Mutations in STT3A and STT3B cause two congenital disorders of glycosylation

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We describe two unreported types of congenital disorders of glycosylation (CDG) which are caused by mutations in different isoforms of the catalytic subunit of the oligosaccharyltransferase (OST). Each isoform is encoded by a different gene (STT3A or STT3B), resides in a different OST complex and has distinct donor and acceptor substrate specificities with partially overlapping functions in N-glycosylation. The two cases from unrelated consanguineous families both show neurologic abnormalities, hypotonia, intellectual disability, failure to thrive and feeding problems. A homozygous mutation (c.1877T>C) in STT3A causes a p.Val626Ala change and a homozygous intronic mutation (c.1539_1540G>T) in STT3B causes the other disorder. Both mutations impair glycosylation of a GFP biomarker and are rescued with the corresponding cDNA. Glycosylation of STT3A- and STT3B-specific acceptors is decreased in fibroblasts carrying the corresponding mutated gene and expression of the STT3A (p.Val626Ala) allele in STT3A-deficient HeLa cells does not rescue glycosylation. No additional cases were found in our collection or in reviewing various databases. The STT3A mutation significantly impairs glycosylation of the biomarker transferrin, but the STT3B mutation only slightly affects its glycosylation. Additional cases of STT3B-CDG may be missed by transferrin analysis and will require exome or genome sequencing.

INTRODUCTION

Rare inherited glycosylation disorders show broad and diverse clinical phenotypes that cover nearly every glycosylation pathway (1–3). Many of the defects can impact multiple glycosylation pathways, while others are quite specific to a single pathway (1,4,5). A good example of the latter is the collection of congenital disorders of glycosylation (CDG), affecting the biosynthesis of the lipid-linked oligosaccharide (LLO) precursor and transfer of the glycan chain to newly synthesized proteins in the ER. These disorders are called Type I CDG and often detected by abnormal glycosylation of serum transferrin where one or both N-glycan chains are missing (6). The oligosaccharyltransferase (OST) is a heteroligomeric protein complex composed of seven to eight non-identical subunits that carry out glycan chain transfer to proteins in the ER (7). To date, mutations in only three of the subunits DDOST-CDG [MIM 614507], TUSC3-CDG [MIM 611093] and MAGT1-CDG [MIM 300716] are reported to cause CDG, but only one, DDOST, causes abnormal transferrin glycosylation.

STT3 is the highly conserved catalytic subunit of OST, and transfers oligosaccharides onto the asparagine residues of sequons (N-X=P-T/S/C) in nascent glycoproteins (8–10). Two alternate STT3 proteins, STT3A [MIM 601134] and STT3B [MIM 608605] are widely expressed in human tissues, encoded by different genes, exist in distinct OST complexes with different kinetic properties, substrate preferences and partially overlapping roles in glycosylation (10,11). STT3A isoform of the OST is located next to the protein translocation channel (11) to insure co-translational glycosylation of NXT/S sites on nascent chains (13), while STT3B mediates post-translational glycosylation of the sites that elude the STT3A complex (11). Prosaposin glycosylation is particularly sensitive to depletion of STT3A (11) while β-glucuronidase and sex hormone binding globulin contain STT3B-specific glycosylation sites (14).

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Here, we describe two new CDG cases with mutations in either STT3A or STT3B. We use both isoform-specific substrates and a glycosylation-sensitive GFP construct to show the isoform-specific impact on protein glycosylation.

RESULTS

Clinical description and identification of the defects

Family 1

Family 1 is of Pakistani ancestry where the parents are first cousins (Fig. 1). Two of the four children were diagnosed in infancy with an unidentified form of type I CDG based on abnormal transferrin glycosylation. The male and female are now 13 years old and both have developmental delay, failure to thrive, hypotonia and seizures (Table 1). The male sibling was more severely affected in nearly all clinical features. He is unable to sit up without assistance, has weak visual tracking and intractable seizures.

Given the consanguinity, we performed homozygosity mapping on the affected male (Case-1) using an Affymetrix 6.0 SNP array which showed 10 regions of homozygosity of >10 MB not seen in other samples with similar ancestry. Based on the abnormal type I transferrin profile, we focused on genes that are known to cause CDG or genes known to be involved in N-glycosylation. From the 10 regions, only two had candidate CDG genes. The first interval was a 50.2 MB region on chromosome 1 (61,449,520-111,655,528) containing ALG6 and ALG14. The second was a 17.6 MB interval on chromosome 11 (115,796,044-133,437,543) where DPAGTI and STT3A reside.

Figure 1. Identification of two consanguineous families with Sanger sequence confirmed mutations in STT3A and STT3B. (A) Family-1 pedigree shows two affected individuals from a consanguineous marriage where homozygosity mapping was performed on Case-1. Lower panel shows Sanger sequence confirmation of the STT3A c.1877T > C mutation (p.Val626Ala) in the affected male. Sample from the affected female was not available for testing. (B) Family-2 pedigree shows consanguinity and homozygosity mapping was performed on the affected male Case-2. Lower panel shows Sanger sequence confirmation of the STT3B c.1539 + 20G > T mutation. Filled in symbols denote affected individuals and filled in symbol with a line through it means individual is deceased.

Lipid linked oligosaccharide (LLO) analysis for Case-1 showed a normal structure, allowing us to exclude ALG6-CDG [MIM 603147], which accumulates a truncated LLO glycan (15). Sanger sequencing of the remaining three genes showed that only STT3A contained variants of potential significance.

STT3A is the catalytic subunit of the OST complex and deficiencies would very likely produce a type I transferrin profile since transferrin is a STT3A substrate (14,16). Sanger sequencing of STT3A confirmed homozygous c.1877T > C variant that encodes a p.Val626Ala (Fig. 1). Polyphen2 predicts this variant to be possibly damaging with a HumVar score of 0.816/1.0 and probably damaging based on a HumDiv score of 0.973/1.0.

Family 2

Family 2 is of Iraqi ancestry and positive for consanguinity where the paternal grandmother was a sister of the maternal great grandmother (Fig. 1). The mother had a previous miscarriage and one healthy female child. Case-2 had significant congenital and developmental abnormalities (Table 1), but only a very mildly abnormal transferrin of M/D 0.147: A/D 0.004 (Normal cutoff, M/D 0.074: A/D 0.022) (Table 1).

We performed homozygosity mapping on the affected male (Case-2) using an Affymetrix 6.0 SNP array and found seven homozygous regions >10 MB. Using the same approach as for Case-1, we identified two candidate regions. The first was a 46.3 MB region on chromosome 6 (111,604,072-157,944,589) which included NUS1, a nuclear undecaprenyl pyrophosphate synthase 1 homolog known to be involved in dolichol metabolism (17). However, Sanger sequencing failed to reveal any
variants within NUS1. The second region of 22.8 MB on chromosome 3 (9,771,508-32,612,897) contains another OST complex gene, STT3B. Sanger sequencing confirmed a homozygous intronic variant c.1539 + 20G>T (Fig. 1).

Mutations reduce STT3A and STT3B protein

Immunoprecipitation of short-term radiolabeled STT3A from affected and control fibroblasts showed that Case-1 cells express less STT3A protein. This may be an underestimate of the actual reduction in STT3A if the p.Val626Ala is less stable than wild-type STT3A. Protein immuno-blotting showed severe reduction in STT3B in Case-2 fibroblasts (Fig. 2B), which was due to a complete absence of mRNA based on PCR analysis (data not shown). This suggests that the intronic mutation produces an unstable mRNA. Normal amounts of STT3B are seen in Case-1 fibroblasts. The higher expression of STT3A in Case-2 fibroblasts (Fig. 2A) is similar to the results observed in HeLa cells treated with STT3B-specific siRNAs (11). Ribophorin 1 (R1), a subunit common to both STT3A- and STT3B-containing OST complexes (10), is expressed at normal levels in both cases compared with the Sec61 loading control. Similar levels of the ER chaperone BiP in wild-type and glycosylation-deficient cells suggest that the unfolded protein response (UPR) pathway is not strongly induced in the defective cells, in contrast to a 20% increase in BiP expression seen in STT3A-depleted HeLa cells (11).

**Glyc-ER-GFP monitoring**

We verified impaired glycosylation in cells from both cases using Glyc-ER-GFP, a marker that fluoresces in N-glycosylation-deficient cells (16,18). Cells were electroporated to introduce the glycosylation-capable Glyc-ER-GFP or the control ER-GFP, which resides in the ER, but does not contain a glycosylation sequence. Glyc-ER-GFP fluoresced in both defective cell lines confirming incomplete N-glycosylation site occupancy. We complemented these cells by overexpressing wild-type cDNA for STT3A and STT3B. Cells were co-electroporated with the Glyc-ER-GFP and a mock vector, STT3A- or STT3B-containing vectors. As shown in Figure 3A, STT3A-deficient cells complemented by wild-type STT3A cDNA showed up to an 80% reduction in Glyc-ER-GFP fluorescence. STT3B-deficient cells complemented with STT3B cDNA showed ~85% decrease in Glyc-ER-GFP fluorescence (Fig. 3B). Clearly, the defect in each was complemented by the appropriate wild-type STT3 protein. We also tested whether over expression of the alternate STT3 isoform corrects glycosylation in the cells. STT3B cDNA partially complements STT3A-deficient cells, but STT3A cannot substitute for STT3B in Case-2 cells.

**Defects in glycosylation of STT3A and STT3B-specific substrates**

The mutations in STT3A and STT3B clearly cause a general defect in N-linked glycosylation, but we wanted to determine whether the mutations affected glycosylation of STT3A or STT3B-specific substrates. Prosaposin, the precursor of saposins A-D (Fig. 4A), is particularly sensitive to reduced STT3A (11). Control fibroblasts and Case-2 fibroblasts glycosylate

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**Table 1. Clinical and molecular summary of cases**

<table>
<thead>
<tr>
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<th>Case-1</th>
<th>Case-2</th>
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<tr>
<td>Ancestry</td>
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<td>Iraqi</td>
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<td>Age</td>
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<td>Death at 4 years of age</td>
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<td>STT3B (NM_178862.1)</td>
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<td>p.Val626Ala</td>
<td>Severe reduced protein expression</td>
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<tr>
<td>cDNA change</td>
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<td>c.1539 + 20G &gt; T</td>
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<td>0.147: 0.004</td>
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Human Molecular Genetics, 2013, Vol. 22, No. 22
prosaposin normally with five N-glycans. However, in Case-1 fibroblasts, prosaposin lacks up to three N-glycans (Fig. 4B) and resembles the pattern from HeLa cells with an STT3A deficit (Fig. 4B).

We also tested whether the STT3A p.Val626Ala mutant can functionally replace wild-type STT3A when expressed in deficient HeLa cells. A DDK-His tag was appended to the C-terminus of wild-type STT3A or STT3A p.Val626Ala to differentiate between genome and vector encoded STT3A proteins. In addition, silent mutations were introduced in the siRNA target sequence of the STT3A coding sequence to obtain siRNA-resistant STT3A expression vectors. STT3A-DDK-His migrates slower than endogenous STT3A (Fig. 4C, upper panel), and was readily detected when the siRNA-treated HeLa cells were transfected with the siRNA resistant expression vectors. As expected, the siRNA-sensitive STT3A expression vectors could not correct the prosaposin glycosylation defect caused by depletion of endogenous STT3A (Fig. 4C, lower panel). When the two siRNA-resistant expression vectors were tested, the STT3A p.Val626Ala mutant was moderately less effective than wild-type STT3A in restoring normal glycosylation of prosaposin. The incomplete restoration of normal prosaposin glycosylation by wild-type STT3A-DDK-His is probably due to a subpopulation of non-transfected cells. Thus, the STT3A p.Val626Ala allele is less stable than wild-type STT3A, and as a consequence, causes protein hypoglycosylation.

Granulin is an endogenous fibroblast glycoprotein (Fig. 4D), which, is highly sensitive to STT3A-specific siRNA knockdown in HeLa cells (Fig. 4E). Hypoglycosylated variants of the short form of granulin (pGran-2) resolved clearly, unlike hypoglycosylated pGran-1 that electrophoresed as a diffuse band. Control and Case-2 fibroblasts primarily synthesized fully glycosylated granulin, unlike the Case-1 fibroblasts that synthesized pGran-2 glycoforms lacking one to three of the four oligosaccharides.

Next, control, Case-1 and Case-2 fibroblasts were transfected with expression vectors for STT3B-dependent substrates.
Sex hormone binding globulin (SHBG) has two glycosylation sites located at the extreme C-terminus of the protein (Fig. 5A) and both are STT3B-dependent (14). Partial glycosylation of the N380RS site in SHBG is responsible for the presence of the mono- and di-glycosylated forms of SHBG that are synthesized by HeLa cells, Case-1 cells and control fibroblasts (Fig. 5B). Case-2 fibroblasts and STT3B-depleted HeLa cells synthesize non-glycosylated and mono-glycosylated SHBG. A β-glucuronidase derivative containing a single C-terminal glycosylation site (β-GUSΔ123) was used as a second STT3B-dependent assay substrate (Fig. 5C). Roughly half of β-GUSΔ123 was glycosylated in HeLa cells and control fibroblasts (Fig. 5D). Glycosylation of the N631ET site in β-GUSΔ123 was much lower in Case-2 fibroblasts and STT3B-depleted HeLa cells.

**DISCUSSION**

We report two previously unreported types of CDG caused by mutations in different genes that encode alternate forms of STT3, the catalytic subunit of the OST complex. In trypanosomes, the OST consists of only a monomer, but in fungi and mammals, it is a hetero-octamer (7). Homologues of STT3 are responsible for N-glycosylation in archaea and certain bacteria (8). In mammals, the complex task of insuring complete N-glycosylation resulted in the duplication of the ancient homolog and specialization for donor and acceptor substrate preference (19). STT3A is thought to carry out co-translational N-glycosylation just as the nascent protein emerges from the translocon complex. However, some proteins are N-glycosylated up to 30 min after translation is finished and this is especially common for proteins containing...
glycosylation sites within ~50 amino acids of the C-terminus. These proteins appear to be preferential substrates for STT3B (14). Recently, STT3B was shown to N-glycosylate misfolded transthyretin, a liver-derived plasma protein, which contains a cryptic N-glycosylation site that becomes exposed only on misfolding (20). Addition of the N-glycan guides the misfolded protein toward ERAD destruction. It is unknown whether other non-glycosylated, misfolded proteins become targets for STT3B-glycosylation and destruction.

STT3A and STT3B both appear to glycosylate Glyc-ER-GFP, since a deficiency in either one produces a hypoglycosylated, fluorescent protein. Over-expression of the deficient OST subunit fully rescues glycosylation in both cases. OST complexes containing STT3A co-translationally glycosylate proteins, while STT3B-containing complexes can glycosylate proteins post-translationally. This places STT3B ‘downstream’ of STT3A and enables it to glycosylate those sites missed by STT3A. Partial rescue of STT3A-deficiency by STT3B overexpression is consistent with STT3B acting as a glycosylation failsafe downstream of STT3A. The failure of STT3A to rescue STT3B deficiency is also consistent with the downstream role of STT3B. It also suggests that, like many other proteins Glyc-ER-GFP molecules can be glycosylated by both complexes. Another view is that a subset of Glyc-ER-GFP molecules is normally only modified by STT3B-containing complexes. Those molecules would not have access to the STT3A-containing OST complexes.

Transferrin is an STT3A-dependent substrate and only slightly abnormal in STT3B-deficient Case-2, while the STT3A-deficient Case-1 showed an abnormal glycosylation pattern typical of CDG cases. This may mean that future potential cases of STT3B-CDG may be missed by transferrin testing, and may require whole exome or genome sequencing for identification. Mutations in the two other OST subunits TUSC3, and MAGT1 cause non-syndromic mental impairment (21,22). As seen in Case-2, mutations in TUSC3 do not cause hypoglycosylation of transferrin. The latter observation suggests that TUSC3 and MAGT1 may also be involved in glycosylation of a subset of total glycoproteins.

In summary, we have identified two cases in which one of the two isoforms of STT3, the catalytic subunit of the OST complex, is depleted. This causes hypoglycosylation of selected proteins in fibroblasts and mimics the results seen in STT3A- and STT3B-deficient HeLa cells. Transferrin analysis may not detect additional rare cases of STT3B deficiency.

MATERIALS AND METHODS

LLO analysis

LLO analysis performed as previously described (23).

Homozygosity mapping

Was performed using an Affymetrix 6.0 SNP Array according to manufacturer’s procedure (Affymetrix). For Case-1 several regions of homozygosity of >10 MB were present, but given the number and sizes of the candidate regions, we opted to perform Sanger sequencing on already known CDG genes and genes likely to be involved in N-glycosylation. For Case-2, a similar scenario occurred but with fewer candidate regions. However, Sanger sequencing of several candidates identified a candidate gene. In both cases, the presence of known glycosylation genes and genes involved in CDG did occur, primarily in the synthesis of the LLO. We eliminated these as candidates by both Sanger sequencing and analysis of the LLO.

Cell culture and plasmid or siRNA transfection

Primary skin fibroblasts from patients and controls were cultured as described previously (23). Fibroblasts were seeded at 90% confluence in 100 mm dishes and grown for 24 h prior to transfection with expression plasmids (18 μg) using Lipofectamine LTX with PLUS reagent in Opti-MEM (GIBCO) using a
protocol from the manufacturer (Life Technologies). Procedures for the culture and siRNA-mediated depletion of STT3A and STT3B in HeLa cells have been described (11, 14).

The STT3A complementation experiments were conducted by cotransfecting HeLa cells with an STT3A expression vector and the STT3A siRNA. The STT3A coding sequence was inserted into the pCMV6-AC-DDK-His (Origene) and modified by PCR to introduce the p.Val626Ala mutation into STT3A-DDK-His, and to create siRNA resistant STT3A mRNA by replacing the siRNA target sequence (GGCCGT TTCTCTCACCGG) with GGAAGATTAGCCATAGG.

Radiolabeling and immunoprecipitation of glycoproteins

The procedures for pulse-labeling of cells and immunoprecipitation of glycoproteins have been described (14). HeLa cells were treated with SIN for 48 h prior to transfection with the SHBG or β-GUSΔ123 expression vectors (14), and cells were pulse-labeled 24 h later. Antibodies were obtained from the following sources: anti-DDK (Sigma, anti-FLAG), anti-SHBG (R&D Systems), anti-granulin (R&D Systems) and anti-SapD (gift from Prof. Konrad Sandhoff, University of Bonn). As indicated, immunoprecipitated proteins were digested with endoglycosidase H (New England Biolabs). Dry gels were exposed to a phosphor screen (Fujifilm) and scanned in Typhoon FLA 9000.

Protein immunoblots and pulse labeling of STT3A

Expression of STT3B, ribophorin I, Sec61 and BiP in human fibroblasts and HeLa cells was analyzed by protein immunoblotting as described previously (11). Due to the presence of non-specific background bands that are in the vicinity of STT3A on protein immunoblots, STT3A expression was evaluated by immunoprecipitation of STT3A from total cell extracts from cells pulse-labeled for 50 min with 35S Trans-label (200 μCi/ml of media).

Electroporation and microscopy

Fibroblasts were cultured up to 85% confluence, then trypsinized, spun down and counted. A total of 500,000 cells were used per reaction. Cells were suspended with 18 μl of supplement buffer + 82 μl of Nucleofector buffer (Lonza) and then placed in contact with 4 μg of the DNA of interest (STT3A DDK-His or STT3B Myc-DDK, Origene). Cells were transferred to the electroporation cuvette and submitted to the Nucleofector Program U-23. Cells were resuspended in 4 ml of fresh medium and distributed on coverslips. After 2 days, cells were fixed 30 min at room temperature with paraformaldehyde 4%/Sucrose 4% in PBS, permeabilized 5 min by 5% Triton-X100 in PBS, blocked 20 min at room temperature by paraformaldehyde 4%/Sucrose 4% in PBS and incubated with primary antibodies polyclonal anti-GFP at 1/400 (Sigma) overnight at 4°C, washed three times with PBS and incubated with secondary antibody anti-Rabbit IgG Cy3 at 1/400 and anti-mouse IgG-Alexa 569 at 1/400 (Jackson immunochemicals) 1 h at room temperature. Coverslips were mounted on slides with Vectashield-DAPI mounting medium.

WEB RESOURCES

1000 Genomes Project, http://www.1000genomes.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/.

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Conflict of Interest statement. None declared.

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