog8-deficient patient. The images were processed with ADOBE PHOTOSHOP 7.0.
We analyzed the localization of both Golgi mannosidase II and GM130 at varying time points (5–11 min) after BFA treatment (Fig. 8D). In untreated patient and control cells, Golgi mannosidase II and GM130 exhibit co-localization at the Golgi (data not shown). In control cells, the loss of the Golgi distribution of mannosidase II was rapid (Fig. 8D, open squares) (81% decrease in 5 min, virtually all lost after 7 min). In the patient’s cells, however, the BFA-induced redistribution of Golgi mannosidase II was significantly delayed at all times investigated (Fig. 8D, filled squares). This raises the possibility that Cog8 and a normal COG complex may influence the mechanism underlying the Golgi-to-ER retrograde trafficking of glycosylation enzymes induced by BFA.

**DISCUSSION**

In the present study, we identified a mutation in the gene encoding the Cog8 subunit of the COG complex, which causes a new type of CDG-II. The Cog8-defective patient suffers from cerebellar atrophy, mental and motor retardation, hypotonia, growth delay and short stature. When we compare the clinical features with Cog7 and Cog1 deficiencies reported earlier (6,7), the clinical phenotype of the Cog8-defective patient overlaps with that of the Cog1-deficient patient. Indeed, both have a much milder clinical phenotype than the Cog7-deficient patients who died at 5 and 10 weeks after birth. Not only does the Cog8-defective patient share clinical phenotypes with other COG-deficient CDG patients, including defective N- and O-glycosylation, she also exhibits some clinical phenotypes similar to those observed in mitochondrial disorders (22).

Despite the similar clinical phenotype of the Cog1- and Cog8-deficient patients, some of the accompanying cellular (fibroblast) phenotypes are distinct. The abnormalities in N-glycosylation in the Cog8-defective patient’s cells were substantially less severe (minor reductions in sialic acid and galactose) than those in cells from the Cog1 and apparently the Cog7-deficient patients. The molecular basis for this must be, therefore, sought in the nature of the specific mutations in each of the COG subunits and how they affect the COG complex’s architecture and function. The connectivity map of the COG complex has been extensively studied, leading to the suggestion that the COG complex is formed by two subcomplexes, lobe A (Cog1–4) and lobe B (Cog5–8) (2,9). However, the link by which these two subcomplexes interact is still not well defined. Currently, two related models have been proposed. In the first model, in mammalian cells, the heteromeric subassemblies (Cog2–4 and Cog5–7) are bridged by a heterodimeric unit containing the Cog1 and Cog8 subunits. Here, Cog1 is directly associated with the Cog2–4 subcomplex, whereas Cog8 interacts with the Cog5–7 subcomplex (11,16). The second model, based on studies in yeast, differs in that Cog1 directly interacts with subunits from lobe A (Cog2–4) and lobe B (Cog5, 6 and 8) (10).

The Y537 X allele, for which the patient is homozygous, encodes a C-terminal truncated protein whose abundance in fibroblasts was only 25% of wild-type Cog8 in control cells. It is likely that protein instability, rather than reduced synthesis, is responsible for the reduction in steady-state levels of Cog8 protein, as well as reductions in the levels of other COG subunits in this patient’s cells (24 and 80% of control for Cog1 and Cog6, respectively). It is also likely that protein instability is the source of the low abundance of the truncated Cog1 in Cog1-deficient patient (16% of control) and the associated reductions of other COG subunits (51–75% for Cog2–4 and 68% for Cog8) (7). The reciprocal instabilities in cells of either Cog1 or Cog8, due to the truncation of one of these subunits, support that Cog1 and Cog8 are in direct physical contact. Moreover, our data clearly indicate that this interaction is mediated through the C-terminal domain of both subunits. The integrity of the Cog1–Cog8 interaction is of general importance for the assembly and stability of the COG complex. This is highlighted by our observations that Cog8 instability results in (i) mislocalization of a substantial fraction of both Cog2 and Cog6 in the fibroblasts of the Cog8-defective patient, suggesting that these two subunits could be more closely linked to the central Cog1–Cog8 dimer and (ii) the loss of octameric COG complexes.

Variations observed in the fibroblasts from Cog1-, Cog8- and Cog7-deficient patients in either the absolute or relative amounts of the intact COG complex and its subcomponents may be responsible for the variations in cellular and consequently the clinical phenotype that have been observed. The most severe abnormal phenotypes were seen in the Cog7-deficient patients and the least severe in the Cog8-defective patient.

It seems likely that the glycosylation defects associated with mutations in the unit genes are a consequence of the role that COG plays in controlling the intracellular trafficking and/or stability of a variety of Golgi-associated glycosylation enzymes (7,18–20,23). For example, a subset of Golgi type II proteins called GEARs was found mislocalized and/or unstable in COG-deficient cells lines (19). Among these proteins, four Golgi glycosylation enzymes, including the Golgi mannosidase II, the β1,4-galactosyltransferase I, the sialyltransferase hST3Gal1 and the GlcNAc transferase I, are mislocalized and/or abnormally rapidly degraded in COG-deficient cells (6,7,18–20,23). The mechanism by which COG influences the stability of Golgi glycosyltransferases has not yet been determined, but substantial evidence suggests that the COG complex may act by directly influencing retrograde intra-Golgi vesicular trafficking. This model is supported by the fact that, in both yeast and mammalian cells, deficiencies in individual COG subunits can lead to accumulation of vesicles (18,23,24). Furthermore, GEARs and other glycosylation enzymes have been found in COG-complex-dependent vesicles that form when Cog3 levels in HeLa cells are reduced by RNAi (18). It is possible that individual COG subunits or subcomplexes play distinct roles in controlling vesicular trafficking in different regions of the Golgi (23). Our data (7, this study) suggest that the COG complex could play different roles in the vesicular Golgi trafficking of different Golgi glycosyltransferases because of differential effects of different COG subunits on these enzymes. Indeed, from our results, we hypothesize that in the Cog1 patient, the loss of the lobe A subunits essentially causes defects in early Golgi glycosylation, as demonstrated by the low steady-state level of the cis/median Golgi.
glycosyltransferase Golgi mannosidase II, whereas in the Cog8-deficient patient, a mutation in a lobe B subunit causes more trans-Golgi glycosylation defects. This is supported by the detailed analysis of the O-glycan structures showing mainly a sialylation deficiency. However, the precise nature of how COG controls membrane trafficking and Golgi structure remains to be established.

The identification of a new Cog8 deficiency associated with a CDG-II case together with previous mutations in Cog1 and Cog7 raises the possibility that defects in COG will contribute to a substantial fraction of all CDG-II cases. Thus, analysis of the COG subunit genes and proteins may contribute to the diagnosis of COG-deficient CDG. At the molecular level, COG mutations in the CDG-II patients are significantly contributing to our understanding of the architecture and functions of the COG complex. According to the current CDG nomenclature, this new case could be classified as CDG-IIh. However, in light of our previous proposal (7), we suggest that this case be named CDG-II because of a Cog8 deficiency (CDG-II/Cog8).

MATERIAL AND METHODS

Cell culture and transfections

Primary fibroblasts from patient and controls were cultured at 37°C under 5% CO2 in DMEM/ F12 (Life Technologies, Paisley, UK) supplemented with 10% Fetal Clone III (HyClones). Cells were transfected at 75–90% of confluence using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions.

Antibodies

Affinity-purified or crude serum rabbit polyclonal antibodies (pab) were used against the Cog1–8 subunits (7,11) in dilutions ranging from 1:1000 to 1:5000 for western blotting and 1:50 to 1:200 for indirect immunofluorescence microscopy. Anti-Cog3 pab was a gift from V. Lupashin (University of Arkansas for Medical Sciences, Little Rock, AK, USA). Anti-α-mannosidase II pab and anti-β-1,4 galactosyltransferase I were gifts from K. Moremen (University of Georgia, Athens) and E. G. Berger (University of Zürich, Zürich), respectively. Anti-GM130 was from BD Biosciences (Franklin Lakes, NJ, USA). Anti-HA raw ascites was from CRP Inc. (Berkeley, CA, USA).

Plasmids

The GenBank accession numbers for the COG subunit cDNAs used were as follows: COG1, NM_018714; COG8, NM_032382. The pcDNA3.1+ (Invitrogen, Carlsbad, CA, USA) vector was used to generate untagged proteins, whereas the pMH vector (Roche Diagnostics) served as a template for HA-tagged proteins. Point mutations corresponding to the mutations found in the patients (Cog1:2660iC and Cog8:C1611G) were introduced using the Quick Change Mutagenesis protocol (Stratagene, La Jolla, CA, USA).

Immunoblotting

Proteins were analyzed by SDS–PAGE and immunoblotted with the indicated antibodies at the concentrations described previously (7). Signals were detected using the ECL Plus detection kit (Amersham Biosciences, UK) according to the manufacturer’s instructions.

Mass spectrometric analysis of O-linked glycans released from total plasma proteins

Release of O-linked glycans An aliquot of 10 μl of serum was subjected to reductive elimination. Sodium hydroxyde solution (200 μl) containing 1 M NaBH4 was added to 10 μl of serum and incubated at 45°C for 16 h. After terminating the reaction with glacial acetic acid, the sample was purified on a column (7 × 0.5 cm) of Dowex 50X-8 (H+ form) and the unbound material was then lyophilized. Borates salts were removed by several evaporations with methanol containing 5% (v/v) acetic acid and freeze-dried.

Permethylylation of glycans Permethylolation of the freeze-dried glycans was performed. The reaction was terminated by adding 1 ml of ultra-pure water, followed by three extractions with 500 μl of chloroform. The pooled chloroform phases (1.5 ml) were then washed eight times with ultra-pure water. The methylated derivative-containing chloroform phase was finally dried under a stream of nitrogen and the extracted products were further purified on a C18 Sep-Pak. The C18 Sep-Pak was sequentially conditioned with methanol (5 ml) and water (2 × 5 ml). The derivatized glycans dissolved in methanol were applied on the cartridge, washed with 3 × 5 ml water, 2 ml of 10% (v/v) acetonitrile in water and eluted with 3 ml of 80% (v/v) acetonitrile in water. Acetonitrile was evaporated under a stream of nitrogen and the samples were freeze-dried.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry MALDI-TOF-MS experiments were carried out on Voyager Elite DE-STR Pro instrument (Persptive Biosystems, Framingham, MA, USA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode by delayed extraction with an accelerating voltage of 20 kV and a pulse delay time of 200 ns and a grid voltage of 66%. All spectra shown represent accumulated spectra obtained by 400–500 laser shots. Permethylated glycans were co-crystallized with 2,5-dihydroxybenzoic acid (DHB) as matrix [10 mg/ml of DHB in methanol/water solution (50:50)].

Mass spectrometric analysis of N-linked glycans released from total plasma proteins

Analysis of N-linked glycans on plasma glycoproteins was carried out by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (Ciphergen, Fremont, CA, USA) essentially as described by Mills et al. (14) for MALDI-TOF-MS. Analysis of acidic N-linked glycans was performed in a negative linear ion mode. The total N-linked
glycans from plasma proteins were released enzymically as described by Mills et al. (14). The reference samples were anonymous plasma samples from patients without CDG or a lysosomal storage disease.

**Cytosol fractionation**

The patient and control cells were cultured in 75 cm dishes and harvested in 1 ml of 20 mM HEPES-KOH buffer, pH 7.4, supplemented with a proteinase inhibitor mixture (Roche Diagnostics) on ice. Cells were then homogenized by using a ball-bearing-type homogenizer. Membrane and cytosol were separated by ultracentrifugation (100 000g, 1 h, 4°C). Cytosol was layered onto a 12 ml linear 10–30% glycerol (w/v) gradient in 20 mM HEPES-KOH, pH 7.4. The gradients were centrifuged in a Beckman SW40 rotor at 120 000g for 15 h with slow acceleration and deceleration. Fractions (1 ml) were collected through a hole punched in the bottom of the tube and concentrated by CHCl₃/MeOH/H₂O (3/2/1) precipitation. Proteins were then electrophoresed and visualized by immunoblotting.

**In vitro co-translation and immunoprecipitation**

**In vitro** co-translation and immunoprecipitation was performed as described (16). In brief, mRNAs corresponding to wild-type and mutant COG subunits were prepared with the mMessage mMachine T7 kit (Ambion, Austin, TX, USA) and added to rabbit reticulocyte lysate (Promega, Madison, WI, USA) for in vitro translation in the presence of 35S-methionine (in vitro translation grade; GE Healthcare, Piscataway, NJ, USA).

For immunoprecipitation, 20 μl of a translation reaction was centrifuged for 5 min and added to a final volume of 200 μl IP buffer (125 mM KCl, 30 mM Tris pH 8.0, 0.5% Tween-20) containing 2 μl of HA.11 antibody (raw ascites, CRP Inc.) and 0.5 mM PMSF. After 1 h incubation and another 5 min centrifugation, the mixture was added to 20 μl of a 50% slurry of protein G–Sepharose (Sigma, St Louis, MO, USA) and incubated for another 25 min. The beads were then washed three times in spin columns, and the bound proteins eluted in SDS–PAGE sample buffer. The samples were analyzed by SDS–PAGE on 20 cm long slab gels, followed by autoradiography.

**Immunofluorescence staining**

Cells were grown on glass coverslips for 12–24 h, washed once with phosphate-buffered saline (PBS) and fixed by incubating for 25 min with 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at room temperature. The coverslips were rinsed twice with 0.1 M glycine in PBS for 15 min. Cells were then blocked in blocking solution (0.1% Triton/1% bovine serum albumin (BSA)/2% normal goat serum in PBS) for 30 min. In the case of COG subunit immunostaining, the cells were additionally treated with 6 M urea in PBS, followed by four washes of 5 min in PBS and 15 min in the blocking solution. The fixed cells were incubated for 1 h with primary antibodies diluted in the blocking solution. After washing with PBS, Alexa 488- or Alexa 568-conjugated secondary antibodies (Molecular Probes) diluted in blocking solution were applied for 1 h. For cells transfected with (Cog8-pMH) plasmid encoding Cog8 with an HA tag at the C-terminus, cells were fixed in 2% formaldehyde in PBS and then incubated in blocking and permeabilization buffer (1% BSA/0.2% saponin in PBS). Immunostaining was detected through an inverted Diaphot 300 (Nikon) microscope connected to a confocal microscope (MRC 1024; Bio-Rad). Data were collected using LASERSHARP 3.0 and finally processed in ADOBE PHOTOSHOP 7.0 (Adobe Systems, San Jose, CA, USA).

**Mutation analysis**

Total RNA was isolated from the patient’s fibroblasts and a normal control using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was then prepared with oligo-dT priming and Superscript II RNase-H reverse transcriptase (Invitrogen) using 3 μg of total RNA in a total volume of 20 μl. To confirm the mutation at the cDNA level, the COG8 sequence of interest was amplified from cDNA with primers COG8-1F (5′-CTCCCAAAGTCTGGGATTA-3′) and COG8-2R (5′-GCTTAGACTTGGCAGTGACA-3′). A 2 μl cDNA sample was used in a total volume of 50 μl with Taq DNA polymerase (Roche Diagnostics). Amplification conditions were 2 min at 94°C, 10 cycles of 20 s at 94°C, 30 s at 65°C (−1°C each cycle) and 1 min at 72°C, followed by 25 cycles of 20 s at 94°C, 30 s at 55°C and 1 min at 72°C. The PCR product was sequenced with BigDye Terminator Ready reaction cycle sequencing kit ver. 3.1 (Applied, Foster City, CA, USA) and analyzed on an ABI3100 Avant (Applied). When transduced with the lentiviral vector coding for COG8, the patient’s cells were washed with PBS and fixed for 15 min in 2% paraformaldehyde in PBS, and then 50 mM NH₄Cl in PBS was added for 20 min to neutralize aldehyde residues. Thereafter, cells were incubated with 5 μg/ml PNA–Alexa 588 conjugate in 0.1% BSA/PBS (Molecular Probes) for 1 h at RT. After three washings with PBS, the coverslips with the labeled cells were mounted on slides with Mowiol 4–88 (Hoechst, Frankfurt, Germany) for observation.
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