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High Residual Activity of PMM2 in Patients' Fibroblasts: Possible Pitfall in the Diagnosis of CDG-Ia (Phosphomannomutase Deficiency)

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Congenital disorders of glycosylation (CDGs) are a rapidly enlarging group of inherited diseases with abnormal N-glycosylation of glycoconjugates. Most patients have CDG-Ia, which is due to a phosphomannomutase (PMM) deficiency. In this article, we report that a significant portion (9 of 54) of patients with CDG-Ia had a rather high residual PMM activity in fibroblasts included in the normal range (means of the controls ± 2 SD) and amounting to 35%–70% of the mean control value. The clinical diagnosis of CDG-Ia was made difficult by the fact that most (6 of 9) of these patients belong to a subgroup characterized by a phenotype that is milder than classical CDG-Ia. These patients lack some of the symptoms that are suggestive for the diagnosis, such as inverted nipples and abnormal fat deposition, and, as a mean, had higher residual PMM activities in fibroblasts (2.05 ± 0.61 mU/mg protein, $n = 9$; vs. controls 5.34 ± 1.74 mU/mg protein, $n = 22$), compared with patients with moderate (1.32 ± 0.86 mU/mg protein, $n = 18$) or severe (0.63 ± 0.56 mU/mg protein, $n = 27$, $P < .001$) cases. Yet they all showed mild mental retardation, hypotonia, cerebellar hypoplasia, and strabismus. All of them had an abnormal serum transferrin pattern and a significantly reduced PMM activity in leukocytes. Six of the nine patients with mild presentations were compound heterozygotes for the C241S mutation, which is known to reduce PMM activity by only ~2-fold. Our results indicate that intermediate PMM values in fibroblasts may mask the diagnosis of CDG-Ia, which is better accomplished by measurement of PMM activity in leukocytes and mutation search in the *PMM2* gene. They also indicate that there is some degree of correlation between the residual activity in fibroblasts and the clinical phenotype.

Introduction

An estimated 300 patients worldwide are known to have congenital disorders of glycosylation type Ia (CDG-Ia [PMM deficiency, MIM 601785]), the most frequent type among the CDGs (Van Schaftingen and Jaeken 1995; Jaeken et al. 1997*a;* Matthijs et al. 1997, 1999, 2000). Often patients can be diagnosed in the neonatal or early infantile period on the basis of a typical morphology (inverted nipples and fat pads), strabismus, axial hypotonia, and hyporeflexia. Recurrent elevated transaminases and failure to thrive often complicate the clinical course (Hagberg et al. 1993; Petersen et al. 1993; Jaeken et al. 1997*b*). A very common feature is cerebellar hypoplasia, which can be documented at or shortly after birth (Jaeken and Carchon 1993; Akaboshi et al. 1995; Jensen et al. 1995). There is a substantial childhood mortality of ~20%, due to severe infections or organ failure (Hagberg et al. 1993; Petersen et al. 1993; Hutchesson et al. 1995; Garcia Silva et al. 1996; van der Knaap et al. 1996; Kristiansson et al. 1998; Matthijs et al. 2000).

At a later age, the impairment of the neurological system becomes more evident, with a variable degree of mental retardation, cerebellar dysfunction, and retinitis pigmentosa (Andreasson et al. 1991; Fiumara et al. 1994; Di Rocco et al. 2000). Some children develop seizures and/or strokelike episodes, probably because of the particular coagulopathy with marked decrease of factor XI, antithrombin, and proteins C and S (Okamoto et al. 1993; Van Geet and Jaeken 1993; Stibler et al. 1996). In adulthood, nonprogressive ataxia, stable mental retardation, and peripheral neuropathy are the main characteristics of the disease (Stibler et al. 1994; Barone et al. 1999). Most patients are wheelchair bound (Stibler et al. 1994). Electrophysiological investigations document decreased sensory and motor nerve–conduction velocities and abnormal somatosensory, auditory, and visual evoked potentials (Itoh et al. 1993; Barone et al. 1999). Adult female patients typically present with hypergonadotropic hypogonadism (Ohzeki et al. 1993; de Zegher and Jaeken 1995; Kristiansson et al. 1995).

The clinical suspicion of CDG can be substantiated by isoelectric focusing (IEF) of serum transferrin (Jaeken

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et al. 1984; Stibler and Jaeken 1990). Initially, IEF of serum transferrin and PMM measurements in fibroblasts were performed mainly in patients presenting with a "classical" and typically severe clinical picture of CDG-Ia (Van Schaftingen and Jaeken 1995), and often a profound PMM deficiency was documented (Kjaergaard et al. 1998; Imtiaz et al. 2000). Thanks to increasing awareness of CDGs and broader screening for N-glycosylation defects, the number of patients with a less typical presentation is growing, including children with a much milder clinical presentation and nearly normal psychomotor development (Di Rocco et al. 2000; van Ommen et al. 2000). Because we identified among these cases patients with high residual PMM activity, we decided to look for mutations in the *PMM2* gene in all patients who had a type 1 pattern in IEF and clinical symptoms suggestive of CDG-Ia. The aims of the study were to determine the percentage of patients with high to normal residual PMM2 activity in fibroblasts and to determine whether some cases could not be better diagnosed by PMM assays in leukocytes rather than in fibroblasts. After careful analysis of the clinical data, a correlation between the severity of clinical expression and residual PMM activity became apparent, to the extent that we have been able to identify the key symptoms that best allow the identification of CDG-Ia (very mild to severe cases) in patients. A comparison of the genotype was made, to find out whether the high PMM residual activities are typically associated with particular mutations.

Subjects and Methods

Subjects

Among 150 patients with a clinically suspected CDG and a CDG type 1 pattern in serum transferrin IEF, the diagnosis of CDG-Ia was confirmed in 50 patients by identification of PMM deficiency in fibroblasts and by mutation analysis. In the remaining patients, the clinical data were reviewed and 14 patients with a clinical picture compatible with CDG-Ia were identified. In spite of PMM activities in fibroblasts within the normal range in four of these patients, the diagnosis was confirmed by the identification of *PMM2* mutations. Thus, a total of 54 patients were included in this study. Clinical data were collected via a questionnaire (including family and patient history, biochemical data, and clinical symptoms), by literature review, and through personal communication. In addition, PMM values were measured in patients' and parents' leukocytes, if available.

Isoelectrofocusing of Serum Transferrin

Analysis of transferrin isoforms was performed in serum and dried blood spots by IEF as described elsewhere (Stibler and Cederberg 1993; de Jong et al. 1994).

PMM Measurement in Fibroblasts and Leukocytes

PMM was measured in fibroblasts and leukocytes as described elsewhere (Jaeken et al. 1997*a*). The means of the three groups were compared by ANOVA analysis, followed by a post hoc test for pairwise comparison, using the Bonferroni correction for multiple testing.

Mutation Studies

Genomic DNA was isolated from fibroblasts or white blood cells by means of standard laboratory procedures. Mutations were identified by a combination of SSCP analysis and direct sequencing, as described elsewhere (Matthijs et al. 1998*b*).

Results

Figure 1 shows the PMM values in fibroblasts from 54 patients with CDG-Ia and from 22 control subjects. There is obviously a wide scatter of values in both groups, with overlap. If the mean control value (5.34 mU/mg protein) ± 2 SD (2 × 1.74 mU/mg protein) is taken to define the normal range, CDG-Ia would not have been diagnosed in as many as 9 of 54 patients. In four cases the PMM values were 35%, 41%, 62%, and 70% of the mean control value, clearly within the normal range. These patients received diagnoses only when we decided to search for mutations in patients with high PMM values.

Figure 2 shows the values of the PMM activities in leukocytes. There is no overlap between the values for the control subjects and for patients with CDG-Ia. In particular, the patients with high residual activities in fibroblasts displayed activities in leukocytes that were

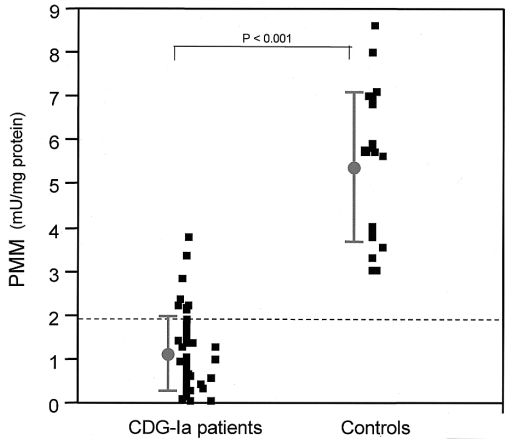

Figure 1 Phosphomannomutase values measured in fibroblasts of CDG-Ia patients ($n = 54$) and control subjects ($n = 22$). The mean value and SD (5.34 ± 1.74 mU/mg protein) in the control group is indicated. The scattered line gives the mean −2 SD (1.86 mU/mg protein).

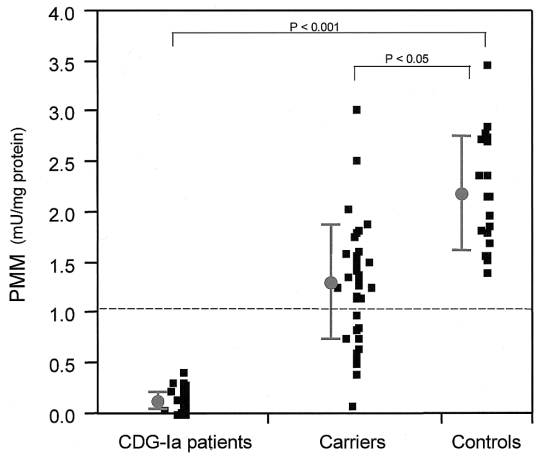

Figure 2 Phosphomannomutase values measured in leukocytes of patients with CDG-Ia ($n = 37$), heterozygotes ($n = 38$), and control subjects ($n = 19$). The mean value and standard deviation (SD) (2.19 ± 0.57 mU/mg protein) in the control group is indicated. The scattered line reflects the mean −2 SD (1.05 mU/mg protein).

in the range of 0.01–0.32 mU/mg protein (>3 SD below the mean control value) (see table 1). Figure 2 also shows the distribution of the values of the obligate heterozygotes (parents of CDG-Ia patients). Although there is an overlap with the controls, 72% of the values are >2 SD below the mean control value.

Table 1 lists the residual PMM activities in fibroblasts from the CDG-Ia patients included in this study (in mU/mg protein; the corresponding leukocyte values are given when available). In an attempt to characterize the variable phenotype of the CDG-Ia patients, the clinical outcome was graded as mild, moderate, or severe on the basis of the predominant clinical symptoms. Patients with the classical CDG-Ia phenotype, including failure to thrive, recurrent infections, multiorgan involvement, convulsions, retinitis pigmentosa, severe developmental delay and inability to walk, peripheral neuropathy, or scoliosis were classified as having "severe" clinical expression. This group includes the patients who died early in childhood. Patients with mild developmental delay but who learned to sit and walk and had no failure to thrive, no feeding difficulties, no visceral organ problems, or no disturbance of clotting factors and transaminases were classified as having "mild" clinical expression. Between these extremes were the patients with "moderate" clinical expression. In all patients, psychomotor retardation (to a very variable degree), strabismus, hypotonia, and cerebellar hypoplasia were documented. There is a clear overrepresentation of (very) mild cases in the group with residual PMM activities >25% of normal, measured in fibroblasts. Overall, there is a correlation between the severity of clinical presentation and the decrease of PMM activity in fibroblasts: in the 9 patients with mild expression, 2.05 ± 0.61 mU/mg protein PMM activity was measured; in the 18 patients with moderate expression, 1.32 ± 0.86 mU/mg protein; and in the 27 severely affected patients, 0.63 ± 0.56 mU/mg protein ($P < .001$ for a comparison of the means of the three groups; P values for comparisons of mild vs. moderate, moderate vs. severe, and mild vs. severe are .034, .004, and <.001, respectively) (control subjects 5.34 ± 1.74 mU/mg protein; $n = 22$).

Table 2 summarizes the frequency of typical symptoms in the general CDG-Ia population and compares it with the occurrence of these symptoms in the group of patients with mild cases of CDG (as available to the authors). Remarkably, inverted nipples, fat pads, failure to thrive, and convulsions are absent in the latter group. On the other hand, all patients present with strabismus, hypotonia, cerebellar hypoplasia, and mental retardation, although to a very variable degree.

Table 1 also lists the mutation data for the CDG-Ia patients included in this study, which allows for a comparison between the phenotype, the PMM activity, and the genotype. Among the plethora of missense mutations observed in this group, several mutations are more frequent. First, the common R141H mutation is observed in 33 of 54 cases. Second, two mutations are frequently associated with the milder phenotype. Mutation C241S in combination with R141H or with F157S was found only in patients with relatively high residual PMM activity and a (very) mild clinical picture. Mutation T237M, again in combination with R141H, is also more frequently found among the patients with higher residual activity, whereas, in the patients with low residual activity, it is found in combination with P113L and T237R. On the other hand, the phenotype in six of seven patients who are compound heterozygous for V231M and R141H was severe. One child died in early childhood because of liver failure. Finally, patients who are compound heterozygous for F119L and R141H are found only in the CDG-Ia patient group with a low residual activity of PMM in fibroblasts, and all patients were severely affected. One patient homozygous for F119L had a more favorable clinical outcome. The clinical presentation in five pairs of siblings was only slightly different. The PMM activities were in the same range but not necessarily in direct relation to the severity of the disease (e.g., in patients 18 and 7).

Discussion

CDG-Ia occurs worldwide, and patients can present with a wide range of symptoms. By using IEF of serum transferrin as a screening test for N-glycosylation defects, we have found several patients with an abnormal pattern that did not present the full diagnostic criteria for CDG-Ia. Enzyme analysis showed, in many of these patients, a PMM deficiency, establishing the diagnosis of CDG-

Table 1

Characteristics of 54 Patients with CDG-Ia

Patient Number,[a] Sex	Genotype	PMM in Fibroblasts (mU/mg protein)	Passage[b]	PMM in Leukocytes (mU/mg protein)	Phenotype
5, F	R141H/C241S	2.19	(4)	.32	Very mild[c]
8, F	R141H/C241S	2.1	3 (3)	.22	Very mild
18[1], M	R141H/C241S	1.41	(3)	.22	Very mild[d]
2, M	L32R/R141H	3.35	(2)	.20	Mild
4, M	R123Q/T226S	2.36	5 (5)	.28	Mild
7[1], M	R141H/C241S	2.15	(3)	.16	Mild
9[2], M	F157S/C241S	1.88	6 (2)	.13	Mild
14[2], F	F157S/C241S	1.62	(1)	.24	Mild
20, F	R141H/T237M	1.35	4 (4)	.14	Mild
3[3], F	R239W/F157S	2.8	9 (6)	.08	Mild–moderate
11, F	R141H/T237M	1.68	(3)	…	Mild–moderate
21, M	R141H/T237M	1.34	(4)	.19	Mild–moderate
26, F	R141H/H195R	.94	(2)	…	Mild–moderate
37, M	E151G/R141H	.54	(2)	…	Mild–moderate
1, M	R141H/R162W	3.75	(3)	…	Moderate
13[3], F	R239W/F157S	1.65	7 (5)	.07	Moderate
16, F	R141H/E197A	1.49	4 (4)	.23	Moderate
17, F	R141H/T237M	1.45	4 (4)	.31	Moderate
19, F	R141H/D65Y	1.38	(2)	…	Moderate
23, M	R141H/V231M	1.25	(3)	.27	Moderate
28, M	N216I/N216I	.9	5 (5)	.14	Moderate
31, M	P113L/F157S	.77	(5)	.10	Moderate
33, F	D65Y/D65Y	.64	4 (4)	…	Moderate
40, M	I132T/R141H	.41	(3)	…	Moderate
51, M	F119L/F119L	.13	(3)	…	Moderate
15, F	F157S/T237M	1.52	4 (4)	…	Moderate–severe
24, M	IVS3+2C→T/H218L	1.03	(3)	.00	Moderate–severe
6, M	R123Q/I153T	2.19	(3)	.01	Severe[e]
10, M	V44A/R123Q	1.8	(3)	…	Severe
25[4], F	R141H/V231M	.98	(3)	…	Severe
29, F	R141H/V231M	.9	(3)	…	Severe
32, M	R141H/V231M	.64	(3)	.02	Severe
34, F	P69S/R141H	.6	(3)	…	Severe
36, M	V44A/R141H	.57	(3)	.42	Severe
38, M	F119L/R141H	.49	6 (6)	…	Severe
39[5], F	P113L/T237M	.43	(2)	…	Severe
41, M	R141H/V231M	.33	(3)	…	Severe
42, M	P113L/R141H	.32	(3)	…	Severe
44, F	R141H/T237M	.28	5 (5)	…	Severe
45[4], F	R141H/V231M	.27	(4)	…	Severe
46, F	F119L/R141H	.26	(4)	…	Severe
47[5], F	P113L/T237M	.19	(2)	…	Severe
48, F	D188G/R141H	.17	7 (7)	…	Severe
49, M	F119L/R141H	.17	(3)	…	Severe
50, F	F119L/R141H	.14	(4)	…	Severe
52, F	P113L/R123Q	.09	(4)	…	Severe
53, F	T237M/T237R A233T[f]	.04	(0)	…	Severe
12, M	Y76C/F206T	1.67	(4)	…	Severe[g]
22, F	V129M/IVS3+2C→T	1.25	(4)	…	Severe[h]
27, F	D65Y/R123Q	.91	(2)	…	Severe[h]
30, M	R141H/D65Y	.81	(3)	…	Severe[h]
35, M	D65Y/F157S	.57	(3)	…	Severe[h]
43, F	R141H/V231M	.3	5 (5)	…	Severe[h]
54, F	D188G/R141H	.02	7 (7)	…	Severe[h]

NOTE.—Patients with CDG-Ia were listed according to the clinical presentation but numbered according to the residual activity measured in fibroblasts. Definitions of "mild," "moderate," and "severe" are given in the Results section.

[a] Patients were numbered according to the residual PMM activity in fibroblasts (patient 1 had the highest activity; patient 54 had the lowest activity). Sib pairs are indicated by superscript numbers.

[b] The total passage number is given if known; the number of passages between arrival in the cell culture facility in Leuven and the enzymatic measurements is given in parentheses.

[c] Patient attends normal school.

[d] Estimated full-scale IQ: 80.

[e] Child of a drug addict.

[f] This patient has A233T on the same allele as T237R.

[g] Premature at 32 gestational weeks; died at age 13 years.

[h] Died during the first year of life.

Ia. Since high PMM activities (up to 25% of the normal activity) had been reported in fibroblasts or lymphoblasts from some patients (Matthijs et al. 1998*b*), mutation search in the *PMM2* gene was also performed in patients with fibroblast PMM values in the normal range, leading to the diagnosis of additional CDG-Ia cases. Thus, the data presented in this study show that the residual PMM activities in fibroblasts from patients with two allelic mutations in the *PMM2* gene could be quite high, reaching 70% of the normal value in one patient. The problem with partial deficiencies has not been encountered in assays performed in leukocytes. Thus, in patients with high residual PMM activity in fibroblasts, analysis of PMM activity in leukocytes can substantiate the diagnosis of CDG-Ia.

The difference between the residual activity in fibroblasts and in leukocytes points to a cell-specific effect. Analysis of the mutant proteins in vitro has shown that they have altered kinetic properties, with V_{max} values ranging from 0.4% to ~50% of the wild-type enzyme, and that they are less stable than the latter (Kjaergaard et al. 1999; Pirard et al. 1999; Vuillaumier-Barrot et al. 2000). This difference in stability is most likely responsible for the fact that the residual activities are, as a rule, higher in fibroblasts, which are rapidly dividing cells with active protein synthesis, than in leukocytes, most of which are terminally differentiated cells with little or no protein synthesis. This problem may be compounded by the fact that a higher PMM2 residual activity may lead to a selective advantage in culture condition and to overgrowth. In this way, the passage number of the cells may affect the PMM values. However, there is no apparent influence of passage number on the PMM values (see table 1), although it was not possible to retrieve the passage number for all fibroblast cultures. Alternatively, the growth phase of the cells might affect the measurement, although this is unlikely for the fibroblasts, because they were all grown to confluency.

Measurement of PMM activity in leukocytes of obligate carriers showed that this enzyme activity was below the mean value −2 SD in 70% of the cases. Thus, PMM assays in leukocytes were also useful for the identification of carriers. In practice, when no material is left from the proband, indirect evidence can be obtained from the PMM activities in leukocytes of the parents (Matthijs et al. 1998*a*).

It is obvious that hypotonia, strabismus, cerebellar hypoplasia, and mental retardation are consistent features in CDG-Ia and are therefore reliable diagnostic criteria, whereas convulsions and/or strokelike events and visceral complications are less common in the milder cases. Importantly, typical symptoms such as inverted nipples, abnormal fat pad distribution, and failure to thrive are missing in these patients.

The PMM2 protein is encoded by the *PMM2* gene, located on chromosome 16p13 (Matthijs et al. 1997). The open reading frame of 738 nucleotides predicts a protein of 246 amino acids, whose three-dimensional structure is not yet known. PMM2 belongs to the haloacid dehalogenase superfamily of proteins, which are characterized by the conservation of three different motifs that are most likely involved in the catalytic activity (fig. 3) (Aravind et al. 1998). The reaction mechanism of the enzyme involves the phosphorylation of the first aspartate in an extremely conserved DXDXT/V sequence that is close to the amino-terminus of the enzyme (Asp-12; see fig. 3) and is also found in a series of other phosphatases and phosphomutases (Collet et al. 1998).

Mutation analysis in CDG-Ia patients reveals a plethora of mutations, mainly missense (Matthijs et al. 1997, 1998*b,* 2000; Kjaergaard et al. 1998; Bjursell et al. 2000; Imtiaz et al. 2000; de Lonlay et al. 2001). R141H, the most frequent mutation among whites, is present in 33 of 54 cases in this series, and this frequency does not differ from the overall frequency of this mutation among patients described elsewhere (Congenital Disorders of Glycosylation Web site). However, R141H is a severe mutation with virtually no residual activity, as shown by overexpression of the mutant protein (Kjaergaard et al. 1999; Pirard et al. 1999), which might explain why homozygosity for this mutation has not been observed (Kjaergaard et al. 1998; Matthijs et al. 1998*b;* Schollen et al. 2000). Thus, whenever the R141H mutation is found in patients, differences in the clinical presentation and in the residual activity in fibroblasts are likely to correlate with the mutations in the second allele.

In this series, one mutation—C241S—is particularly frequent in the milder phenotypes (see table 1). The C241S mutation is in the C-terminal part of the protein, in a nonconserved region, and seems to impair the pro-

Table 2

Frequency of Reported Clinical Symptoms in All CDG-Ia Cases in This Series and in the Cases Considered "Mild"

Clinical and Biochemical Features	All Cases (%)	Mild Cases (%)
Inverted nipples	10/26 (38)	0/9 (0)
Fat pads	10/26 (38)	0/9 (0)
Failure to thrive	13/30 (43)	0/9 (0)
Elevated transaminases	13/25 (52)	0/9 (0)
Clotting factor deficiency	17/23 (75)	2/9 (22)
Convulsions or strokes	10/27 (37)	0/9 (0)
Strabismus	33/33 (100)	9/9 (100)
Hypotonia	33/33 (100)	9/9 (100)
Cerebellar hypoplasia	33/33 (100)	9/9 (100)
Mental retardation	33/33 (100)	9/9 (100)

NOTE.—Data are no. of cases with characteristic/total no. of cases for which data are available.

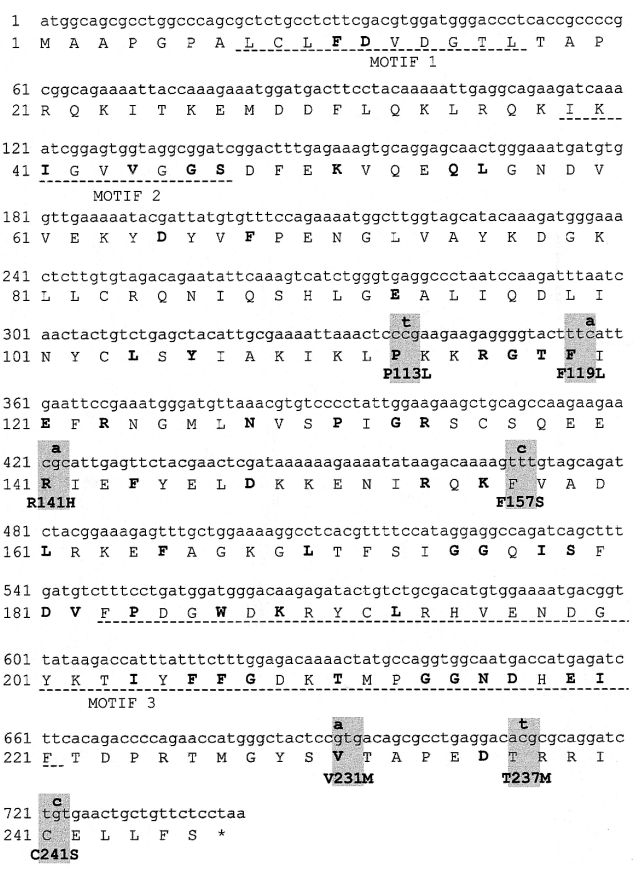

Figure 3 Nucleotide and amino acid sequence of PMM2. Amino acid residues that are phylogenetically strictly conserved are indicated in boldface type. The mutations that are discussed in the context of this study are indicated. Amino acid 241 is not conserved at all, whereas amino acid 237 is conserved between human and yeast but not between human/yeast and *Babesia bovis* (see also Matthijs et al. 2000). On the basis of structural data obtained with P-type ATPases, Aravind et al. (1998) have delineated three motifs, in the phosphomannomutases, that may be directly involved in the catalytic activity. They are underlined.

tein activity to a minor extent, since it decreases the activity by only 50% (Vuillaumier-Barrot et al. 2000). The combination of R141H/T237M is also observed more frequently in the patients with higher residual activity in fibroblasts, but the phenotype ranges from mild to severe. T237M affects a conserved amino acid of the protein, and one can assume that the mutated protein has only limited residual activity. When T237M is combined with either P113L, T237R or F157S, the clinical outcome is severe. Because the enzyme acts as a (homo)dimer, the differences between the effects of these combinations of mutations might be due to molecular "interactions" between the mutated proteins.

V231M is also frequent in the group of patients described here. The V231M mutation has a residual activity of 38.5%, but the mutated protein is extremely unstable (Pirard et al. 1999), which might explain why the compound heterozygous patients with R141H/V231M have, in general, a less favorable clinical outcome than patients with the R141H/C241S mutation. F119L is the second most common *PMM2* mutation in CDG-Ia, because of a founder effect in the Scandinavian population (Bjursell et al. 1997; Kjaergaard et al. 1998). Its presence among the CDG-Ia patients in this series who are heterozygous for F119L/R141H was limited to severe cases. In expression studies of F119L in *Escherichia coli,* a residual activity of 25% was measured and the mutated protein showed a marked decrease of substrate affinity (Kjaergaard et al. 1999; Pirard et al. 1999). The other mutations are too rare for conclusions about their phenotypic impact to be drawn.

In conclusion, patients with high to normal residual activity of PMM in fibroblasts may harbor mutations in *PMM2*. Most of these patients have mild to moderate clinical expression, significantly broadening the spectrum of CDG-Ia.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Congenital Disorders of Glycosylation Web site, Centre for Human Genetics, University of Leuven, http://www.kuleuven.ac.be/med/cdg/ (for *PMM2*)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CDG-Ia [MIM 212065] and phosphomannomutase [MIM 601785])

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