A new congenital disorder of glycosylation caused by a mutation in SSR4, the signal sequence receptor 4 protein of the TRAP complex

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Received July 30, 2013; Revised September 17, 2013; Accepted October 28, 2013

Nearly 50 congenital disorders of glycosylation (CDG) are known, but many patients biochemically diagnosed with CDG do not have mutations in known genes. Here, we describe a 16-year-old male who was born with microcephaly, developed intellectual disability, gastroesophageal reflux and a seizure disorder. We identified a de novo variant in the X-linked SSR4 gene which encodes a protein of the heterotetrameric translocon-associated protein (TRAP) complex. The c.316delT causes a p.F106Sfs*53 in SSR4 and also reduces expression of other TRAP complex proteins. The glycosylation marker Glyc-ER-GFP was used to confirm the underglycosylation in fibroblasts from the patient. Overexpression of the wild-type SSR4 allele partially restores glycosylation of the marker and of the other members of the TRAP complex. This is the first evidence that the TRAP complex, which binds to the oligosaccharyltransferase complex, is directly involved in N-glycosylation.

INTRODUCTION

Most proteins synthesized in the endoplasmic reticulum (ER) undergo co- and/or posttranslational N-glycosylation (1). Congenital disorders of glycosylation (CDG) are a group of genetic defects characterized by a deficiency in the addition or processing of glycans. To date, over 50 types of CDG have been described (2,3). Type I defects are caused by deficiency in the biosynthesis of the glycan precursor or its addition to the proteins. Such defects result from mutations in genes involved in the synthesis of the lipid-linked precursor glycan or in the oligosaccharyltransferase complex. These patients are usually detected by incomplete N-glycosylation site occupancy in serum transferrin, a convenient glycosylation biomarker. Here, we describe a new type of CDG involving a protein associated with the translocon (TRAP) complex.

The translocon is involved in proteins’ translocation across the ER membrane and is formed by the association of the Sec61p complex, the TRAM protein and the TRAP complex (4,5). The TRAP complex is composed of four signal sequence receptor proteins (SSR1–4) (6). Although the exact function has not been defined, the TRAP complex associates with the OST complex through interaction with two of its subunits, STT3A and DAD1 (7). Association of SSR4 and DAD1 also seems to play a role in beta cell survival in Type 2 diabetes (8). TRAP also appears to associate with misfolded proteins in the ER and to be involved in ER-associated degradation (ERAD), since all four of the subunits are induced with ER stress and accelerate ER degradation (9). Conversely, knockdown of the TRAP subunits by RNAi destroys the complex and slows ERAD (9).

Despite TRAP interaction with these OST subunits there is no evidence that it participates in or affects N-glycosylation. Using exome sequencing, we identify a CDG type I patient carrying a de novo mutation in X-linked SSR4 gene. The absence of SSR4 in patient’s cells reduces the level of other SSR proteins. The patient’s cells underglycosylate the biomarker Glyc-ER-GFP (10) and overexpression of SSR4 partially restores the glycosylation efficiency. This is the first evidence that TRAP is actively involved in the addition of N-glycans.

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RESULTS

Patient clinical information

CDG-050 is currently a 16-year-old male, born to healthy non-consanguineous parents (Figure 1), and initially presented at birth with microcephaly, dysmorphic features including excess skin around the neck and micrognathia. He displayed increased fat pads, mild hypoplasias and clinodactyly of the fourth and fifth toes bilaterally. He also experienced two episodes of respiratory distress. Over the next few months, he was hospitalized for possible apnea and persistent stridor. By 7 months of age, he was developmentally delayed, had become hypotonic, developed gastroesophageal reflux and a seizure disorder. The gastroesophageal reflux was treated with multiple medications and he was eventually weaned of medication without further complications. His seizure disorder was mild and the parents opted to forego treatment. During the initial 7-month period, he was noted to have not cried. He had problems with persistent bleeding and easy bruising, which was later confirmed to be Von Willebrand disease, but it should be noted that this may not be related to his CDG since this is more common than glycosylation disorders.

All biochemical and genetic testing was found to be normal, except for a mildly abnormal carbohydrate deficient transferin profile suggestive of a type I CDG. This was confirmed by multiple isoelectric focusing and electrospray mass spectrometry analyses.

Follow-up analyses for all known CDG defects were performed without finding a candidate gene. Subsequently, we performed whole-exome sequencing, which identified a de novo variant c.316delT resulting in a p.F106SfsX53 in the X-linked gene SSR4 (Figure 1).

Immunodetection of the signal sequence receptor protein complex

The SSR4 protein expression from control fibroblasts cell lines and from the patient CDG-050 was then checked by western blot (Figure 2). Since SSR4 exists as a heterotetrameric complex with SSR1, 2 and 3, we also checked their expression levels to see if the complex was destabilized. Figure 2 shows total absence of SSR4 from the patient’s cells using either monoclonal or polyclonal antibodies. Interestingly, SSR1 and SSR3 are decreased by half compared with their level in control cells and SSR2 is decreased by 90%. These blots were reproduced multiple times using different control fibroblasts to confirm the pathogenic effects of the SSR4 mutation on the TRAP complex.

Gene complementation, Glyc-ER-GFP phenotype rescue

In order to verify that mutation in SSR4 is responsible of this glycosylation deficiency, we expressed SSR4-Flag in the patient’s cells. As shown in the immunoblot in Figure 2, the SSR4-Flag protein was overexpressed and also increased the levels of other SSR subunits (Figure 2), presumably allowing formation of functional SSR complex.

To demonstrate the effects of the SSR4 mutation on glycosylation in the patient’s fibroblasts, we used an engineered Glyc-ER-GFP, a fluorescent biomarker whose fluorescence is lost when the molecule is N-glycosylated due to steric hindrance by the glycan (10). After expression in patient fibroblasts, the Glyc-ER-GFP has fluorescence comparable with the ER-GFP, a control whose fluorescence is independent of glycosylation. The high fluorescence of Glyc-ER-GFP in the patient’s cells indicates an N-glycosylation deficiency (11,12).

Figure 3 shows the quantitation of the relative green fluorescence of the Glyc-ER-GFP compared with the anti-GFP signal (red) before and after complementation. As expected, the fluorescence observed in the patient’s cells electroporated with Glyc-ER-GFP and SSR4-Flag is significantly decreased, ~48%, compared with those transfected by the Glyc-ER-GFP and the control vector. These results confirm the involvement of SSR4 in the N-glycosylation deficiency in this patient.

DISCUSSION

Our results present a new unreported cause of CDG due to a de novo mutation in the X-linked gene-encoding SSR4, which is a part of the TRAP complex and associated with the translocon (6). SSR4 consists of six exons (13) and alternative splicing of exon 5 has been detected both Xenopus laevis and Mus musculus, but not in human SSR4 (14). A functional link between the TRAP complex, translocon and glycosylation has never been clearly established, although some interaction between TRAP proteins and the oligosaccharyltransferase complex were described (7). In particular, Shibatani et al. described the interaction of SSR proteins with five OST complex proteins, STT3A, DAD1, OST48 (DDOST), ribophorin I and II (7). Recently, our team reported defects in the two homologues of STT3 and in OST-48 (DDOST) leading to underglycosylation of the proteins (11,12). STT3A takes part in co-translational N-glycosylation when the nascent protein emerges from the translocon complex. DDOST and DAD1 are modulators of OST stability. DAD1 is required for the structural integrity of the OST complex as demonstrated by Sanjay et al. in a baby hamster kidney cell lines expressing a mutant temperature sensitive form of DAD1. In this cell line, loss of DAD1 leads...
not only to a decrease in the amount of ribophorins and OST-48 but also the TRAP complex (SSR1) and Sec61 translocon proteins (7).

Nagasawa et al. (9) showed that knockdown of one TRAP subunit destroys the entire complex. Our western blots results show reduced SSR1, SSR2 and SSR3 proteins in patient’s cells. Since the TRAP complex interacts with multiple OST subunits, we hypothesized that disruption of TRAP complex impairs OST glycosylation efficiency.

Nagasawa et al. also demonstrated that ER stress induces the TRAP complex which then preferentially associates with misfolded proteins. Knockdown of the TRAP complex reduces

Figure 2. Protein expression levels of the signal sequence receptor proteins complex. (A) The immunodetection after western blot of the SSR1, 2, 3 and 4 proteins in control cell lines, in the patient’s cells (CDG-050) and in patient’s cells-expressing SSR4-Flag. Note that the SSR4 band in Lane 3 has a higher mass due to the presence of the tag. (B) The relative band intensity quantitation obtained by Quantity One (Bio-Rad) of the blots of (A).

Figure 3. Detection by microscopy of the Glyc-ER-GFP in patient’s cells overexpressing wild-type SSR4. We show the relative amount of green fluorescence in patient cells electroporated with ER-GFP and the Glyc-ER-GFP using a control vector (PMM2) or SSR4-Flag. Anti-Flag antibody identifies transfected cells, which are detected by a far-red linked secondary antibody. Total GFP expression was monitored by a GFP antibody followed by a Cy3-linked secondary antibody. Images were monitored by fluorescent microscopy and processed using ImageJ software. The quantitation of the relative GFP fluorescence per cell is shown in the histogram and was calculated from an average of two experiments. Error bars show standard deviation.
ERAD efficiency (9). In parallel, an increased ER stress was shown to correlate with increased mannose-6-phosphate concentration in fibroblasts and a destruction of the lipid-linked oligosaccharides (LLO) (15). In the case of the SSR4 defect, the concentration in fibroblasts and a destruction of the lipid-linked oligosaccharides was shown to correlate with increased mannose-6-phosphate concentration (R01DK55615) and ‘The Rocket Fund’.

This work was supported by National Institutes of Health grant (R01DK55615) and ‘The Rocket Fund’.

**MATERIALS AND METHODS**

**Exome sequencing**

Exome sequencing was done as previously described (16).

**Cell culture and plasmid**

Fibroblasts from the patient and controls were cultured as described previously (12). The plasmid pCMV6-SSR4-Flag was purchased from Origene. Fibroblasts were seeded at 70% confluency in 15 cm dishes 24 h prior electroporation by 6 µg of DNA (see Electroporation and Microscopy).

**Protein immunoblots**

Lysis buffer (Tris 62.5 mM, pH 6.8, SDS 2%) was added to fibroblasts cells pellets and sonicated for few seconds on ice and samples were boiled in presence of Laemmli buffer. Expression of SSR1, SSR2, SSR3 and SSR4 proteins was analyzed by western blot as described previously (10). Antibodies were obtained from mAb anti-TRAP alpha (SSR1) 4C7 (Novus Biologicals), pAb anti-SSR2 (Novus Biologicals), pAb anti-SSR3 (Novus Biologicals), pAb anti-signal sequence receptor delta (SSR4) (Novus Biologicals) and mAb anti-SSR4 clone 2D3 (Sigma). Secondary antibodies linked to HRP were purchased from Amersham Biosciences. Band intensity quantization was performed using the Quantity One (Bio-Rad) software.

**Electroporation and microscopy**

Cells were electroporated by 6 µg of pCMV6-SSR4-Flag and prepared for microscopy as described previously in Shrimal et al. (12). Primary antibodies used were polyclonal anti-GFP at 1/400 (Invitrogen) and monoclonal anti-Flag at 1/400 (Sigma). Secondary antibodies used were anti-rabbit IgG Cy3 at 1/400 and anti-mouse IgG-Alexa 569 at 1/400 (Jackson Immunochemicals).

**FUNDING**

This work was supported by National Institutes of Health grant (R01DK55615) and ‘The Rocket Fund’.

**REFERENCES**