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Quality control of glycoproteins bearing truncated glycans in an *ALG9*-defective (CDG-IL) patient

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We describe an *ALG9*-defective (congenital disorders of glycosylation type IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation. This patient presented with psychomotor retardation, axial hypotonia, epilepsy, failure to thrive, inverted nipples, hepatomegaly, and pericardial effusion. Due to the *ALG9* deficiency, the cells of this patient accumulated the lipid-linked oligosaccharides $Man_6GlcNAc_2$-PP-dolichol and $Man_8GlcNAc_2$-PP-dolichol. It is known that the oligosaccharide structure has a profound effect on protein glycosylation. Therefore, we investigated the influence of these truncated oligosaccharide structures on the protein transfer efficiency, the quality control of newly synthesized glycoproteins, and the eventual degradation of the truncated glycoproteins formed in this patient. We demonstrated that lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ are transferred onto proteins with the same efficiency. In addition, glycoproteins bearing these $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures efficiently entered in the glucosylation/deglucosylation cycle of the quality control system to assist in protein folding. We also showed that in comparison with control cells, patient's cells degraded misfolded glycoproteins at an increasing rate. The $Man_8GlcNAc_2$ isomer C on the patient's glycoproteins was found to promote the degradation of misfolded glycoproteins.

Keywords: congenital disorders of glycosylation/ER/quality control/*N*-glycosylation

Introduction

N-Glycosylation of proteins is a widespread posttranslational modification, crucial for the development, growth, function, and survival of organisms (Varki 1993). The *N*-linked glycosylation pathway starts with the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor on a P-dolichol carrier in the Endoplasmic Reticulum (ER) membrane. Once synthesized, this structure is transferred onto nascent polypeptide chains by the oligosaccharyltransferase (OST) complex. Subsequently, the $Glc_3Man_9GlcNAc_2$ oligosaccharide is deglucosylated by the combined action of glucosidase I and II, leaving a $Man_9GlcNAc_2$ oligosaccharide on the proteins (Parodi 2000).

The fate of newly synthesized glycoproteins is determined during the quality control system in the ER lumen. This quality control system ensures that (1) only correctly folded glycoproteins leave the ER, (2) misfolded glycoproteins are assisted in their folding, and (3) irreversibly misfolded proteins are retro-translocated to the cytosol for degradation. One of the key steps in the quality control of newly synthesized glycoproteins is mediated by the action of UDP-Glc:glycoprotein glucosyltransferase (UGGT) and glucosidase II. UGGT, considered as a sensor for protein folding, recognizes hydrophobic regions on incorrectly folded glycoproteins and reglucosylates the protein-linked $Man_9GlcNAc_2$ oligosaccharide. This reglucosylation event allows the resulting $Glc_1Man_9GlcNAc_2$ to interact with molecular chaperones such as calnexin and calreticulin, which assist in protein folding. Finally, glucosidase II removes again the glucose residue to allow the release of the folded glycoproteins. Correctly folded glycoproteins then leave the ER and are transported to the appropriate destination, while partially folded glycoproteins enter once more in the glucosylation/deglucosylation cycle until folding is complete (Parodi 2000).

If glycoproteins fail to fold correctly after several cycles of reglucosylation and deglucosylation, the $Man_9GlcNAc_2$ oligosaccharide is converted into the $Man_8GlcNAc_2$ isomer B by the action of ER mannosidase I. The formation of this $Man_8GlcNAc_2$ isomer B signals the unfolded glycoproteins for degradation via the ER-associated degradation (ERAD) pathway (Jakob et al. 1998; Cabral et al. 2001). This ERAD pathway constitutes the retro-translocation of misfolded glycoproteins from the ER to the cytosol, followed by the release of the oligosaccharides by a peptide *N*-glycanase activity (Cacan and Verbert 2000; Suzuki and Funakoshi 2006). The resulting free oligosaccharides bear two GlcNAc residues at their reducing ends (called OSGn2). Further degradation of these free oligosaccharides in the cytosol occurs via the trimming by a chitobiase, resulting in the formation of oligosaccharides having one GlcNAc residue at their reducing ends (called OSGn1). Moreover, these OSGn1 species are converted into a specific $Man_5GlcNAc_1$ structure by a cytosolic mannosidase (Kmiécik et al. 1995; Cacan et al. 1996).

The oligosaccharide structures play an important role during the glycosylation pathway. On the one hand, the integrity of the lipid-linked oligosaccharides (LLO) influences the protein transfer rate, and therefore the appearance of hypoglycosylated protein forms: nonglucosylated oligosaccharide structures are

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20- to 25-fold less efficiently transferred onto proteins in comparison with their fully glucosylated forms (Turco et al. 1977; Trimble et al. 1980; Karaoglu et al. 2001). On the other hand, the structure of the protein-bound oligosaccharide has a profound effect on the protein quality control and the recycling of misfolded glycoproteins in the glucosylation/deglucosylation cycle. It has been shown that UGGT and glucosidase II act poorly on substrates with trimmed mannose branches (Grinna and Robbins 1980; Sousa et al. 1992), while extensive mannose trimming retained the chaperone binding activity (Spiro et al. 1996).

The importance of glycosylation is dramatically illustrated by a group of inherited human disorders called congenital disorders of glycosylation (CDG). Up to now, 31 different CDG types have been identified, of which 14 are caused by deficiencies in oligosaccharide precursor formation. These defects in oligosaccharide precursor formation were formerly classified as CDG type I (CDG-I) (Eklund and Freeze 2006; Leroy 2006; Freeze 2007; Jaeken and Matthijs 2007).

In 2004, Frank and co-workers described a new type of CDG, called CDG-IL, in an infant with neuro-developmental impairment, seizures, and hepatomegaly (Frank et al. 2004). CDG-IL was caused by a mutation (c.1567G>A; p.E523K) in the *ALG9* gene, which codes for the α1,2 mannosyltransferase catalyzing the addition of the seventh and ninth mannose residue during oligosaccharide precursor formation. One year later, Weinstein and collaborators described a second CDG-IL patient who was homozygous for the p.Y286C (c.860A>G) mutation, differing from the originally described mutation (Weinstein et al. 2005). Due to recent changes in nomenclature, CDG-IL is now called *ALG9* deficiency (CDG-IL) (Jaeken et al. 2008).

In this study, we described an *ALG9*-defective (CDG-IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation. Due to the accumulation of both $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ on LLO, the patient's fibroblasts were used to study *in vivo* the protein transfer efficiency of these assembly intermediates, the quality control of newly synthesized glycoproteins, and the eventual degradation of the truncated glycoproteins formed in this patient. We demonstrated that lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ are transferred onto proteins with the same efficiency. In addition, glycoproteins bearing these $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures efficiently entered in the glucosylation/deglucosylation cycle of the quality control system to assist in protein folding. We also showed that in comparison with control cells, patient's cells degraded misfolded glycoproteins at an increasing rate. The $Man_8GlcNAc_2$ isomer C on the patient's glycoproteins was found to promote the degradation of misfolded glycoproteins.

Results

Clinical phenotype

The patient is the daughter of Caucasian parents that are distantly related. She was admitted at 3 months with failure to thrive, vomiting, and watery stools. Further investigations at 6.5 months revealed mild dysmorphy, mild central hypotonia, and psychomotor retardation. She was on nasogastric feeding with good catch-up growth and had less frequent episodes of unconsolable crying. In addition, she suffered from several seizures; echocardiography showed a small resolving pericardial effusion and CT scan of the brain was normal. Metabolic investigations were normal.

Biochemical and molecular diagnosis

Iso-electric focusing of serum transferrin revealed a type 1 sialotransferrin pattern in the patient, confirming the clinical suggestion of CDG-I (Figure 1A). Moreover, PMM2 deficiency (CDG-Ia), the most frequent type of CDG-I, was excluded on the basis of normal phosphomannomutase activity in the patient's fibroblasts.

In order to detect a possible defect during oligosaccharide precursor formation in the ER, LLO analysis was performed. Compared to control fibroblasts, an accumulation of the assembly intermediates $Man_6GlcNAc_2$-PP-dolichol and $Man_8GlcNAc_2$-PP-dolichol was detected in the patient's fibroblasts (Figure 1, panels B and C), suggesting a deficiency in the *ALG9* α(1,2)-mannosyltransferase. Sequencing of the patient's DNA revealed indeed a homozygous missense mutation c.860A>G (p.Y286C) in the *ALG9* gene, and both parents were shown to be carriers for this mutation (Figure 1D).

Transfer of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ onto proteins

Due to the accumulation of both lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$, the fibroblasts of this patient provided a good model to analyze and compare *in vivo* the transfer rate of these intermediates onto proteins. Hence, the structure of the oligosaccharides transferred onto proteins was analyzed after metabolic labeling of the patient's fibroblasts. $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, and $Glc_1Man_9GlcNAc_2$ structures were found on the glycoproteins of control fibroblasts, whereas mainly $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were detected on the patient's glycoproteins (Figure 2, panels A and B). Moreover, kifunensin treatment did not influence the patient's HPLC profile (Figure 2C). We thus concluded that the species accumulating on LLO were transferred onto proteins, although they were not optimal substrates for the OST complex.

Intriguingly, both species were transferred with the same efficiency. By comparing the ratios of $Man_6GlcNAc_2$/$Man_8GlcNAc_2$ on LLO and glycoproteins, a value of 2.98 ($\pm$0.27; $n = 3$) was obtained for the LLO, while a value of 2.99 ($\pm$0.14; $n = 3$) was found on the glycoproteins (for the calculation of the ratios, see *Material and methods*). The addition of kifunensin during these metabolic labelings was essential to avoid a mixture of the transferred $Man_8GlcNAc_2$ structure with the $Man_8GlcNAc_2$ formed by demannosylation of the $Man_9GlcNAc_2$ structure. The obtained ratios suggested an equal protein transfer rate of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ species. Changing the duration of metabolic labeling from 1 h to 30 min, 2 h, or 4 h did not influence the obtained ratios (data not shown).

Reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ on proteins

Besides the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharide structures observed in Figure 2B, two other peaks (X_1 and X_2) were detected on the patient's glycoproteins. Peaks X_1 and X_2 migrated as $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$, respectively. To confirm their identity, a metabolic labeling in presence of kifunensin and castanospermin, the latter being an inhibitor of glucosidase I and II, was carried out. In the presence

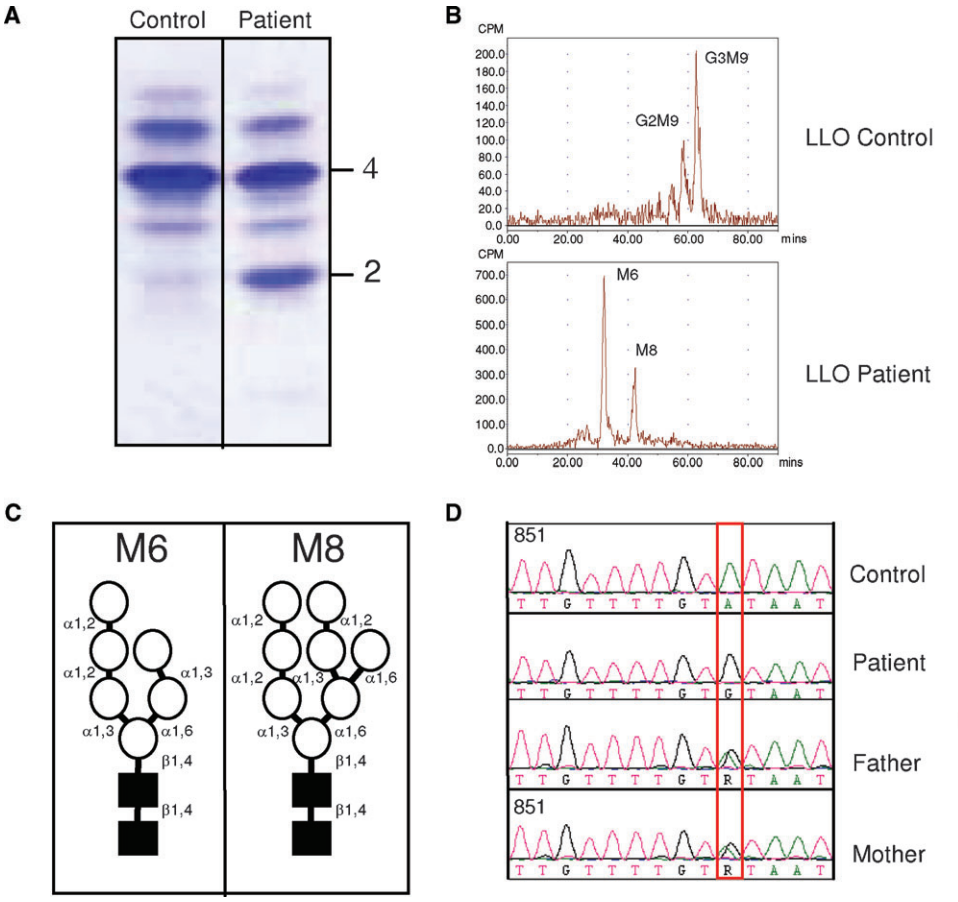

Fig. 1. (**A**) Isoelectric focusing of serum transferrin in control and patient. The number of negative charges is indicated on the right. (**B**) HPLC analysis of the lipid-linked oligosaccharides in control and patient's fibroblasts. (**C**) Structure of the lipid-linked oligosaccharides accumulating in the patient's fibroblasts. (**D**) Sequence alignment of the *ALG9* cDNA fragment in control, patient, and the patient's parents. The patient is homozygous for an A–G transition at position c.860, while both parents are heterozygous for this base pair change. Symbols: 4, tetrasialotransferrin forms; 2, disialotransferrin forms; G2–3M9, $Glc_{2-3}Man_9GlcNAc_2$-PP-dolichol; M6–8, $Man_{6-8}GlcNAc_2$-PP-dolichol; squares represent GlcNAc residues and circles represent Man residues.

of kifunensin and castanospermin, mainly $Glc_3Man_9GlcNAc_2$ was found on the glycoproteins of control fibroblasts (data not shown), whereas only a small amount of $Glc_3Man_9GlcNAc_2$ was detected in the patient. However, the levels of X_1 and X_2 increased significantly in the glycoproteins fraction of the patient (Figure 2D). Additionally, treatment of the glycoproteins with glucosidase II resulted in a significant decrease in the levels of X_1 and X_2 in combination with an increase in $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ (Figure 2E). From these results, we concluded that X_1 and X_2 referred to $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$, respectively.

Because $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ can be formed by deglucosylation of $Glc_3Man_6GlcNAc_2$ and $Glc_3Man_8GlcNAc_2$, respectively, we checked the presence of these latter species on the LLO and/or glycoproteins in the patient's fibroblasts. In the presence of kifunensin and castanospermin, triglucosylated oligosaccharides (X_3: potentially $Glc_3Man_8GlcNAc_2$ and X_4: potentially $Glc_3Man_6GlcNAc_2$) did appear on the LLO and the glycoproteins, although at a very low level (Figure 2, panels D, F, and G). This result excluded that $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ oligosaccharides were formed by deglucosylation of triglucosylated species. Moreover, we did not detect monoglucosylated $Man_6GlcNAc_2$ or $Man_8GlcNAc_2$ species on the LLO after metabolic labeling in the presence of kifunensin and castanospermin (Figure 2, panels F and G).

All together, these results demonstrated that $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ structures were formed by reglucosylation of the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures transferred onto proteins. When comparing the efficiency of reglucosylation, only minor differences in the reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were detected. A comparison of the ratios $Glc_1Man_6GlcNAc_2/Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2/Man_8GlcNAc_2$ in the presence of castanospermine revealed a value of 1.25 ($\pm$0.12; $n = 3$) and 1.78 ($\pm$0.08; $n = 3$) respectively (for the calculation of the ratios, see *Material and methods*). These data suggested that the $Man_6GlcNAc_2$ structure was only 30% less glucosylated than the $Man_8GlcNAc_2$ structure.

Preferential degradation of $Man_8GlcNAc_2$

To study the fate of the truncated glycoproteins in the patient, we analyzed the free oligosaccharides, which are the degradation products of the misfolded glycoproteins (Cacan and Verbert 2000; Suzuki and Funakoshi 2006). First, we compared the total amount of free oligosaccharides formed in control and patient's fibroblasts. After metabolic labeling, patient's cells incorporated 14.20% ($\pm$0.58; $n = 3$) of total radioactivity in the

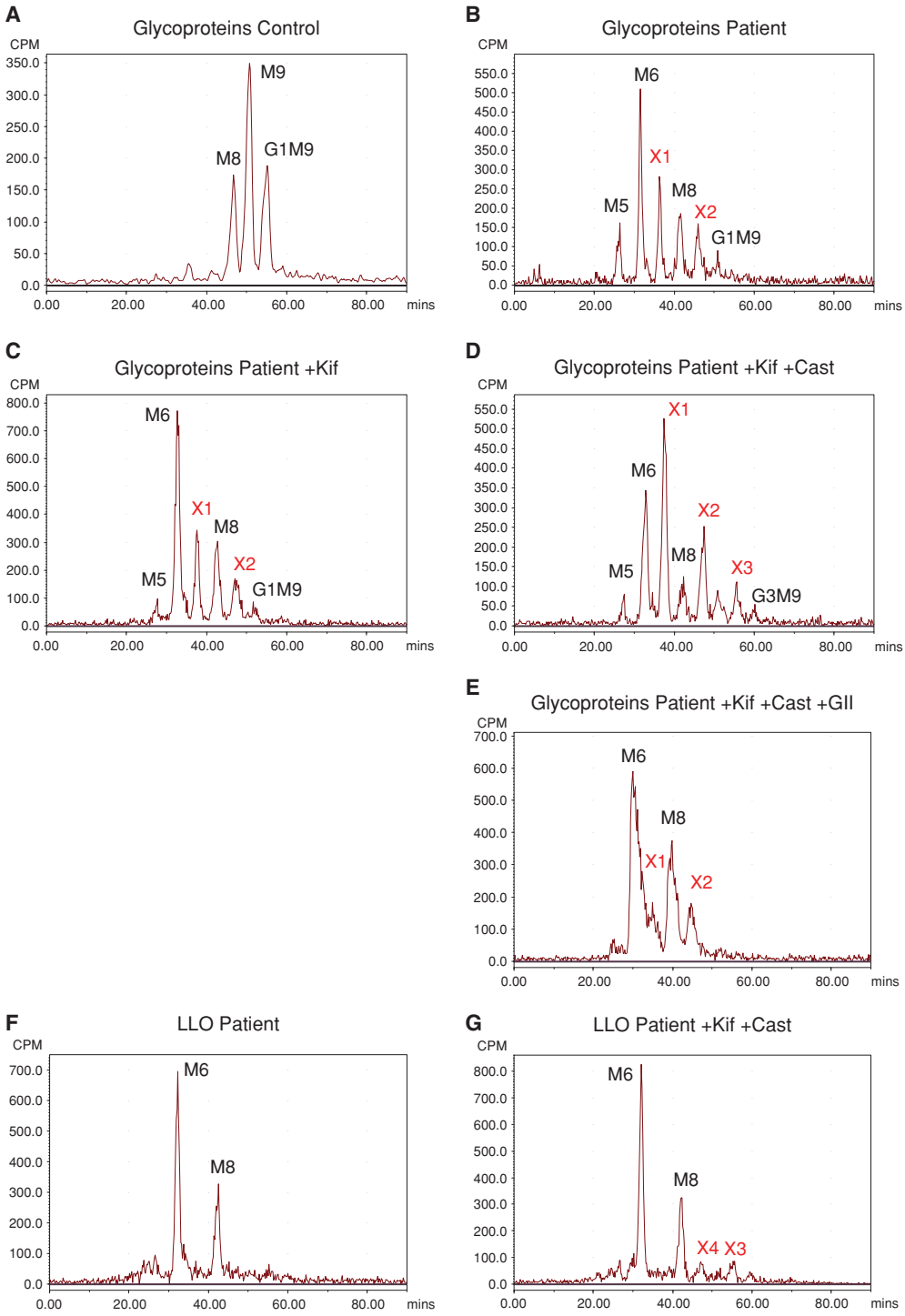

Fig. 2. (**A–E**) HPLC analysis of the oligosaccharides linked to proteins. (**A**) Control fibroblasts. (**B**) Patient's fibroblasts. (**C**) Patient's fibroblasts in the presence of the ER mannosidase I inhibitor kifunensin. (**D**) Patient's fibroblasts in the presence of kifunensin and castanospermin, the latter being an inhibitor of glucosidases I and II. (**E**) Profile D after treatment with glucosidase II. (**F**) HPLC analysis of the lipid-linked oligosaccharides in the patient's fibroblasts. (**G**) HPLC analysis of the lipid-linked oligosaccharides in the patient's fibroblasts in the presence of kifunensin and castanospermin. Symbols: M5–9, $Man_{5-9}GlcNAc_2$; G1–3M9, $Glc_{1-3}Man_9GlcNAc_2$; and X1–X4, peaks of unknown identity shown in red.

free oligosaccharides, while only 6.98% (±0.54; $n = 3$) of total radioactivity was found in control-free oligosaccharides (Figure 3). In addition, this increased formation of free oligosaccharides in the patient resulted in a proportional decrease in the glycoproteins fraction, while the LLO remained unchanged (Figure 3). Subsequently, the structure of the free oligosaccharides was analyzed by HPLC. The free oligosaccharide fraction of control fibroblasts contained mainly $Man_8GlcNAc_{1/2}$ and $Man_9GlcNAc_{1/2}$ structures, which was in accordance with the glycan structures found on the glycoproteins (Figure 4A).

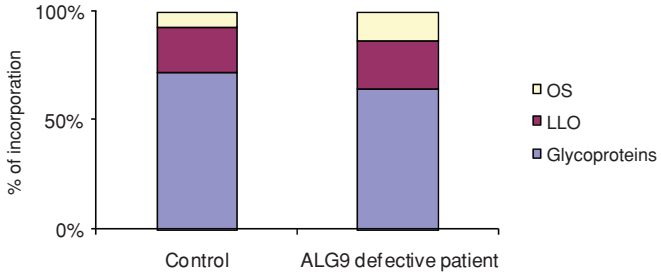

Fig. 3. Distribution of the incorporated radioactivity between the various fractions (LLO, glycoproteins and free oligosaccharides) after metabolic labeling of control and patient's fibroblasts.

In contrast, $Man_6GlcNAc_{1/2}$ and $Man_8GlcNAc_{1/2}$ structures were found in the free oligosaccharides of the patient's fibroblasts, reflecting the glycan structures on the patient's glycoproteins (Figure 4B). However, $Man_8GlcNAc_{1/2}$ species were better represented in the free oligosaccharide fraction. The ratio $Man_6GlcNAc_{1/2}/Man_8GlcNAc_{1/2}$ reached indeed a value of 2.02 (± 0.05; $n = 3$) in the free oligosaccharide fraction, which is in contrast to the ratio of 2.99 (± 0.14; $n = 3$) obtained for the glycoproteins (for the calculation of the ratios, see *Material and methods*).

Discussion

In this paper, we described an *ALG9*-defective (CDG-IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation, like the patient described by Weinstein et al. (2005). The new *ALG9*-defective (CDG-IL) patient has a Canadian nationality, like the first patient, which could point to a founder effect of the p.Y286C mutation. Both patients showed very similar clinical pictures including psychomotor retardation, axial hypotonia, epilepsy, failure to thrive, inverted nipples, hepatomegaly, and pericardial effusion. In addition, the patient reported by Weinstein et al. (2005) showed intermittent esotropia and splenomegaly, while the present patient had more, albeit mild dysmorphic features. The *ALG9*-defective (CDG-IL) patient reported by Frank et al. (2004) had psychomotor retardation, axial hypotonia, epilepsy, and hepatomegaly in common with the other two patients. Other features were not mentioned in this very short report, which does not exclude their presence. Altogether, the reported *ALG9*-defective (CDG-IL) patients did not show a specific clinical picture.

Due to the accumulation of both $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ on LLO, the patient's cells were used to analyze and compare *in vivo* the transfer rate of these accumulating intermediates onto proteins. Analysis of the patient's glycoproteins revealed the transfer of the accumulating $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides onto proteins (Figure 2B). Assembly intermediates have a low affinity for the OST complex. However, transfer of accumulating assembly intermediates onto proteins has been repeatedly reported in different CDG patients (Korner et al. 1999; Kranz et al. 2001; Chantret et al. 2002, 2003; Grubenmann et al. 2002; Frank et al. 2004). Though, a comparison of the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ transfer rate has never been done. Analysis of the glycoproteins in the *ALG9*-defective patient revealed an equal transfer rate for both accumulating oligosaccharides. This proved that the OST had no preference for the most entire assembled intermediate, which is the $Man_8GlcNAc_2$ oligosaccharide. The donor-substrate activation model for the OST complex only favors the selective utilization of the fully assembled oligosaccharide precursor. In this model, the OST has a regulatory binding site that favors the transfer of the fully assembled oligosaccharide precursor as a function of the donor population. For this reason, the transfer of the fully assembled oligosaccharide precursor is promoted, even when this structure is not abundant (Karaoglu et al. 2001).

To address whether or not the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides were transferred onto proteins in their triglucosylated forms, a metabolic labeling in the presence of kifunensin and castanospermin was performed. Only minor triglucosylated $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were detected on the LLO and glycoproteins (Figure 2). These triglucosylated forms could therefore not account for the transfer of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ onto proteins. Furthermore, the absence of triglucosylated $Man_7GlcNAc_2$ was reported in *ALG12*-defective (CDG-Ig) fibroblasts labeled in the presence of castanospermin (Chantret et al. 2002; Grubenmann et al. 2002). From these results, we could postulate that the accumulating assembly intermediates $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$ were not good substrates for the α1,2 glucosyltransferase I and that the presence of nine mannose residues was essential for its catalytic activity (Figure 5A). Glucosyltransferase I catalyzes the addition of the first glucose residue during oligosaccharide precursor formation.

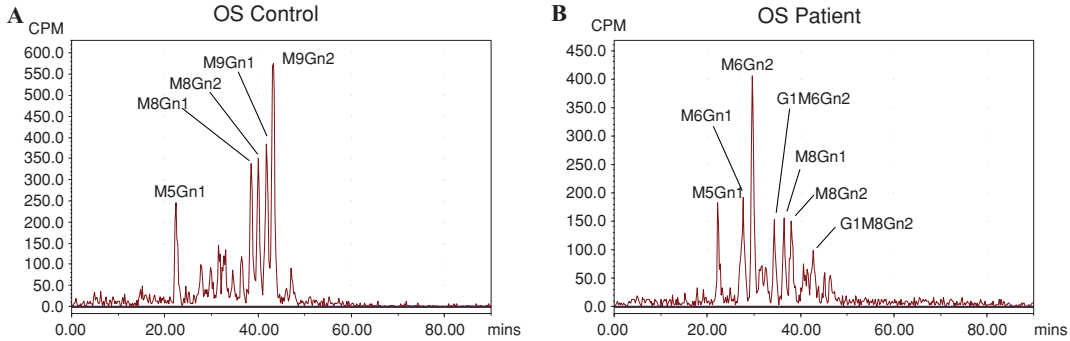

Fig. 4. HPLC analysis of the free oligosaccharides in control (**A**) and patient (**B**). Symbols: M5–9Gn1, $Man_{5-9}GlcNAc_1$; M5–9Gn2, $Man_{5-9}GlcNAc_2$; G1M6–9Gn2, $Glc_1Man_{6-9}GlcNAc_2$; and G1M8Gn1, $Glc_1Man_8GlcNAc_1$.

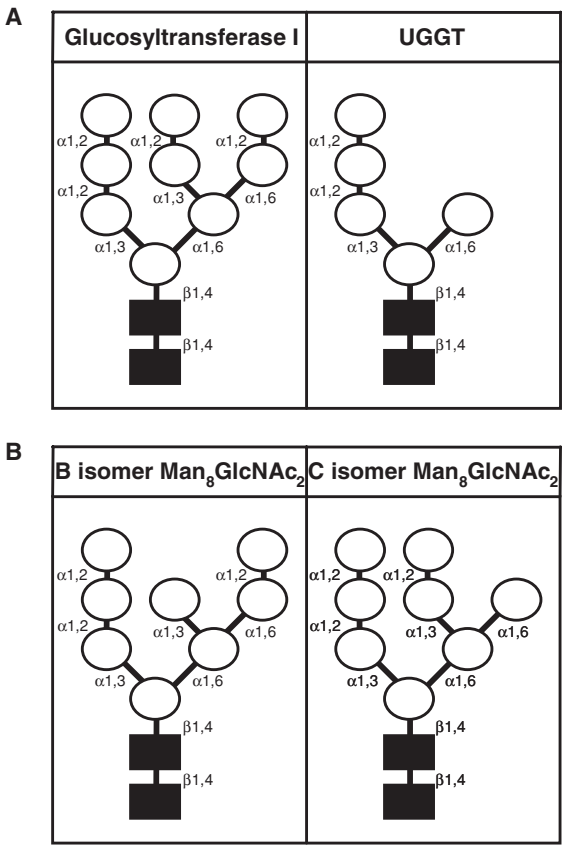

Fig. 5. (**A**) Model of the minimal structure necessary for the catalytic activity of the glucosyltransferase 1 and UDP-Glc:glycoprotein glucosyltransferase (UGGT). (**B**) Structure of the B and C isomer of $Man_8GlcNAc_2$. Symbols: squares represent GlcNAc residues and circles represent Man residues.

Analysis of the patient's glycoproteins after metabolic labeling also revealed the presence of $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ (Figure 2B). Due to the absence of monoglucosylated and triglucosylated species on LLO, we hypothesized that the monoglucosylated structures ($Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$) observed onto proteins were formed by the reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures. While UGGT reglucosylates oligosaccharide structures transferred onto protein, these results showed that the truncated glycoproteins bearing $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were incorrectly folded and entered into the glucosylation/deglucosylation cycle to improve folding. In addition, we demonstrated that UGGT efficiently reglucosylated small oligosaccharide structures such as $Man_6GlcNAc_2$: $Man_6GlcNAc_2$ was only 30% less glucosylated in comparison with $Man_8GlcNAc_2$. This is in contrast to the report of Sousa et al. (1992), where they reported a strong diminution in UGGT activity when smaller oligosaccharides were used. In this study, the relative rates for reglucosylation of $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc_2$ were 1.0, 0.5, and 0.15, respectively. We could thus hypothesize that, in contrast to glucosyltransferase I, mannose trimming does not impair the catalytic activity of UGGT. Moreover, studies in CHO demonstrated that $Man_4GlcNAc_2$ could not be reglucosylated (Duvet et al. 2000). Only a fully assembled α1,3 branch is thus essential for reglucosylation by UGGT (Figure 5A).

In parallel, analysis of the patient's glycoproteins revealed that the reglucosylated $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were also efficiently deglucosylated. In absence of castanospermin, only a small fraction of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were glucosylated. On the other hand, the addition of castanospermin caused a marked increase in the fraction of reglucosylated oligosaccharides. These results tended to prove that $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were also efficiently deglucosylated, which is in contrast to the report of Grinna and Robbins (1980). In this study, a marked decrease in deglucosylation efficiency was obtained for substrates with trimmed mannose residues. Thanks to the efficient reglucosylation and deglucosylation of truncated glycoproteins, the cell ensures that every truncated glycoprotein can enter into the quality control system and can be recycled in the glucosylation/deglucosylation pathway. In addition, the specific activity of glucosyltransferase I ensures that only correctly assembled LLO are glucosylated during oligosaccacharide precursor assembly.

We also investigated the fate of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ bearing glycoproteins by analyzing the free oligosaccharides, the degradation products of misfolded glycoproteins. Analysis of the total amount of free oligosaccharides formed in control and patient's fibroblasts revealed an increased formation of free oligosaccharides in the patient. These results showed that the degradation of misfolded glycoproteins was increased in this patient, reflecting a higher amount of misfolded glycoproteins. In addition, metabolic labeling of the patient's fibroblasts revealed that the oligosaccharide structure influences the degradation rate of misfolded glycoproteins. Glycoproteins bearing $Man_8GlcNAc_2$ oligosaccharides were shown to be 34% more efficiently degraded than glycoproteins bearing a $Man_6GlcNAc_2$ oligosaccharide. In a yeast study, comparing carboxypeptidase Y (CPY) bearing $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ oligosaccharides, the $Man_8GlcNAc_2$ oligosaccharide also promoted degradation of the misfolded glycoproteins (Jakob et al. 1998). However, the $Man_8GlcNAc_2$ structure detected in our patient is not identical to the one reported by Jakob et al. (1998): in yeast, the B-isomer of $Man_8GlcNAc_2$, generally known as the degradation signal, was investigated, while we analyzed the C isomer of $Man_8GlcNAc_2$ (Figure 5B). Based on these results, we could postulate that the C isomer of $Man_8GlcNAc_2$ also promoted degradation via the ERAD pathway or that the C-isomer is the degradation signal of another degradation pathway, like for example the nonproteasomal degradation of misfolded glycoproteins. These data were reinforced by the recent publication of Clerc et al. (2009). They reported that *N*-glycans bearing a terminally α1,6-linked mannosyl residue, such as $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ isomer C, were a determinant directing misfolded glycoproteins to degradation.

Material and methods

Cell culture

Primary fibroblasts from controls and patient were cultured at 37°C under 5% CO_2 in DMEM/F12 (Life Technologies, Paisley, UK) supplemented with 10% Fetal Clone III (HyClones, UT, USA).

Metabolic radiolabeling of cells

For this purpose, 8×10^6 fibroblasts were grown overnight in a 175 cm^2 tissue culture flask. The next day, cells were pre-incubated in 0.5 mM glucose for 45 min and then pulse-radiolabeled with 150 μCi of 2-[^{3}H] mannose (16 Ci/mmol, Amersham Biosciences, UK) at the same glucose concentration for 1 h. Where appropriate, glycosidase inhibitors kifunensin (20 μM, Calbiochem, CA, USA) and castanospermin (50 μg/mL, Sigma, St. Louis, MO, USA) were added to the cells at the beginning of the pre-incubation step and lasted throughout the experiment. After metabolic labeling, cells were scraped with 1.1 mL MeOH/H_2O (8:3; v/v) followed by the addition of 1.2 mL $CHCl_3$. Sequential extraction of oligosaccharide materials was performed as previously described (Cacan and Verbert 1997).

Analysis of oligosaccharide material

Free oligosaccharide fractions obtained after sequential extraction of the oligosaccharide material were desalted on Bio-Gel P2 and eluted with 5% (v/v) acetic acid. Glycoprotein fractions obtained at the end of the sequential extraction were digested overnight at room temperature with trypsin (1 mg/mL; Sigma) in the 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were then treated with 0.5 U PNGase F (Roche Diagnostics, Indianapolis, IN, USA) in the 50 mM phosphate buffer, pH 7.2, for 4 h to release the oligosaccharides from the protein. Purification of the released oligosaccharides was performed as described above. Lipid-linked oligosaccharide fractions obtained after the sequential extraction were subjected to a mild acid treatment (0.1 M HCl in THF) at 50°C for 2 h. Purification of the released oligosaccharides was performed as described above.

The oligosaccharides were separated by HPLC on a polymer-based amino column (Asahipak NH_2P-50 column; 250 mm × 4.6 mm; Asahi, Kawasaki-ku, Japan) applying a gradient of acetonitril/H_2O ranging from 70:30 (v/v) to 50:50 (v/v) at a flow rate of 1 mL/min over 90 min. Oligosaccharides were identified on the basis of their retention times compared to well-defined standards (Foulquier et al. 2002). Elution of the radiolabeled oligosaccharides was monitored by continuous flow detection of radioactivity with a flo-one β detector (Packard, Les Ullis, France).

Calculation peak counts

HPLC chromatograms were analyzed using the ProFSA software (Perkin Elmer, MA, USA). The counts in each peak were calculated on the basis of the peak area and normalized against the total number of counts in the injected sample. As protein-linked $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ are formed by reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$, the peak counts for the glucosylated forms were taken into account for calculating the $Man_6GlcNAc_2/Man_8GlcNAc_2$ ratios on the glycoproteins and free oligosaccharides. In addition, the difference in mannose residues between $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were taken into account.

Mutation analysis

Primers were designed to amplify the 15 exons of *ALG9*, including at least 50 bp of the flanking intronic regions, based on the genomic sequence of *ALG9* (NM_024740). Primer sequences are available on request. The exons were amplified using standard PCR conditions, subsequently sequenced with Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applera, Foster City, CA, USA), and analyzed on an ABI3100 Avant (Applera).

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Conflict of interest statement

None declared.

Abbreviations

CDG, congenital disorders of glycosylation; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; GlcNAc, *N*-acetylglucosamine; LLO, lipid-linked oligosaccharides; Man, mannose; OST, oligosaccharyl transferase complex; UGGT, UDP-glucose:glycoprotein glucosyltransferase.

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