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A Novel Disorder of N-Glycosylation Due to Phosphomannose Isomerase Deficiency

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Three siblings suffered from an unusual disorder of cyclic vomiting and congenital hepatic fibrosis. Serum transferrin isoelectric focusing showed increased asialo- and disialotransferrin isoforms as seen in the carbohydrate-deficient glycoprotein (CDG) syndrome type I. Phosphomannomutase, which is deficient in most patients with type I CDG syndrome, was found to be normal in all three patients. Structural analysis of serum transferrin revealed nonglycosylated, hypoglycosylated, and normoglycosylated transferrin molecules. These findings suggested a defect in the early glycosylation pathway. Phosphomannose isomerase was found to be deficient and the defect was present in leucocytes, fibroblasts, and liver tissue. Phosphomannose isomerase deficiency appears to be a novel glycosylation disorder, which is biochemically indistinguishable from CDG syndrome type I. However, the clinical presentation is entirely different.

Key Words: **cyclic vomiting; congenital hepatic fibrosis; carbohydrate-deficient glycoproteins; CDG syndrome; phosphomannose isomerase deficiency.**

Carbohydrate-deficient glycoprotein (CDG) syndromes are a group of inherited multisystemic disorders with hypoglycosylation of glycoproteins. The disorder was first described by Jaeken et al (1). At least four subtypes have been recognized on the basis of different isoelectric focusing patterns of serum transferrin (2). CDG syndrome type I is the most frequent form and patients are characterized by dysmorphism, severe neurological features and organ dysfunction. In the majority of patients with CDG syndrome type I phosphomannomutase (PMM) was deficient (3). Mutations have been reported in a PMM gene located on chromosome 16p13 (4).

The biosynthesis of oligosaccharide chains of glycoproteins is a complex process, starting with the building of an oligomannose structure. These mannoses originate from mannose-6-phosphate, which is produced from glucose via a series of reactions including hexokinase, glucose-phosphate isomerase, phosphomannose isomerase and phosphomannomutase. Phosphomannomutase deficiency causes a disturbance of the biosynthesis of GDP-mannose and this subsequently leads to a reduced N-glycosylation of proteins (Fig. 1).

We describe a family with three sibs known with cyclic vomiting with onset in the newborn period and congenital hepatic fibrosis. Hypoglycosylation of serum transferrin was present with a predominance of asialo- and disialotransferrin identical to CDG syndrome type I. However, phosphomannomutase activity was normal. It could be demonstrated that another enzyme in the fructose-mannose interconversion pathway, viz phosphomannose isomerase, was deficient in these patients.

PATIENTS, MATERIALS, AND METHODS

Case Histories

The patients were from a consanguineous Turkish family, the parents were first-cousins and had themselves unremarkable medical histories. Three sibs in this family were affected by the disorder; one girl was without symptoms.

Abbreviations used: ALAT, alanine aminotranfserase; ASAT, aspartate aminotransferase; CDG, carbohydrate-deficient glycoprotein; PMI, phosphomannose isomerase; PMM, phosphomannomutase.

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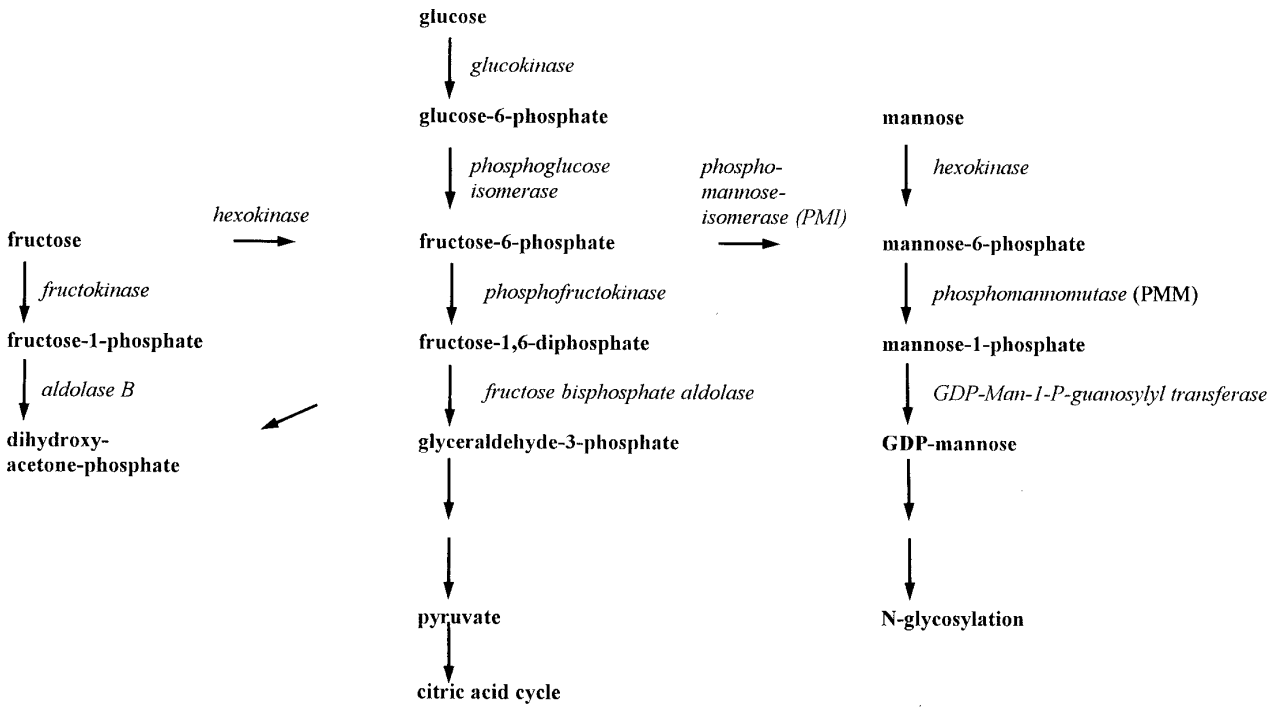

FIG. 1. Early steps in the interconversion of fructose, glucose and mannose.

Case 1. This patient was a 19-year-old girl. Initial presentation was at the age of 10 months with vomiting, diarrhea and dehydration. She was admitted to hospital 24 times in 19 years because of episodic vomiting, sometimes associated with diarrhea leading to dehydration. Physical examination during attacks revealed signs of dehydration and a mildly enlarged liver, and no other abnormalities. Routine laboratory examination showed persistently low albumin (19-34 g/L, normal 35-45) and elevated aminotransferases during attacks (ASAT 42-493 u/L, normal 15-35, ALAT 45-403 u/L, normal 15-35). There were no signs of renal or gastro-intestinal loss of protein. On one occasion at the age of 11 years she presented with severe liver failure. A liver biopsy at the age of 14 years showed minimal histological abnormalities consistent with 'ductal plate malformation.'

Case 2. This patient was an 18-year-old girl, presented at the age of 2 months an acute unexplained episode of multiorgan failure. After the initial presentation the girl was admitted 8 times in 18 years with episodes of persistent vomiting sometimes associated with diarrhea leading to dehydration. Physical examination invariably showed mild to moderate signs of dehydration, a mildly enlarged liver and no other abnormalities. Routine laboratory examination showed persistently low albumin (30-34 g/L) and elevated aminotransaminases in periods of attacks (ASAT 46-182 u/L, ALAT 45-191 u/L). At the age of 18 months a liver biopsy was taken and showed abnormalities consistent with congenital hepatic fibrosis. At the age of 18 years she appeared to suffer from a chronic active hepatitis B infection.

Case 3. This patient was a 14-year-old male. Symptoms started at the age of 10 months with persistent vomiting. After this first episode he was found to have recurrent episodes of vomiting sometimes associated with diarrhea leading to dehydration. For each episode he had to be admitted to hospital for rehydration therapy, a total of 13 admissions in 14 years. Laboratory investigations during attacks showed isotonic or hypotonic dehydration and moderately elevated aminotransaminases (ASAT 27-108 u/L, ALAT 16-218 u/L). Albumin was consistently low, also in symptom free periods (albumin 23-31.5 g/L). A liver biopsy at the age of 3 years showed the histological features of congenital hepatic fibrosis.

The mental and motor development of the three patients were entirely normal; they did not have dysmorphic features or unusual fat distribution. The healthy girl in this family never had any clinical problems necessitating hospitalisation.

Isoelectric Focusing

Isoelectric focusing of serum transferrin was run in PhastSystem (Pharmacia LKB, Uppsala, Sweden) with Phast Gel IEF 4-6.5 (5).

Sample Preparation

Serum or plasma (20 μl) were mixed with 2 μl 10 mM Fe (III)-citrate, 2 μl 0.5 M $NaHCO_3$ solution and 60 μl 10 mM $NaHCO_3$ solution. This mixture was incubated for 2 hours at room temperature. Before analysis the mixture was centrifuged in an Eppendorf centrifuge at 13,000 $\times$ g for 10 min.

Visualization of the Isotransferrins

For the visualization of the separated transferrin bands 40 μl anti-transferrin immunoglobulin (Dako, Copenhagen, Denmark) was diluted with 40 μl 0.9% NaCl solution. The diluted anti-transferrin was spread over the surface of the gel immediately after the end of the isoelectric focusing run. After 2 hours incubation at 4°C the reaction was stopped by soaking the gel in 0.9% NaCl solution; the latter solution was refreshed three times. The gel was left overnight in this solution while gently shaking. Fixation of the bands was carried out by soaking the gel in 12% trichloroacetic acid in water (w/v). Staining of the bands was performed with 0.1% Coomassie Brilliant Blue R250 (Sigma, St. Louis, USA) solution in ethanol/acetic acid (0.29 gram Coomassie Brilliant Blue in 250 ml of a mixture ethanol/acetic acid/water, prepared by mixing 350 ml ethanol and 100 ml acetic acid glacial followed by dilution with water to 1000 ml).

Structural Analysis of Transferrin

Isolation and desalting. Transferrin fractions were isolated according to van Noort *et al.* (6). The samples were desalted by gel filtration chromatography on a HiTrap column (3x, Pharmacia) using an FPLC system with water as eluent at a flow rate of 3 ml/min and UV detection at 214 nm. The void volume fraction was collected and lyophilized.

Monosaccharide analysis. Monosaccharide composition analysis was carried out by gas chromatography (GLC) on a CP Sil 5CB column (25m × 0.32 mm, Chrompack, Middelburg, The Netherlands), using a Chrompack CP 9002 gas chromatograph (temperature program: 140 − 240°C at 4°C/min) and flame-ionization detection (7).

Trimethylsilylated methyl glycosides were prepared by methanolysis (1.0 M HCl/methanol, 24 h, 85°C), re-N-acetylation (acetic anhydride, 24 h, room temperature) and trimethylsilylation (pyridine/HMDS/TMCS = 5:1:1, 30 min. room temperature).

Electrospray-Mass Spectrometry

Positive-ion electrospray-mass spectrometry (ES-MS) spectra of 5 μl samples (~6-10 pmol/μl 0.1% formic acid) were recorded on a MicroMass Platform ES1$^+$ mass spectrometer with a capillary voltage of 2.5-4 kV and a cone voltage of 60 V. Scanrange 1600 − 3000 m/z. Calibration was done with myoglobin, M = 16951; scan range 600-2200 m/z.

Phosphomannose Isomerase and Phosphomannomutase Assays

Phosphomannose isomerase (PMI; E C 5.3.1.8) and phosphomannomutase (PMM; E C 5.4.2.8) were determined in stopped assays derived from the kinetic method of Van Schaftingen en Jaeken (8); the [NADP$^+$] was increased to 0.6 mM and for PMM the [mannose-1-P] was increased to 0.4 mM, reaction mixtures were incubated for 1 h at 37°C and stopped with 0.5 M sodium carbonate/bicarbonate buffer pH 10.7 (25 and 20 μl, respectively). PMI reaction mixtures (200 μl) contained two different amounts of protein (for leucocytes: 40 and 20 μg; for fibroblasts and liver :20 and 10 μg). For PMM the incubation volume was 125 μl and the amount of protein was 1.25 times higher.

RESULTS

Isoelectric focusing of serum transferrin of the three cases showed abnormal patterns, and normal patterns for the parents and the unaffected sib (Fig. 2). For cases 1, 2, and 3 the amount of tetrasialotransferrin (tTf) was reduced whereas disialotransferrin (dTf) and zerosialotransferrin (zTf) were strongly increased. The patterns of the isoforms of transferrin in the plasmas of the cases 1 and 3 were identical to that of CDG type I. The pattern found in case 2 showed less predominance of asialo and disialotransferrin. The percentual compositions of the various transferrin isoforms in plasma of the three patients, that of the parents, a healthy sib, two normal controls and of a CDG type I patient are depicted in Table I.

From blood of case 1 three isoforms of transferrin were isolated on a preparative scale *viz* tetrasialotransferrin (tTf), disialotransferrin (dTf) and zerosialotransferrin (zTf).The results of monosaccharide composition

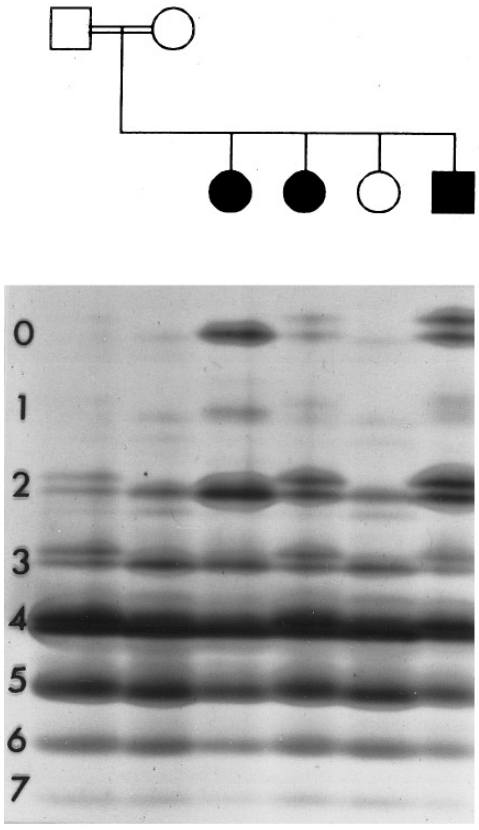

FIG. 2. Transferrin isoelectric focusing of the parents, the three cases and the healthy sib.

analysis of the three isoforms of transferrin are given in Table II.

From this composition analysis one can conclude that zTf does not contain oligosaccharide chains. The data of dTf and tTf are in accordance with biantennary sugar chains. Investigation by electrospray-mass spectrometry revealed the following molecular masses of the isoforms tTf, dTf and zTf: 79,573, 77,368 and 75,164, respectively. The differences in mass between tTf and dTF and between dTf and zTf are 2205 and 2204, respectively. This is in accordance with the mass of a disialobiantennary sugar chain (9). This means that tTf carries two sialylated biantennary sugar chains; dTf carries one sialylated biantennary chain and zTf is nonglycosylated, as is also observed in plasma of CDG type I patients. This was suggestive of a defect in one of the early steps of N-glycosylation, possibly an inability to form the oligomannoside chains.

Phosphomannomutase activity was normal in leucocytes of all patients and in a liver biopsy of two patients. Phosphomannose isomerase was found to be deficient in all three patients (Table III). The deficiency could be demonstrated in leucocytes, fibroblasts and liver tissue of the patients. The patients had considerable residual activity in all tissues investigated. The parents

TABLE I

Isoelectric Focusing of Serum Transferrins; Percentual Compositions of the Various Transferrin Isoforms

Transferrin isoform	Control 1	Control 2	CDG type I	Case 1	Case 2	Case 3	Father	Mother	Healthy sib
7-sialo Tf	<1	<1	<1	<1	2	<1	<1	<1	<1
6-sialo Tf	6	3	3	2	7	1	3	3	5
5-sialo Tf	22	20	14	11	20	8	18	17	19
4-sialo Tf	51	63	33	40	42	45	64	60	54
3-sialo Tf	14	10	7	7	10	8	9	14	14
2-sialo Tf	7	4	27	27	14	28	3	4	5
1-sialo Tf	<1	<1	2	2	1	2	<1	<1	<1
0-sialo Tf	<1	<1	13	11	3	8	<1	<1	<1

Note. The patterns of cases 1 and 3 were indistinguishable from that of a patient with phosphomannose mutase deficiency (CDG syndrome type I). In case 2 the asialo and disialo isoforms were less prominent as compared to her sibs. No differences were observed between controls and heterozygotes.

showed enzyme activities in leucocytes below the control range, compatible with heterozygosity for the enzyme defect. The healthy sib showed normal activity.

DISCUSSION

The patients in the present report presented with an unusual disorder with cyclic vomiting, hepatomegaly and the histological abnormalities of congenital hepatic fibrosis. Cyclic vomiting is a clinical syndrome with multiple episodes of nausea and vomiting leading to dehydration (10). Some metabolic disorders were found in patients with complaints of cyclic vomiting, but none of these conditions could be detected in our cases (11). Congenital hepatic fibrosis can be associated with a large number of genetic syndromes of which cystic kidney disease is most frequent (12). None of these disorders were present in our cases. Hypoglycosylation of proteins was present in the three affected sibs as demonstrated by the abnormal transferrin isoelectric focusing. Transferrin isoelectric focusing in case 2 revealed a somewhat different pattern as compared to her sibs. This could be the result of her hepatitis B infection.

TABLE II

Carbohydrate Composition of the Oligosaccharide Chains of the Various Sialotransferrins Isolated from the Serum of Case 1

Monosaccharide	tTf	dTf	zTf
Fucose	Trace	Trace	—
Mannose	3.0[a]	3.0[a]	Trace
Galactose	1.9	2.1	—
N-Acetylglucosamine	2.7 (+1)	2.6 (+1)	Trace
N-Acetylneuraminic acid	2.0	1.8	—

Note. Virtually no carbohydrate was detected in the asialo transferrin (zTf) whereas a normal distribution was observed in disialotransferrin (dTf) and tetrasialotransferrin (tTf).

[a] Calculation based on 3 mol of mannose/mol oligosaccharide chain.

Transferrin isoelectric focusing was performed because of persistently low albumin, without signs of renal or gastrointestinal loss of albumin. Low albumin is a characteristic feature of the CDG syndrome (13). Abnormal or hypoglycosylation can be due to a primary glycosylation defect or is secondary to liver dysfunction or a metabolic disorder as galactosemia or hereditary fructose intolerance (14, 15). The predominance of asialo- and disialotransferrin as in our cases is identical to CDG syndrome type I. The clinical phenotype of CDG syndrome type I comprises severe neurological abnormalities, dysmorphism and organ dysfunction (13). In the majority of these patients phosphomannomutase (PMM) is deficient causing a defect in the early mannose pathway (3). However, phosphomannomutase activity was normal in our cases. Therefore structural analysis of three isoforms of transferrin was performed. This showed nonglycosylated, hypoglycosylated and normoglycosylated transferrin identical to CDG syndrome type I. This suggested a defect in the early glycosylation pathway and phosphomannose isomerase (PMI) was found deficient. This enzyme catalyzes the reaction from fructose-6-phosphate to mannose-6-phosphate as shown in Figure 1. A deficiency of phosphomannose isomerase will lead to compromised GDP-mannose formation, causing hypoglycosylation. Our data indicate that the enzyme deficiency was clearly present in different tissues such as fibroblasts, leucocytes and liver tissue. Although a deficiency of phosphomannose isomerase causes identical biochemical abnormalities as in CDG syndrome type I, the clinical manifestations were very different. PMI deficiency can not be discriminated from PMM deficiency by transferrin isoelectric focusing and therefore, direct enzyme assays are needed for a correct diagnosis.

PMI deficiency has been reported in yeast which gives rise to a conditional lethal phenotype due to glycosylation defects (16). These mutants are unable to secrete wall-associated proteins. Addition of D-mannose

TABLE III

Activities of Phosphomannose Isomerase (PMI) and Phosphomannomutase (PMM) in Various Tissues

	PMI (nmol/h/mg protein)			PMM (nmol/h/mg protein)	
	Leucocytes	Fibroblasts	Liver	Leucocytes	Liver
Case 1	120	250	70	252	230
Case 2	130		130	228	320
Case 3	180			221	
Healthy sib	1610			286	
Father	710			221	
Mother	680			228	
Control range	860–1800 n = 15	1370–2500 n = 13	1550–1770 n = 3	160–290 n = 9	150–710 n = 3

led to a correction of the secretion defect by restoring glycosylation. Treatment with mannose infusion in a patient with phosphomannomutase deficiency has been attempted, but was not very effective in correcting the biochemical abnormalities (17). However, in phosphomannose isomerase deficiency GDP-mannose can still be synthesized from mannose (Figure 1). Mannose is converted to mannose-6-phosphate by hexokinase and the latter compound will lead to GDP-mannose formation. This may be of use in the treatment of patients with PMI deficiency and correcting the clinical and biochemical abnormalities.

The disorder described here has many similarities with the clinical symptoms of hereditary fructose intolerance (fructose-1-P-aldolase def.). Untreated fructose intolerance causes defective glycosylation (15). In our cases 1 and 2 fructose-1-phosphate-aldolase activity was normal in liver (data not shown). One may speculate that tissue-specific expression of glycosylation defects causes different phenotypes and that in the case of phosphomannose isomerase deficiency the defect is mainly expressed in tissues with specific fructose pathway as liver and intestine. It was shown by Jaeken et al. (18),) that accumulation of fructose-1-P inhibits phosphomannose isomerase, explaining the hypoglycosylation in fructose intolerance. Defective glycosylation can be a common factor in the pathogenesis of both fructose intolerance and the symptoms of cyclic vomiting in our cases.

In conclusion, we present three patients with an unique disorder of cyclic vomiting, congenital hepatic fibrosis and carbohydrate-deficient glycoproteins, due to a novel enzyme deficiency of phosphomannose isomerase.

Note: When preparing the manuscript we noticed an abstract about phosphomannose isomerase deficiency associated with protein losing enteropathy (T. Marquart *et al., Eur. J. Pediatr.* **157,** 174, 1998).

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