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Research Article

Carbohydrate-deficient glycoprotein syndromes (CDGS) type I are a group of genetic diseases characterized by a deficiency of N-linked protein glycosylation in the endoplasmic reticulum. The majority of these CDGS patients have phosphomannomutase (PMM) deficiency (type A). This enzyme is required for the synthesis of GDP-mannose, one of the substrates in the biosynthesis of the dolichol-linked oligosaccharide Glc3Man9GlcNAc2. This oligosaccharide serves as the donor substrate in the N-linked glycosylation process. We report on the biochemical characterization of a novel CDGS type I in fibroblasts of four related patients with normal PMM activity but a strongly reduced ability to synthesize glucosylated dolichol-linked oligosaccharide leading to accumulation of dolichol-linked Man9GlcNAc2. This deficiency in the synthesis of dolichol-linked Glc3Man9GlcNAc2 oligosaccharide explains the hypoglycosylation of serum proteins in these patients, because nonglucosylated oligosaccharides are suboptimal substrates in the protein glycosylation process, catalyzed by the oligosaccharyltransferase complex. Accordingly, the efficiency of N-linked protein glycosylation was found to be reduced in fibroblasts from these patients.

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A Novel Carbohydrate-deficient Glycoprotein Syndrome Characterized by a Deficiency in Glucosylation of the Dolichol-linked Oligosaccharide

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Abstract

Carbohydrate-deficient glycoprotein syndromes (CDGS) type I are a group of genetic diseases characterized by a deficiency of N-linked protein glycosylation in the endoplasmic reticulum. The majority of these CDGS patients have phosphomannomutase (PMM) deficiency (type A). This enzyme is required for the synthesis of GDP-mannose, one of the substrates in the biosynthesis of the dolichol-linked oligosaccharide $Glc_3Man_9GlcNAc_2$. This oligosaccharide serves as the donor substrate in the N-linked glycosylation process. We report on the biochemical characterization of a novel CDGS type I in fibroblasts of four related patients with normal PMM activity but a strongly reduced ability to synthesize glucosylated dolichol-linked oligosaccharide leading to accumulation of dolichol-linked $Man_9GlcNAc_2$. This deficiency in the synthesis of dolichol-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide explains the hypoglycosylation of serum proteins in these patients, because nonglucosylated oligosaccharides are suboptimal substrates in the protein glycosylation process, catalyzed by the oligosaccharyltransferase complex. Accordingly, the efficiency of N-linked protein glycosylation was found to be reduced in fibroblasts from these patients. (*J. Clin. Invest.* 1998. 102:647–652.)
Key words: glycosylation • dolichol • oligosaccharyltransferase • endoplasmic reticulum

Introduction

Carbohydrate-deficient glycoprotein syndromes (CDGS)[1] are a group of genetic diseases mostly associated with major involvement of the central nervous system (1–3). Patients with these syndromes have a worldwide occurrence (4, 5). The diagnosis is mostly made by isoelectro focusing of serum transferrin showing an altered cathodal migration of transferrin due to undersialylation of the protein (6). Two distinct types of CDGS have been well characterized due to different glycosylation pattern of serum transferrin. The CDGS type II has been described in two patients and was found to be caused by a deficiency of the Golgi-processing enzyme *N*-acetylglucosaminyltransferase II (1, 4, 7, 8). The much more common type I CDGS is characterized by a partial or complete absence of entire glycans on glycoproteins (9–11), and it is assumed that hypoglycosylation of proteins is the cause for the diverse symptoms observed in CDGS type I patients. This reduced glycosylation efficiency is best explained by an abnormal biosynthesis of the dolichol-linked oligosaccharide, the substrate of the *N*-glycosylation process in the endoplasmic reticulum (12–14). The assembly of the dolichol-linked oligosaccharide occurs at the ER membrane, where nucleotide-activated and dolichol-linked sugars serve as substrates (15). Indeed, a deficiency in phosphomannomutase (PMM) activity, which is required for the biosynthesis of the sugar donor guanosine diphosphate (GDP)-mannose, was found in ~ 80% of CDGS type I patients, and a number of mutations in the PMM2 locus have been identified (16, 17). For other CDGS type I cases, a reduced polyprenol reductase activity was suggested to be the primary defect; however, a reduced phosphomannomutase activity was also observed in cells of these patients (18). Recently, the CDGS type Ib was described and assigned to a deficiency in phosphomannoisomerase, an enzyme required in GDP-mannose biosynthesis before phosphomannomutase (19).

In this report, we present biochemical data on four related patients with the CDGS type I serum sialotransferrin pattern but normal levels of PMM activity. The clinical symptomatology was milder as compared with CDGS patients with impaired PMM activity. Fibroblasts from all four patients showed a specific deficiency in the assembly of the dolichol-linked oligosaccharide, namely an impaired glucosylation of the dolichol-linked oligosaccharide leading to accumulation of dolichylpyrophosphate-linked $Man_9GlcNAc_2$.

Methods

Patients and cells. Fibroblasts and sera from four members of an inbred Dutch family (3 girls, 1 boy; 3–7 years of age), as well as from sex- and age-matched controls were studied. The most striking clini-

The contributions of the first two authors should be considered as equivalent.

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1. *Abbreviations used in this paper:* CDGS, carbohydrate-deficient glycoprotein syndromes; DMEM, Dulbecco's minimal medium; LLO, lipid-linked oligosaccharides; PMM, phosphomannomutase.

cal feature of these patients was a neurological involvement but much less pronounced as compared with previously reported CDGS patients. Detailed clinical features and laboratory data are reported elsewhere (de Rijk-van Andel, manuscript in preparation). Fibroblasts were cultivated in Dulbecco's minimal medium (DMEM/F12; Gibco BRL, Grand Island, NY) supplemented with 10% FCS.

Isoelectro focusing of serum transferrin. Isoelectro focusing of serum transferrin was performed as described (6).

Metabolic labeling of fibroblasts with [^{3}H]-mannose and preparation of dolichol-linked oligosaccharides. Subconfluent cultures of fibroblasts grown in DMEM/F12 medium with 5% dialyzed FCS on 24-cm plates were rinsed once with PBS and incubated for 90 min in Optimem medium (Gibco BRL, Grand Island, NY) supplemented with 0.5 mM glucose (low glucose), 5% dialyzed FCS, and 2 mM glutamine. After this incubation, cells were labeled with 150 μCi [^{3}*H*]-mannose (20 Ci/mmol; ICN Pharmaceuticals) for 60 min in the same low glucose medium. After removal of radioactive medium, cells were washed twice with ice-cold PBS and collected with a rubber policeman in 10 ml of ice-cold 0.1 mM Tris-HCl pH 7.4: methanol (8:3, vol/vol) (20). To the suspension, 10.9 ml chloroform was added; the mixture was extensively vortexed and centrifuged. Cells were collected at the interphase of the upper aqueous phase and the lower organic phase and resuspended in chloroform:methanol:water (10:10:3, vol/vol/vol). Extraction of dolichol-linked oligosaccharides was performed as described (21). The radiolabeled oligosaccharides were analyzed by HPLC (22). The different species of oligosaccharides were identified by comigration with defined oligosaccharides derived from specific yeast mutant strains, which accumulate characterized intermediates in the lipid-linked oligosaccharides (LLO) biosynthesis.

Phosphomannomutase assay. The phosphomannomutase assay was performed as described elsewhere (16).

Metabolic labeling of fibroblasts with [^{3}H]mannose and [^{35}S]methionine. Fibroblasts were cultured in DMEM/F12 medium with 5% dialyzed FCS in T75 flasks. Cells were metabolically labeled for 60 min with [^{3}H]mannose (16 μCi/ml) and [^{35}S]methionine (3 μCi/ml) in methionine-free MEM medium containing 0.5 mM glucose and 5% dialyzed FCS. The procedure to determine the incorporation rate of [^{3}H]-mannose and [^{35}S]methionine into protein has been described (12).

Results

Type I serum sialotransferrin pattern but normal phosphomannomutase activity in patient fibroblasts. Four children of healthy consanguineous parents presented moderate symptoms similar to the carbohydrate-deficient glycoprotein syndrome (de Rijk-van Andel, manuscript in preparation). Identical twin girls (patients J and N, at five years of age) and two other related children, patient R, a seven-year-old boy and his two-year-old sister L, were studied. In all four cases similar clinical symptoms were observed; however, much less pronounced neurological involvement as compared with previously reported CDGS patients was noted (de Rijk-van Andel, manuscript in preparation). We analyzed the electrophoretic mobility of serum transferrin in one of the patients (Fig. 1). In healthy individuals, the major transferrin species is the tetrasialo-transferrin with low amounts of trisialo and pentasialo-forms (lanes *1* and *5*; 9). In contrast, analysis of serum transferrin from patient J revealed that the disialo- (two) and tetrasialo-forms (four) were the most abundant glycoforms; low amounts of asialo-transferrin (zero) were also detected (Fig. 1, lane *2*). These alterations in the transferrin profile are very similar to those observed in a CDGS type I patient with a known deficiency in phosphomannomutase activity (Fig. 1, lane *3*). However, the pattern of serum transferrin is clearly different from

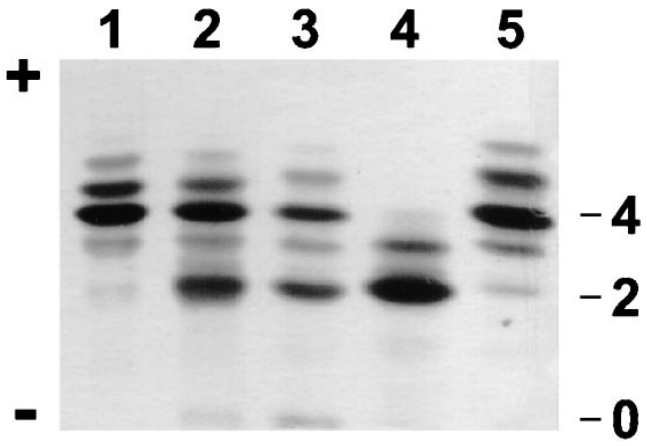

Figure 1. Isoelectro focusing of transferrin immunoisolated from serum of CDGS patients and healthy individuals. Transferrin from CDGS patients and healthy individuals was purified and saturated with iron, then subjected to isoelectro focusing electrophoresis and stained after electrophoresis as indicated in Methods. Lane *1* and *5*, transferrin from healthy individuals; lane *2*, one of the present patients (pt J); lane *3*, CDGS type I patient (phosphomannomutase deficiency); lane *4*, CDGS type II patient (*N*-acetylglucosaminyltransferase II deficiency). The numbers given at the right indicate the number of sialic acid residues present on each sialotransferrin fraction.

the one observed in serum from a CDGS type II patient with a defect in *N*-acetylglucosaminyltransferase II (Fig. 1, lane *4*; 8).

The hypoglycosylation of transferrin observed in a majority of CDGS type I patients is caused by a strongly reduced phosphomannomutase activity (3, 16, 17). We measured the PMM activity in leukocytes of one of the four patients (patient J: 2.3 mU/mg protein; see also Fig. 1, lane *2*) and compared it with the PMM activity found in leukocytes either from healthy individuals (range of 2.2–6.4 mU/mg protein) or CDGS type I patients with PMM deficiency (range of 0–1.4 mU/mg protein; CDGS type I patient analyzed in Fig. 1, lane *3*: 0.1 mU/mg protein). Due to the normal levels of PMM activity found, we conclude that these consanguineous patients present a novel type of carbohydrate deficient glycoprotein syndrome type I characterized by the hypoglycosylation of transferrin but normal levels of PMM activity.

Altered biosynthesis of the dolichol-linked oligosaccharide in patient fibroblasts. Hypoglycosylation of transferrin as well as the almost equal distribution of disialoform (two) and tetrasialoform (four) of transferrin and low amounts of asialotransferrin (zero) in the present patients is best explained by a reduced efficiency of N-linked glycosylation of proteins in the endoplasmic reticulum (9). A reduced efficiency of protein glycosylation is either due to a reduced oligosaccharyltransferase activity or an impaired synthesis of the dolichol-linked oligosaccharide substrate (as in the case of the CDG type I patients). To detect alterations in the biosynthesis of the dolichol-linked oligosaccharide, we have analyzed the composition of dolichol-linked oligosaccharides in fibroblasts derived from healthy individuals and from the patients. Cells were labeled with [^{3}H]mannose, dolichol-linked oligosaccharides were extracted, the oligosaccharides were released from the lipid car-

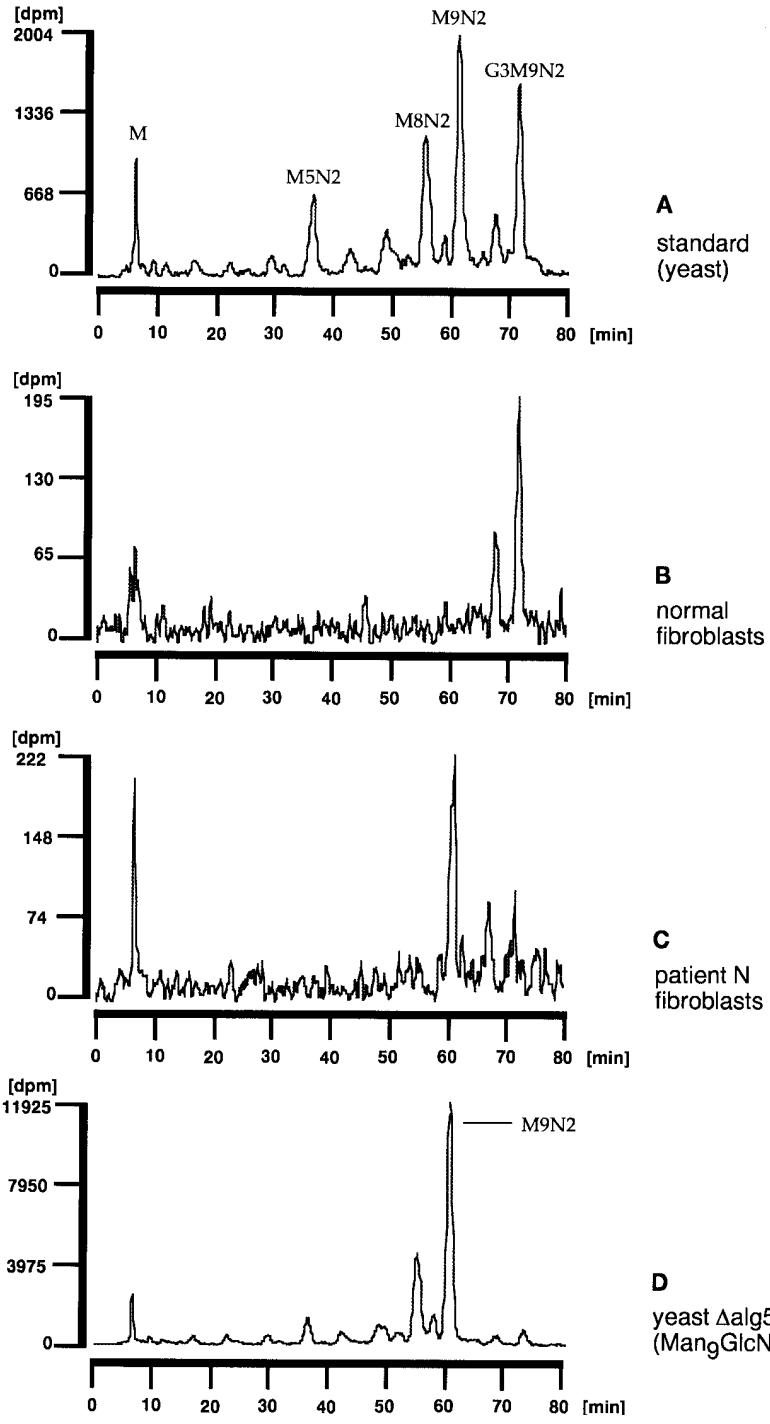

Figure 2. Patient derived fibroblasts synthesize incompletely assembled dolichol-linked oligosaccharide intermediate. Fibroblasts were labeled with 50 mCi/ml [^{3}H]mannose for 1 h; dolichol-linked oligosaccharides were isolated and the oligosaccharide portion analyzed by HPLC. Elution profiles recorded by a radioactivity flow detector are shown. Oligosaccharides derived from yeast wild-type cells (*A*) and yeast *Δalg5* cells accumulating $Man_9GlcNAc_2$ (*M9N2*) (*D*) served as standards. Dolichol-linked oligosaccharide derived from normal fibroblasts was essentially composed of $Glc_3Man_9GlcNAc_2$ (*B*), whereas LLO derived from fibroblasts of patient N was mainly in the form of $Man_9GlcNAc_2$ (*C*). The position of mannose (*M*), $Man_5GlcNAc_2$ (*M5N2*), $Man_8GlcNAc_2$ (*M8N2*), $Man_9GlcNAc_2$ (*M9N2*) and $Glc_3Man_9GlcNAc_2$ (*G3M9N2*) are indicated.

rier dolichylpyrophosphate and analyzed by HPLC. This type of analysis makes it possible to separate the individual biosynthetic intermediates of the dolichol-linked oligosaccharides. As markers for the different oligosaccharides, we used oligosaccharides derived from yeast strains deficient in defined steps of the assembly pathway. In such strains, the substrate of the affected reaction accumulates (23–26). In fibroblasts derived from healthy individuals, we detected predominantly the fully assembled dolichol-linked oligosaccharide consisting of three glucose-, nine mannose-, and two *N*-acetylglucosamine-residues ($Glc_3Man_9GlcNAc_2$; 15). Small amounts of an oligosaccharide lacking the terminal α-1,2-linked glucose ($Glc_2Man_9GlcNAc_2$) were also detected (Fig. 2 *B*). In contrast, the dolichol-linked oligosaccharides accumulating in fibroblasts from the present patients were different: a major fraction of the oligosaccharides consisted of $Man_9GlcNAc_2$, lacking the three terminal glucose residues (Fig. 2 *C* and Table I). This was confirmed by comparison of the retention time by using LLO from a *Δalg5* yeast strain lacking dolichylphosphoglucose synthase activity and therefore accumulating Man_9-

Table I. [^{3}H]Mannose Incorporation into Dolichol-linked Oligosaccharides in Fibroblasts Indicating the Relative Amounts of the Dolichol-linked Oligosaccharide Species

Subjects	Experiment	% M_8N_2	% M_9N_2	% $G_1M_9N_2$	% $G_2M_9N_2$	% $G_3M_9N_2$
Patient N	1	35	49	—	—	16
	2	30	70	—	—	—
	3	—	61	—	23	16
	4	19	60	—	—	21
Patient J	1	—	31	—	22	47
	2	—	43	—	—	57
Patient L	1	—	45	—	—	55
Patient R	1	—	44	—	23	33
Controls	1	—	—	—	21	79
	2	—	—	—	33	67
	3	—	—	—	35	65

$GlcNAc_2$ (26, 27; Fig. 2 *D*). As reported in Fig. 2 *C* and *D*, oligosaccharide species derive either from patient N or the *Δalg5* yeast strain had the same retention time. The accumulation of $Man_9GlcNAc_2$ was observed in the four patients analyzed; however, the relative amount of this oligosaccharide species was found to vary between assays of the cells from the same patient as well as between the cells derived from the four patients (Table I). Beside the predominant $Man_9GlcNAc_2$ oligosaccharide, we detected variable amounts of $Glc_3Man_9GlcNAc_2$ and $Glc_2Man_9GlcNAc_2$ oligosaccharides, suggesting only a partial deficiency in the assembly of the complete dolichol-linked oligosaccharide in these cells. But most importantly, we did not observe the accumulation of $Man_9GlcNAc_2$ oligosaccharide in non-CDGS cells (Table I). These results show that fibroblasts derived from the present patients have a strongly reduced capacity to glucosylate dolichol-linked oligosaccharides. This defect most likely explains the hypoglycosylation of glycoproteins in these patients because nonglucosylated oligosaccharides are suboptimal substrates for the oligosaccharyltransferase and are therefore transferred to proteins with a reduced efficiency (20, 28).

Altered efficiency of N-linked protein glycosylation in patient fibroblasts. To confirm our hypothesis that the altered biosynthesis of the LLO in the patients affects N-linked protein glycosylation, we measured the incorporation of [^{3}H]mannose into glycoproteins of fibroblasts derived from CDGS patients and control individuals. To determine the efficiency of N-linked protein glycosylation, we compared the incorporation of [^{3}H]mannose with that of [^{35}S]methionine into TCA-precipitable material (Fig. 3). Fibroblasts from patient N (glucosylation deficiency) and a PMM-deficient CDGS type I patient incorporated significantly less mannose into proteins as compared with control fibroblasts. Incorporation of [^{3}H]mannose into glycoproteins is affected more strongly in PMM-deficient CDGS type I cells (fivefold reduction) as compared with fibroblasts derived from the glucosylation-deficient patients (threefold reduction). As reported previously (12, 14), we observed a strongly reduced incorporation of [^{3}H]mannose into LLO in PMM-deficient cells (data not shown). However, the extent of [^{3}H]mannose incorporation into LLO was not significantly affected in the fibroblasts of glucosylation-deficient patient N (Fig. 2). We conclude that the reduced incorporation of [^{3}H]mannose into glycoproteins in glucosylation-deficient CDGS fibroblasts is due to a reduced transfer rate of the oligosaccharide from the lipid carrier dolichylpyrophosphate to protein. These results support our hypothesis that the hypoglycosylation of glycoproteins in this novel CDGS type I is the result of impaired glucosylation in LLO biosynthesis.

Discussion

The patients studied represent a novel subtype of the CDGS type I. As described for other cases of CDGS type I, an altered N-linked glycosylation process was observed in these patients. This alteration is reflected by a specific hypoglycosylation pattern of serum transferrin. A similar distribution of transferrin glycoforms is also observed in CDGS type I patients affected in PMM activity and is compatible with the hypothesis that hypoglycosylation of proteins results from a reduced efficiency of the N-linked glycosylation process in the endoplasmic reticulum. In contrast to patients with CDGS type I syndromes, normal PMM activity was observed in the patients analyzed in this study. Therefore, we determined the composition of dolichol-linked oligosaccharides in normal and patient fibroblasts and found that the intermediate $Man_9GlcNAc_2$ of the dolichol-linked oligosaccharide biosynthesis accumulated in all four different patient cell lines tested. The accumulation of this biosynthetic intermediate was not detected in control fibroblasts. However, we also observed low and variable amounts of complete $Glc_3Man_9GlcNAc_2$ oligosaccharide in the patient-derived cells lines, suggesting a partial deficiency in the synthesis of glucosylated oligosaccharide. A variability in the relative amount of dolichol-linked oligosaccharide species has been observed before (12, 14); however, no explanation to account for this observation could be given. A defective biosynthesis of the dolichol-linked oligosaccharide explains the hypoglycosylation phenotype observed in these patients. As proposed for the PMM-deficient CDGS type I patients, incomplete dolichol-linked oligosaccharide is assembled in the ER and serves

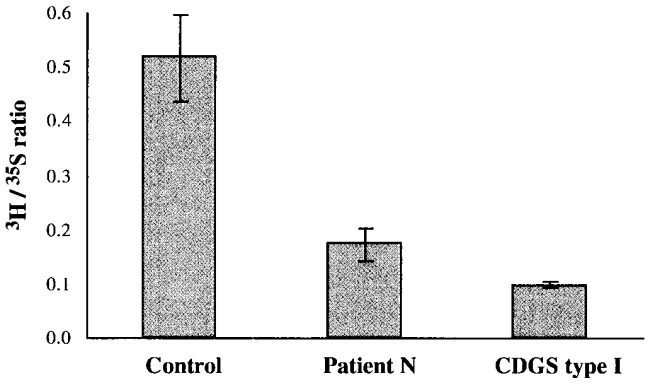

Figure 3. Incorporation of [^{3}H]mannose and [^{35}S]methionine into protein. Incorporation of radioactivity in TCA-insoluble material was determined in fibroblasts after metabolic labeling for 60 min. The ratio of [^{3}H] vs. [^{35}S] incorporation is shown. The mean value of at least three independent experiments is given. Standard deviations are indicated by vertical bars.

as a suboptimal substrate for the oligosaccharyltransferase reaction (12, 13). Studies both in higher eukaryotic cell lines (20, 28, 29) and in yeast (23–26, 30) have shown that incompletely assembled dolichol-linked oligosaccharide can be transferred to protein, albeit with reduced efficiency. Indeed, we observe a reduced [^{3}H]mannose incorporation into glycoproteins in both glucosylation-deficient and PMM-deficient CDGS type I fibroblasts. This low transfer efficiency results in underglycosylated proteins producing a phenotype also observed in CDGS patients.

At present, we can only speculate about the primary defect in these patient cells. In yeast, both a deficiency in the Dol-P-Glc (Alg5p) synthesizing enzyme or in the first Dol-P-Glc–dependent glucosyltransferase (Alg6p; 24, 26, 27) lead to accumulation of dolichol-linked $Man_9GlcNAc_2$. Determination of these two enzyme activities or the isolation of the corresponding genes will make it possible to define the primary defect in the patient cells. Proteins involved in the assembly of the dolichol-linked oligosaccharide are highly conserved. This will allow us to isolate the appropriate human genes by using the information obtained from the homologous yeast loci.

In CDGS type I derived cells, the glycosylation of many different glycoproteins is affected (11), and for many glycoproteins, the carbohydrate moiety is essential for their activity (31). In line with this hypothesis, we observed that in glucosylation-deficient fibroblasts, the activity of the Golgi-localized α-2,6 sialyltransferase is significantly reduced (Borsig, L., and E.G. Berger, unpublished observations). Indeed, glycosylation of α-2,6 sialyltransferase was reported to be essential for enzymatic activity (32).

It is interesting to note that the PMM-deficient CDGS type I patients and the patients of this study differ in their clinical symptoms; however, the hypoglycosylation of serum proteins as visualized for transferrin is similar (see Fig. 1). Our results offer an explanation to account for this observation. A PMM deficiency leads to reduced biosynthesis of GDP-mannose, which not only affects the process of N-linked glycosylation in the ER but also the biosynthesis of GDP-fucose, the donor substrate for the Golgi-localized fucosyltransferases, as well as the biosynthesis of the GPI anchor, where four mannose residues are required to build up the glycolipid anchor (33). In contrast, a deficiency in glucosylation of the dolichol-linked oligosaccharide does only affect the N-linked glycosylation process and possibly the quality control mechanism of *N*-glycoprotein folding (34). The recently identified CDGS type Ib, which is caused by a deficiency in phosphomannose isomerase activity, again differs in the clinical symptoms observed in such patients but also results in a similar hypoglycosylation of glycoproteins (19). The variety of clinical representations of deficiencies in the N-linked glycoprotein biosynthesis suggests that other disorders might be caused by hypoglycosylation. An extended screening of glycosylation deficiencies might lead to their detection.

Acknowledgments

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