Carbohydrate-deficient glycoprotein syndrome type V: Deficiency of dolichyl-P-Glc:Man$_9$GlcNAc$_2$-PP-dolichyl glucosyltransferase

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ABSTRACT Deficiency of dolichyl-P-Glc:Man$_9$GlcNAc$_2$-PP-dolichyl glucosyltransferase is the cause of an additional type of carbohydrate-deficient glycoprotein syndrome (CDGS type V). Clinically this type resembles the classical type Ia of CDGS caused by the deficiency of phosphomannomutase. As a result of the glucosyltransferase deficiency in CDGS type V nonglucosylated lipid-linked oligosaccharides accumulate. The defect is leaky and glucosylated oligosaccharides are found on nascent glycoproteins. The limited availability of glucosylated lipid-linked oligosaccharides explains the incomplete usage of N-glycosylation sites in glycoproteins. This finding is reflected in the presence of transferrin forms in serum that lack one or both of the two N-linked oligosaccharides and the reduction of mannose incorporation to about one-third of control in glycoproteins of fibroblasts.

METHODS

Isoelectric Focusing and SDS-PAGE. Isoelectric focusing of transferrin was carried out on ultrathin polyacrylamide gels within the pHe range of 4–7 followed by Western blotting as described by Stibler et al. (17). Dilution of patient and control samples was 1:50. Primary antibody from Dako was diluted 1:500. Secondary antibody (Dako) diluted 1:2,000 was labeled with alkaline phosphatase (Bio-Rad). SDS/PAGE was performed on 7.5% SDS-polyacrylamide gels under nonreducing conditions followed by Western blot.

Cell Culture. Human primary fibroblast cultures from controls and patients were obtained from upper arm skin biopsies. Cells were grown at 37°C in the presence of 5% CO$_2$ on DMEM (GIBCO/BRL) supplemented with 10% fetal calf serum (Pansystems, Aidenbach, Germany) and passaged by trypsinisation.

Phosphomannomutase (PMM) Assay. PMM activity was determined by following the conversion of mannose 6-phosphate into mannose 1,6-bisphosphate in the presence of glucose 1,6-bisphosphate as described (18). In brief, 25,000 cpm of [2-3H]mannose 6-phosphate, 1 mM glucose1,6-bisphosphate, and 4 mg of cell extracts were incubated for 30 min at 37°C. [2-3H]mannose 6-phosphate was separated from [2-3H]mannose 1,6-bisphosphate by high-voltage paper electrophoresis in a buffer containing 80 mM pyridine, pH 5.5.

Phosphomannose Isomerase (PMI) Assay. PMI activity was determined in a coupled optical assay according to the method of van Schaftingen and Jaeken (4).

Metabolic Labeling with [2-3H]Mannose and [35S]Methionine. Labeling of fibroblasts with d-[2-3H]mannose or [35S]methionine (Amersham Buchler, Braunschweig, Germany) and determination of radioactivity incorporated into proteins was carried out as described (18). Columns were repeatedly calibrated by authentic oligosaccharide standards to monitor small changes in retention times and resolution.

Extraction and HPLC Analysis of LLO. Oligosaccharides released by mild acid hydrolysis from chloroform/methanol/water (10:10:3) extracts of fibroblasts labeled with [2-3H]mannose were analyzed by HPLC as described (18).

PNGase F Digestion and HPLC Analysis of Protein-Derived Oligosaccharides. Glycoproteins from fibroblasts labeled with [2-3H]mannose were treated with PNGase F followed by analysis of released oligosaccharides by HPLC as described (18).

UDP-Glc:Dol-P Glucosyltransferase (Dol-P-Glc Synthase). Fibroblasts (3.6 × 10$^6$) were plated onto 10-cm culture dishes and grown for 60 h in DMEM. Cells were washed with 10 mM

Abbreviations: CDGS, carbohydrate-deficient glycoprotein syndrome; LLO, lipid-linked oligosaccharides; Dol-P-Glc, dolichyl phosphate glucosyltransferase.

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PBS, pH 7.4, scraped into 2 × 1 ml of PBS, and centrifuged at 1,000 g for 10 min. Pellets were taken up in 500 µl of PBS and homogenized by passing 20 times through a 22-g syringe. After centrifugation for 15 min at 100,000 g, the pellet was taken up in water and the amount of protein was determined following the method of Lowry et al. (19). When indicated exogenous Dol-P (Sigma) was added to the incubation mixture. Five micrometers of Dol-P was dried under nitrogen, taken up into 10 µl of 2.5% Triton X-100, and dissolved by sonification. Reactions took place in a buffer containing 7 mM Tris HCl (pH 7.4), 7 mM MgCl2, 0.1 µCl UDP-[^14]C]Glc (231 mCi/mol), and 100 µg of membrane material in a total volume of 70 µl. The reactions were incubated at 23°C as indicated, stopped by adding 2 × 1 ml of chloroform/methanol (2:1), and vortexed immediately. To remove free UDP[^14]C]Glc, 0.33 ml of 4 mM MgCl2 was added to the reaction mix followed by vortexing and centrifugation. The upper phase was discarded, and the lower phase was washed twice with methanol/water/chloroform (1:1) and subjected to TLC on Silica gel 60 plates (Merck) in chloroform/methanol/water (65:25:4). Dol-P-Glc was quantified by using a flat screen scanner (Berthold, Wildbad, Germany).

**Determination of UDP-Glc.** The endogenous level of UDP-Glc in control and patient fibroblasts was determined according to the method of Grubb et al. (20). In brief, cells were plated and grown on 10-cm culture dishes as described above. After washing twice with ice-cold 10 mM PBS, cells were covered with 80% methanol at 4°C for 60 min. The methanol phase was removed and centrifuged for 10 min at 8,000 g. The supernatant was dried under vacuum. The residue was dissolved in 0.25% Nikkol, 6.3 mM MgCl2, 1.5 mM phosphatidylcholine, 1 mM DTT, and microsomal membrane fraction (0.12 mg of protein) in a total volume of 70 µl. Incubations were carried out at 25°C for 30 min and stopped with 0.4 ml of 20 mM HCl. Release of LLOs by mild acid and their analysis by HPLC was as described (18). Membrane fraction was isolated from fibroblasts as described (21). Radioactive Dol-P[^14]C]Glc was enzymatically synthesized and purified (22, 23) by using membranes from yeast strain DBY 746 (MATa his3-D1 leu2–3,112 ura3–52 trp1–289 pALG5–1).[^3H]Man9GlcNAc2–PP-Dol was isolated from yeast cells YG0092 (MATa ade2–101 his3-D200 ura3–53 Dalg5::HIS3) metabolically labeled with[^3H]mannose (24).

**RESULTS**

**Clinical Phenotype.** The now 7-year-old girl was born at term by caesarian section because of breech position. Birth weight, length, and head circumference were in a normal range. She is the first child from healthy nonconsanguinous parents. Familial history is inconspicuous.

In the first year a convergent squint, recurrent oedema of the upper eye lids, and recurrent infections were noticed. Muscular hypotonia, ataxia, and mental and motor developmental retardation were realized at the age of 6 months. Since the age of 11 months seizures occur during infections.

Nerve conduction velocity was always normal. In the MRI at the age of 4 years a slight general atrophy of the cerebrum and cerebellum was seen. She has an atrophic retinal pigmentation, reduction of retinal vascularization, and hyperopia.

Laboratory diagnostics repeatedly revealed pathological coagulation parameters with prolonged partial thromboplastin time, low antithrombin, low factor VIII, low Ristocetin cofactor, and low von Willebrand factor. Further diagnostic shows low coagulation factors, V, VI, X, XI, and XII. The other serumglycoproteins, parameters for thyroid gland function, and liver enzymes were in the normal range.

**Diagnosis.** The diagnosis of CDGS was established by isoelectric focusing of serum transferrin, which showed the characteristic pattern of CDGS type I with increased frequency of di-, mono-, and asialoforms at the expense of higher sialylated forms of transferrin (Fig. 1, Upper). Size determin-
nation by SDS/PAGE revealed the presence of faster migrating forms of transferrin (Fig. 1, Lower), indicating that some polypeptides lack one or both of the two oligosaccharide side chains normally present in transferrin. Phosphomannomutase and phosphomannose isomerase were normal. The deficiency of either of the two enzymes in CDGS type Ia and type Ib causes a similar change of the transferrin pattern in isoelectric focusing and SDS/PAGE.

Oligosaccharides Derived from Glycoproteins and LLO. In cultured fibroblasts incorporation of $[2^-3H]$mannose in glycoproteins was reduced to about one-third of controls (Table 1). The oligosaccharides were released from newly synthesized glycoproteins by endoglycosidase PNGaseF and size-fractionated. The size pattern of glycoprotein-derived oligosaccharides from controls and the patient fibroblasts was similar with Man$_9$GlcNAc$_2$ and Glc$_1$Man$_9$GlcNAc$_2$ oligosaccharides representing the two major species (Fig. 2, Upper). This finding indicates that the reduced incorporation of $[2^-3H]$mannose does not result from incorporation of oligosaccharides with a lower number of mannose residues, but from a lower number of oligosaccharides transferred onto nascent glycoproteins, a conclusion in agreement with the reduced size of serum transferrin.

Next we examined the size of the oligosaccharide moiety of LLOs isolated from fibroblasts metabolically labeled for 30 min with $[2^-3H]$mannose. In control fibroblasts oligosaccharides corresponding in size to Glc$_3$Man$_9$GlcNAc$_2$ standards represent the major fraction, whereas in the patient's cells the major species had a size as a Man$_9$GlcNAc$_2$ standard (Fig. 3). Trace amounts of the di- and triglucosylated oligosaccharides Glc$_2$Man$_9$GlcNAc$_2$ and Glc$_3$Man$_9$GlcNAc$_2$ were detectable in the patient’s LLOs.

Enzyme Defect. The lack of glucosylated forms of LLOs suggested a defect either in the glucosyltransferase(s), which catalyzes the transfer of glucose from Dol-P-Glc, or in the synthesis of Dol-P-Glc from UDP-Glc and Dol-P. UDP-Glc was determined in methanolic extracts of the cells by HPLC and estimated to be 7.4 and 9.1 nmol/mg of cell protein in control and patient’s fibroblasts, respectively. Also the activity of UDP-Glc:Dol-P-Glc synthase in microsomal extracts determined both by using endogenous or an excess of exogenous

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<td>Patient</td>
<td>117,235</td>
<td>928,777</td>
</tr>
<tr>
<td>Control</td>
<td>520,597</td>
<td>1,415,335</td>
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The values represent the mean of two independent experiments, which deviated by less than 15%.

Fig. 2. Glycoprotein-linked oligosaccharides. Control (Left) and patient’s (Right) fibroblasts were metabolically labeled for 30 min with $[2^-3H]$mannose. (Lower) Cultures shown were incubated in the presence of 5 mM N-methyldeoxynojirimycin (MdNM) for 30 min before and during the labeling. $[^{3H}]$Oligosaccharides were released from glycoproteins with PNGaseF and size-fractionated. (Upper) $M_0$, $G_1$, $G_2$, and $G_3$ refer to the elution of Man$_9$GlcNAc$_2$ and Glc$_1$Man$_9$GlcNAc$_2$ standards.
Dol-P was found to be normal in the patient’s fibroblasts (Fig. 4).

To determine the activity of Dol-P-Glc:Man₉GlcNAc₂-PP-Dol glucosyltransferase, Dol-P-[¹⁴C]Glc and [³H]Man₉GlcNAc₂-PP-Dol labeled in their mannose moieties were prepared. After incubation with microsomal extracts the sugar moieties were liberated by mild acid hydrolysis and separated by HPLC. Microsomal extracts from control fibroblasts converted 32% of the Man₉GlcNAc₂ oligosaccharides into forms with the mobility of Glc₁–₃Man₉GlcNAc₂ standards (Fig. 5B). The ratio of mono-, di-, and triglycosylated forms was variable. Omission of either Dol-P-[¹⁴C]Glc or [³H]Man₉GlcNAc₂-PP-Dol or boiling of microsomal extracts prevented product formation. The ¹⁴C-radioactivity was recovered exclusively in products eluting in the position of mono-, di-, and triglycosylated products (Fig. 5C). No decrease in oligosaccharide size was observed when Man₉GlcNAc₂-PP-Dol was incubated with microsomal extracts in the absence of Dol-P-[¹⁴C]Glc (see Fig. 5A). This result indicates that under the conditions used mannosidases do not trim the acceptor oligosaccharide and that the products with the apparent size of mono- and diglycosylated product do not represent triglycosylated oligosaccharides, of which one or two mannose residues had been trimmed. Microsomal extracts from the patient’s fibroblasts were unable to transfer glucose onto Man₉GlcNAc₂-PP-Dol (Fig. 5D). In mixing experiments with microsomal extracts from fibroblasts of controls and the patient no inhibitory activity was noted. We conclude from these data that the patient’s fibroblasts are deficient in Man₉GlcNAc₂-PP-Dol glucosyltransferase activity.

Transfer of Nonglucosylated Oligosaccharides onto Nascent Glycoproteins. The presence of monoglucosylated oligosaccharides in newly synthesized glycoproteins (see Fig. 2) suggested that the failure to detect glucosyltransferase activity was caused by insufficient sensitivity of the assay procedure to detect low residual activity and that the residual glucosyltransferase activity present in fibroblasts is sufficient to ensure formation and transfer of Glc₃Man₉GlcNAc₂ oligosaccharides. To test this assumption, fibroblasts were metabolically labeled with [2-³H]mannose for 30 min in the presence of 5 mM N-methyldeoxynojirimycin, which inhibits the trimming of glucosidases I and II without affecting the synthesis of glucosylated forms of LLOs (25). The oligosaccharides were released from glycoproteins with PNGaseF and size-fractionated. In glycoproteins from both controls and the patient, Glc₃Man₉GlcNAc₂ oligosaccharides were the major species, and the relative frequency of the different oligosaccharides with no, one, two, or three glucose residues was comparable (Fig. 2, Lower). We therefore conclude that in the patient only glucosylated oligosaccharides are used for N-glycosylation in spite of the accumulation of Man₉GlcNAc₂-PP-Dol.

**DISCUSSION**
In fibroblasts of a patient with the clinical features and the serum transferrin pattern in isoelectric focusing and SDS/
PAGE characteristic of CDGS type I, accumulation of LLOs with oligosaccharides shortened by three hexose units was observed. Theoretically this finding could be explained by a lack of glucose and/or mannose residues in oligosaccharides of the composition Man9GlcNAc2 or Glc1–Man6–GlcNAc2. Because microsomal extracts from fibroblasts of controls but not of the patient were able to transfer glucose from Dol-P-Glc to a Man9GlcNAc2-PP-Dol acceptor, we ascribe the reduction in size to the accumulation of Man9GlcNAc2-PP-Dol. The UDP-Glc level and the activity of Dol-P-Glc synthase were normal in the patient’s fibroblasts. We conclude from these results that deficiency of Dol-P-Glc:Man9GlcNAc2-PP-Dol glucosyltransferase is the cause for the accumulation of the LLOs with truncated oligosaccharide units.

Deficiency of Dol-P-Glc:Man9GlcNAc2-PP-Dol glucosyltransferase represents an additional enzyme defect in CDGS. The current classification of CDGS is based on the isoelectric focusing pattern of serum transferrin, which distinguishes four types so far (9, 10, 26, 27). Although the isoelectric focusing pattern of the form described here is indistinguishable from that of CDGS type Ia and type Ib, we prefer to classify this additional form of CDGS as type V rather than as type Ic. This decision is based on the consideration that any enzyme defect that results in incomplete utilization of N-glycosylation sites, but permits normal processing of the glycoprotein-bound oligosaccharides, will produce the pattern of transferrin seen in CDGS type I. Three unrelated defects in enzymes controlling the de novo synthesis of mannose (phosphomannoisomerase), the formation of nucleotide-activated mannose (phosphomannomutase), or glucosylation of LLO have been shown to produce the CDGS type 1 transferrin pattern. It can be anticipated that many more enzyme defects will be detected than in complete utilization of N-glycosylation sites, but permits normal processing of the glycoprotein-bound oligosaccharides, will produce the pattern of transferrin seen in CDGS type I. Because the clinical phenotype can vary considerably as illustrated by CDGS types Ia and Ib, we propose to classify CDGS according to the gene product that is defective rather than to the isoelectric focusing pattern of serum transferrin.

Studies in porcine aorta indicated that each of the three glucose residues, of which the two inner ones are α1,3 linked and the outer one is α1,2 linked, is transferred onto the acceptor LLO by a separate glucosyltransferase (28). The accumulation of non-glucosylated LLOs suggests that the glucosyltransferase synthesizing Glc1Man9GlcNAc2-PP-Dol is missing in CDGS type V. Trace amounts of LLOs with two or three glucose residues were detectable in the patient's fibroblasts (see Fig. 3), indicating that the glucosyltransferase deficiency is not complete. This finding was further supported by an experiment, in which the deglucosylation of glycoprotein-bound oligosaccharides was inhibited by N-methyl-β-D-glucoside, an inhibitor of glucosidase I and glucosidase II in the endoplasmic reticulum. This inhibition resulted in the accumulation of mainly glucosylated oligosaccharides containing 3–5 glucose residues in glycoproteins from fibroblasts of both control and the patient.

The well-known preference of oligosaccharyltransferase for glucosylated over non-glucosylated forms of LLOs (11–16) easily explains that in the patient’s fibroblasts only glucosylated oligosaccharides are transferred onto nascent glycoproteins in spite of the accumulation of Man9GlcNAc2-PP-Dol. The decreased rate of glucosylation and the preferential utilization of the glucosylated LLOs for N-glycosylation is responsible for the low levels of Glc1Man9GlcNAc2-PP-Dol in the patient’s fibroblasts. The incomplete utilization of N-glycosylation sites as reflected in vivo by the pattern of serum transferrin forms and in vitro by a reduction of mannose incorporation into glycoproteins to about one-third of control demonstrates that the increase in Man9GlcNAc2-PP-Dol concentration cannot

Fig. 5. Dol-P-Glc:Man9GlcNAc2-PP-Dol glucosyltransferase. Microsomal extracts from fibroblasts of controls (A–C) and the patient (D) were incubated for 30 min in the presence of [3H]Man9GlcNAc2-PP-Dol and Dol-P-[14C]Glc. After extraction of LLOs, the oligosaccharides were released by mild acid hydrolysis and size-fractionated. (B and C) The column fractions were separately examined for 3H and 14C-radioactivity. (A) A control extract incubated in the absence of Dol-P-[14C]Glc. For standards M9, G1, G2, and G3 see Fig. 3.
compensate for the decrease in the glucosyltransferase activity.

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