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Lack of Homozygotes for the Most Frequent Disease Allele in Carbohydrate-Deficient Glycoprotein Syndrome Type 1A

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Summary

Carbohydrate-deficient–glycoprotein syndrome type 1 (CDG1; also known as "Jaeken syndrome") is an autosomal recessive disorder characterized by defective glycosylation. Most patients show a deficiency of phosphomannomutase (PMM), the enzyme that converts mannose 6-phosphate to mannose 1-phosphate in the synthesis of GDP-mannose. The disease is linked to chromosome 16p13, and mutations have recently been identified in the *PMM2* gene in CDG1 patients with a PMM deficiency (CDG1A). The availability of the genomic sequences of *PMM2* allowed us to screen for mutations in 56 CDG1 patients from different geographic origins. By SSCP analysis and by sequencing, we identified 23 different missense mutations and 1 single-base-pair deletion. In total, mutations were found on 99% of the disease chromosomes in CDG1A patients. The R141H substitution is present on 43 of the 112 disease alleles. However, this mutation was never observed in the homozygous state, suggesting that homozygosity for these alterations is incompatible with life. On the other hand, patients were found homozygous for the D65Y and F119L mutations, which must therefore be mild mutations. One particular genotype, R141H/D188G, which is prevalent in Belgium and the Netherlands, is associated with a severe phenotype and a high mortality. Apart from this, there is only a limited relation between the genotype and the clinical phenotype.

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Introduction

Carbohydrate-deficient glycoprotein (CDG) syndromes are a series of genetic disorders characterized by defective N-glycosylation of serum and cellular proteins (Jaeken et al. 1980, 1997*b;* Jaeken and Carchon 1993; Jaeken and Casaer 1997). At present, four types of CDG have been described on the basis of serum transferrin isoelectrofocusing (IEF). CDG type 1 (CDG1 [MIM 212065]) is the most frequent type. It is a severe disorder that presents neonatally. There is a life-threatening liver insufficiency (with an overall 20% mortality during the neonatal period), combined with a severe cerebellar dysfunction and peripheral neuropathy, leading to severe psychomotor retardation. These children also have skeletal deformities and a characteristic deposition of adipose tissue (Jaeken and Carchon 1993; Jaeken et al. 1997*b*). CDG type 2 (CDG2), type 3 (CDG3), and type 4 (CDG4) represent only two cases each (Stibler et al. 1993, 1995; Jaeken et al. 1994). CDG2 is caused by a deficiency of UDP-GlcNAc:α-6-D-mannoside β-1,2-N-acetylglucosaminyltransferase 2 (GnT 2), located in the Golgi apparatus, and mutations in the GnT 2 gene (MGAT2) on 14q21 have been identified (Jaeken et al. 1994; Tan et al. 1996). The causes of CDG3 and CDG4 remain unknown.

CDG1 is inherited in an autosomal recessive manner, and its locus has been mapped to chromosome 16p13 (Martinsson et al. 1994). Linkage to the region between D16S406 and D16S500 has been confirmed in 10 of 11 informative families (Matthijs et al. 1996). In one family with two affected siblings, the disease was, however, not linked to chromosome 16p, indicating genetic heterogeneity for CDG1 (Matthijs et al. 1996).

Biochemical evidence has long suggested a basic defect in the synthesis of the dolichol-P-oligosaccharides (synthesis of the asparagine-N-linked oligosaccharides) in the endoplasmic reticulum (ER) (Jaeken et al. 1984; Wada et al. 1992). In 1995, Van Schaftingen and Jaeken (1995) identified a deficiency of phosphomannomutase (PMM) activity in patients with CDG1. This observation has been confirmed in more than 50 CDG1 patients from different geographic origins (Jaeken et al. 1997*a*). We

have recently cloned the human PMM gene *PMM2* and have shown that it is the CDG1 gene (Matthijs et al. 1997*a*). Another PMM gene, *PMM1,* could be assigned to chromosome 22q13 (Matthijs et al. 1997*b*). Both PMM1 and PMM2 have been expressed in *Escherichia coli* and have been found to be active proteins (Pirard et al. 1997; E. Van Schaftingen and M. Pirard, unpublished data). We have previously reported 11 missense mutations in 16 CDG1 patients with a documented PMM deficiency (CDG1A) (Matthijs et al. 1997*a*). These mutations have been identified at the cDNA level, after reverse transcriptase–PCR amplification, followed by SSCP analysis and sequencing. To search for mutations in genomic DNA, the *PMM2* intron/exon structure has been determined, whereby eight exons have been identified (Schollen et al. 1998), and primers flanking each translated exon have been designed. We here describe the results of an exhaustive mutation analysis of the *PMM2* gene in patients with a documented PMM deficiency.

Patients and Methods

Patients

Fifty-six patients from 12 countries were included in the study; all except 2 were of Caucasian origin. A diagnosis of CDG1 was made in all these patients, on the basis of clinical manifestations, and was substantiated by the typical IEF pattern of serum transferrins: there is both a strong reduction in the intensity of the normal tetrasialotransferrin band and a concomitant increase in the disialo- and asialotransferrin concentration. PMM deficiency was documented in most cases (see table 1 and the Results section). The clinical features of CDG1 patients have recently been reviewed by Jaeken and Casaer (1997) and Jaeken et al. (1997*b*). In brief, the neurological picture includes abnormal eye movements, combined with slow head movements in the neonatal period, and axial hypotonia with hyporeflexia. Most children present with an alternating strabismus. There is a severe psychomotor retardation and failure to thrive, with ataxia and, sometimes, deafness. Additional features, presenting after infancy, are hypogonadism, retinitis pigmentosa, joint contractures, and strokelike episodes. Most patients never attain the ability to walk without support, but there is no regression. Other symptoms include mild facial dysmorphism (with large, somewhat dysplastic ears), skeletal deformities, and a typical subcutaneous deposition of adipose tissue ("fat pads"). There is a mild to moderate hepatomegaly, and some infants develop pericardial effusion and/or cardiomyopathy. Approximately 20% of the patients die before the age of 5 years, as a consequence of liver failure, severe infection, cardiac insufficiency, nephrotic syndrome, or status epilepticus. Salient features in some patients are listed in table 1.

The blood samples and/or fibroblast or lymphoblast cultures from patients were provided to us after a request for enzymatic assays and molecular diagnosis, and the referring physicians and the families have been informed about the results. Amniocytes were analyzed in the context of prenatal diagnosis.

SSCP Analysis and Sequencing

DNA was isolated either from fresh blood or from fibroblast or lymphoblast cultures from patients by use of a high-salt-extraction procedure. On the basis of the available sequence, primers were designed for the PCR amplification of nine DNA fragments suitable for SSCP analysis. One primer in each pair was labeled with FITC. The primer sequences are given in table 2. PCR reactions were typically done in 25 μl, and the cycling conditions were 30 s at 95°C, 30 s at 50°–60°C, and 30 s at 72°C, for 32 cycles. A 10–15-μl portion of each PCR product was mixed with an equal volume of formamide and then was denatured for 5 min at 95°C, loaded onto a nondenaturing polyacrylamide gel (0.5 × Hydrolink MDE [J. T. Baker] in 0.6 × TBE [10 × TBE = 1 M Tris, 0.82 M boric acid, and 10 mM EDTA]), and electrophoresed for 10 h at 4°C at 400 V. The gels were directly scanned on a Fluorimager (Vistra), and the signals were analyzed with the ImagequaNT software (Molecular Dynamics).

The PCR fragments were sequenced by cycle sequencing or solid-phase sequencing. Prior to cycle sequencing with the Thermosequenase kit (Amersham), the PCR fragments were purified by use of the Qiaquick-PCR purification kit (Qiagen), and, typically, 50–100 ng was used with 1 pmol of fluorescently labeled primer, for 15–22 cycles. Solid-phase sequencing using a biotinylated template and streptavidin-coated DynaBeads (Dynal) was done according to established procedures.

Results

Fifty-six patients and their affected siblings were included in this series. Included are three pairs of patients in which each pair has at least one common ancestor (table 1). The diagnosis of CDG1 with PMM deficiency was confirmed biochemically in 46 patients for whom fibroblasts, lymphoblasts, or fresh leukocytes were available; in 2 of these patients, a partial deficiency of PMM was found in fibroblasts (patients 47 [SAO] and 48 [GSS]), and, in 1 family (family 4, patients FG and FP, both affected), intermediate values were measured in lymphoblasts. From the remaining 10 patients, whose diagnosis had been made previously on clinical grounds, no cells were available for the enzymatic assay; in 7 of

Table 1

PMM2 Mutations in 57 CDG1 Patients of Different Geographic Origins

Patient(s)	Family	PMM Activity[a] (mU/mg protein)	Mutation(s) 1	Mutation(s) 2	Origin	Special Feature(s)
1. RJM	33	NA[b]	V44A	R141H	Ecuador	
2. AB	37	1.02(f), .1(am)[c]	D65Y	R141H	France	Died at age 4 mo (hepatic insufficiency)
3. SF[d]	16	.13(le)	Y106C	R141H	Belgium	
4. LM[d]	30	.09(le), .50(f)	A108V	R141H	France	
5. PS[d]	17	.10(f)	P113L	R141H	Belgium	Mild dysmorphy (Jaeken et al. 1980; Jaeken and Casaer 1997)
6. KS	21	NA[b]	P113L	R141H	Netherlands	
7. SFX		.32(f)	P113L	R141H	Germany	
8. WC[d]	38	.0(f)	P113L	R141H	Germany	
9. MC[d]		.19(f)	P113L	R141H	France	Severe gastrointestinal symptoms
10. FRL		.26(f)	P113L	R141H	Portugal	
11. HSJ	39	.11(f)	F119L	R141H	Netherlands	
12. SWM	26	.04(le)	F119L	R141H	Netherlands	
13. NP	6	NA[b]	F119L	R141H	Netherlands	
14. PL[d]		.19(f)	F119L	R141H	Netherlands[e]	Died at age 6 years
15. SE[d]	5	NA[b]	F119L	R141H	Netherlands[f]	
16. HR[d]	31	.17(f)	F119L	R141H	Germany	
17. OM	24	.16(f)	F119L	R141H	Germany	
18. PS	28	.26(f)	F119L	R141H	Germany	
19. DD		.03(f)	F119L	R141H	Germany	
20. HE	45	NA, [g].35(am)[c]	F119L	R141H	France	
21. MR	7	NA[g]	F119L	R141H	France	
22. RD[d]	35	.13(f)	F119L	R141H	UK	
23. KB		.17(f)	F119L	R141H	US	
24. MMi[d]	27	.0(f)	F119L	R141H	US	
25. FG, FP[d]	4	2.75(Ly), 2.2(Ly)[h]	V129M	R141H	Italy (Sicily)	
26. LN		.44(f)	V129M	R141H	US	Died
27. BA[d]	9	.05(f)	P131A	R141H	France	
28. MMa		.41(f)	I132T	R141H	Italy	
29. RP[d]		.43(f)	R162W	R141H	UK	
30. BJ	14	.02(f)	D188G	R141H	Belgium	Died
31. BI	2	<.1(f)	D188G	R141H	Belgium[i]	Died
32. LA	2	.17(f)	D188G	R141H	Belgium[i]	
33. AV	47	NA[g]	D188G	R141H	Netherlands	Died
34. JI	1	.21(f)	D188G	R141H	Netherlands	Died (nephrotic syndrome) (Van der Knaap et al. 1996)
35. WH	48	.16(f)	G208A	R141H	US	
36. CF, CA[d]	3	.33(Ly), .25 (Ly)[h]	N216I, 699g/a	R141H	Italy (Sicily)	
37. HSt[d]	15	.10(f)	V231M	R141H	Belgium	Died
38. HN	29	.15(le)	V231M	R141H	Belgium	Severe gastrointestinal syndrome
39. LA, LF		.27(f), .98(f)[h]	V231M	R141H	Peru	
40. IS		.64(f)	V231M	R141H	Italy	
41. CM	22	.47(f)	V231M	R141H	US	
42. HJ		.90(f)	V231M	R141H	US	
43. MCC[d]	12	.0(f)	T237M	R141H	Spain	
44. TLP		.64(f)	D65Y	D65Y	Portugal	
45. RMan	10	NA[b]	N101K	324 del g	Germany	
46. PG		NA[b]	F119L	F119L	Netherlands[e]	
47. SAO		1.80(f)	R123G, 324g/a	V44A	Spain	
48. GSS		.09(f)	R123G, 324g/a	P113L	Spain	Pubertal development (Pineda et al. 1996)

(*Continued*)

Table 1 (continued)

Patient(s)	Family	PMM Activity[a] (mU/mg protein)	Mutation(s) 1	Mutation(s) 2	Origin	Special Feature(s)
49. SN	5	.15(f)	R123G, 324g/a	F119L	Netherlands[f]	
50. RW	32	NA[b]	R123G, 324g/a	R162W	Netherlands	
51. DBE	8	.04(le)	R123G, no polymorphim	F119L	France	
52. HA	20	.29(f)	R123G, no polymorphism	Y229S	Japan	
53. VC		.11(le)	G175R	P113L	Belgium	
54. SN, SJ		.43(f), .19(f)[h]	T237R, A233T	P113L	Germany	
55. LS	43	.04(f)	T237R	T237M, A233T	France	
MM, MY		.83(f), .42(f)[h]	R238P	??	Japan	

[a] f = fibroblasts of index patient; le = leukocytes of index patient; ly = lymphoblasts of index patient; and am = amniocytes. Mean ± SD control activities were 2.46 ± .48 in leukocytes (n = 12); 3.77 ± .86 in fibroblasts (n = 3), and 6.7 ± 1.0 in amniocytes (n = 3).
[b] No cells were available; mutation analysis was done on DNA from the patient.
[c] Obtained from amniocytes of a sibling with the same genotype.
[d] Patient has been reported elsewhere (Matthijs et al. 1997*a*).
[e] Two patients sharing disease alleles through a common ancestor.
[f] Two patients sharing disease alleles through a common ancestor.
[g] No patient material was available; genotype was derived from mutation analysis of parents.
[h] In two siblings with the same genotype.
[i] Two patients sharing disease alleles through a common ancestor.

these cases (patients RJM, KS, NP, SE, PG, RW, and RMan), DNA from the patient was available for analysis, whereas, in the remaining 3 cases (patients HE, MR, and AV), the mutation screening needed to be done on DNA from the parents; in 2 of these 3 cases (patients HE and AV), the clinical diagnosis was substantiated by the measurement of intermediate PMM activities in fresh leukocytes from the parents.

The mutation screening was done by a nonradioactive SSCP analysis of fragments amplified from genomic DNA. The PCR fragments encompassed the individual exons and the flanking sequences. Whenever a fragment revealed an aberrant SSCP pattern, the corresponding exon was sequenced.

With this approach, 20 different mutations were identified by SSCP in 56 patients (by deduction from the parents' genotypes, when applicable). For those patients in whom no or only one mutation was identified by SSCP analysis, the entire coding region was sequenced. Four mutations were not revealed by SSCP analysis under the described conditions but were identified after sequencing; these mutations are 131T→C (V44A), 303C→G (N101K), 623G→C (G208A), and 713G→C (R238P), in exons 2, 4, 7, and 8 respectively. Also, the mutations 484C→T (R162W) and 523G→C (G175R) in exon 6 were not easily identifiable, because of the presence of two frequent polymorphisms in the flanking regions of the exon. In total, mutations were found in 111 (99%) of 112 disease chromosomes. In one Japanese patient, heterozygous for one mutation at the genomic level, a second mutation could not be found. Southern blot analysis has excluded major rearrangements of the gene in

Table 2

Sequence of Oligonucleotide Primers Used in PCR and SSCP Analysis of the Eight Exons of PMM2

Exon	Name	Sequence	Name	Sequence	Annealing Temperature (°C)
1	UP3B	AGCGGCCGAACCCGGAAGTTC	int0R fitc	AGCAGCCGCCGGCCGCCAC	60
2	int1UB	GGTCTCCTGATTATTGTGTGGC	int2Rbis fitc	GGCAGCCTATGATACTTG	55
3	int2FB	GATTCTTTGCATTCTAAGTG	int3R fitc	TCCTAGAGGCATTCATTGTG	50
4	int3F	CTGGGTTTGCTATGAAGCTG	int4R fitc	ACCATGTGACACTACGCTATG	60
5A[a]	intU2	GCACAGAGCTGAGAAACATT	2B fitc	TGCGTTCTTCTTGGCTGCAGC	60
5B[a]	3A fitc	GGTACTTTCATTGAATTCC	int4R	GTGTTGGGATTACAGGCATG	55
6	int5F fitc	CCAGTAGTTAAAACTGTGCT	int6RB	CCAAGTTTGGAACACAGGCA	55
7	int6F	TCAGTGACATATCATTAGCC	int6R fitc	CCATCAAGCGCAAATGC	55
8	int7FB	TCCAGGGTCACATCAGCAATGG	4B1 fitc	GGAGAACAGCAGTTCACAG	52

[a] Exon 5 is analyzed in two fragments.

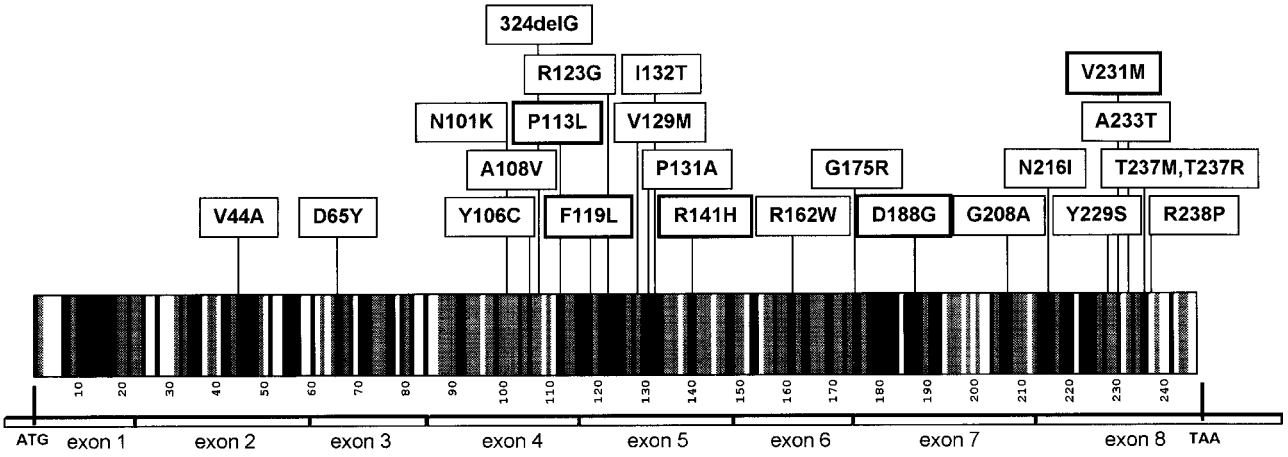

Figure 1 Schematic diagram of *PMM2* cDNA, depicting the location of the various mutations found in the gene, with the exons indicated below the diagram. Because, at present, nothing is known about possible domains in the protein, the degree of similarity with the yeast *SEC53*, *Candida albicans* PMM, and human PMM1 has been represented: the black bands denote amino acid residues that are perfectly conserved among the four proteins; the gray-shaded bands denote partial conservation; and the blank bands denote residues that are not conserved. Only mutation R238P affects a residue that is not conserved. Mutations that have relatively frequently been encountered in this study are indicated in boxes bordered by the thicker lines.

this patient (data not shown). The mutational spectrum thus consists of 23 missense mutations and only one single-base-pair deletion (fig. 1 and table 3). This deletion of a G at position 324 in exon 4 causes a frameshift and premature stop (fig. 2).

Forty-three patients were heterozygous for the R141H mutation, but not a single patient was found to be homozygous for this mutation. Given the frequency of the R141H mutation (the allele frequency, by counting, is 40% in the 54 Caucasians in this study), eight homozygotes are expected in this sample, on the basis of the Hardy-Weinberg equation ($\chi^2 = 23.4$; df = 1; $P < .00001$). The most frequent genotype is the R141H/F119L combination (14 patients). One patient was homozygous for the relatively frequent F119L mutation (fig. 3), which is in accordance with Hardy-Weinberg equilibrium, and one was homozygous for the D65Y mutation; the latter is a rare mutation, and the disease allele was probably inherited from a common ancestor of the parents.

In two patients, three different amino acid substitutions were identified. In patient SN (and in the affected sibling SJ), the A233T and T237R mutations are syntenic, as deduced from the parental genotype. In patient LS, the T237M and A233T mutations are probably on the same chromosome—paternal DNA was not available for analysis, but the mother carried only the T237R mutation.

Discussion

PMM Deficiency and Mutations in the PMM2 *Gene*

Both the localization of the *PMM2* gene, encoding an active PMM, on chromosome 16 and the identification of mutations in this gene that segregate with the disease (Matthijs et al. 1997*a*) gave conclusive support to the biochemical evidence that PMM deficiency is the basis for CDG1A (Van Schaftingen and Jaeken 1995; Jaeken et al. 1997*a*). This conclusion is now strengthened by the fact that, in almost all cases with a documented PMM deficiency, we found mutations in the *PMM2* gene. In one case, a second mutation was not found, despite the fact that all exons were scrutinized by sequencing. The other mutation is probably in a regulatory region of the gene.

Recent work has shown that PMM deficiency is found in a majority of—but not in all—patients with CDG1. In these patients, there is no reason to search for mutations in the *PMM2* gene. Accordingly, in the family in which linkage to chromosome 16 could be excluded (Matthijs et al. 1996), no PMM deficiency was found in the propositus (authors' unpublished results). Because of this heterogeneity, PMM assays are still useful for diagnosis. The only drawback of these measurements is that, in some cases, the activity found in fibroblasts or in lymphoblasts still represents as much as ~30% of the control value (e.g., see patients 2, 25, and 39), whereas in other samples, derived either from the same patients or from other patients with the same genotype, the activity was <5% of the control activity. This problem has not been encountered in assays performed with fresh material such as leukocytes or liver. In the latter cases, a profound deficiency has always been observed. We have, at present, no explanation for these discordant findings. One possibility is that PMM1 or the mutant forms of PMM2 become overexpressed after several passages in culture.

Table 3

Mutations in the PMM2 Gene

Exon	Mutation	Codon Change	Amino Acid Change	No. of Occurrences[a]	Conservation Status[b]
2	131T→C	GTA→GCA	V44A	2	Conserved
3	193G→T	GAT→TAT	D65Y	3	Conserved
4	303C→G	AAC→AAG	N101K	1	Not conserved
4	317A→G	TAC→TGC	Y106C	1	Conserved
4	324 delG	Frameshift	…	1	…
4	323C→T	GCG→GTG	A108V	1	Semiconserved
4	338C→T	CCG→CTG	P113L	9	Conserved
5	357C→A	TTC→TTA	F119L	18	Conserved
5	368G→A	CGA→CAA	R123G	6	Conserved
5	385G→A	GTG→ATG	V129M	2	Semiconserved
5	391C→G	CCT→GCT	P131A	1	Conserved
5	395T→C	ATT→ACT	I132T	1	Conserved
5	422G→A	CGC→CAC	R141H	43	Conserved
6	484C→T	CGG→TGG	R162W	2	Conserved
6	523G→C	GGA→CGA	G175R	1	Conserved
7	563A→G	GAC→GGC	D188G	5	Conserved
7	623G→C	GGA→GCA	G208A	1	Conserved
8	647A→T	AAT→ATT	N216I	1	Conserved
8	686A→C	TAC→TCC	Y229S	1	Conserved
8	691G→A	GTG→ATG	V231M	6	Conserved
8	697G→A	GCG→ACG	A233T[c]	2[c]	Conserved
8	710C→T	ACG→ATG	T237M	2	Conserved
8	710C→T	ACG→AGG	T237R	2	Conserved
8	713G→C	CGC→CCC	R238P	1	Semiconserved

[a] In the series of 112 disease alleles reported in this study.

[b] Compared with human PMM1, yeast PMM (*SEC53*), and *Candida albicans* PMM.

[c] A233T is observed in combination with the T237R mutation (patient 54) and with the T237M mutation (patient 55).

PMM measurements are also useful for the identification of carriers. If cells from an affected child are not available, indirect evidence can be obtained from the PMM activities in leukocytes from the parents. This has proved worthwhile in two cases with an urgent request for prenatal diagnosis, in which we derived information on the PMM deficiency from the parents, before initiating the molecular analysis. In view of the genetic heterogeneity, prenatal testing should be offered only in families with a documented PMM deficiency and with mutations in *PMM2*. Thus far, we have combined enzymatic measurements and mutation analysis for prenatal diagnosis (Matthijs et al. 1998). Molecular analysis is the most dependable test for prenatal diagnosis.

Characteristics of the Mutations

Of the 24 different mutations that were identified, 20 were detectable by SSCP; among these 20 were the most frequent ones, such as R141H, P113L, F119L, V231M, and D188G. In practice, in only 1 of the 56 patients with mutations (patient MM [and the sibling MY]), no aberrant fragment was detected by SSCP analysis, whereas in the other patients at least one mutation was detected by SSCP alone. Given that the gene contains only eight exons, the SSCP analysis represents a fairly simple, reliable, and cost-effective approach for the molecular analysis of CDG1A patients.

Of interest is the fact that the mutations are unequally distributed between the different exons (see fig. 1): six mutations were found in exons 5 and 8, whereas no mutation was found in exon 1. It is unlikely that the first exon would accommodate mutations less easily than would other exons, since its degree of conservation is intermediate between those of exon 5 and exon 8 (Matthijs et al. 1997*a*). It is more probable that the mutation-rate difference among exons is due to their sequence context and position in the genome.

All mutations except one are missense mutations. It is remarkable that, in this collection of mutations, only a single frameshift mutation has been observed. The G175R mutation might also affect splicing. Nineteen of the mutations that were found affect residues that are strictly conserved among PMMs (fig. 1 and table 3). This supports the notion that mutations at these sites are detrimental to the function of the protein. Also, the comparative data in figure 1 suggest that the list of mutations presented here is not exhaustive. An alignment of PMM2 with PMM1, yeast SEC53, and *Candida albicans* PMM has been published (Matthijs et al. 1997*a*). Enzymatic studies of some of the mutant proteins are underway.

The R141H mutation is by far the most frequent (Cau-

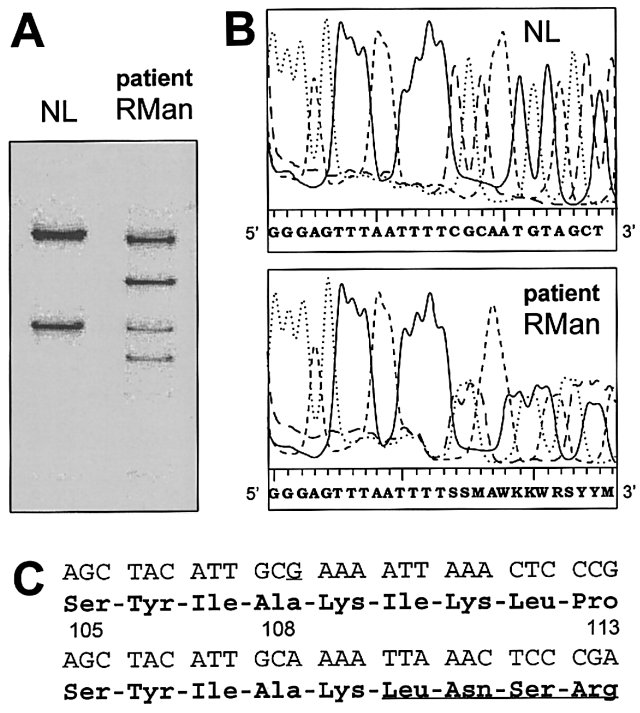

Figure 2 Identification of a single-base-pair deletion in exon 2 of *PMM2*, by SSCP and sequencing in a patient with CDG1A. *A*, SSCP analysis showing an aberrant pattern for patient RMan. *B*, Direct sequencing using the fluorescent primer int4R, revealing a heterozygous deletion of the G at position 324, which is the third base of codon 108. Downstream of the deletion site, the signal is a mixture of two overlapping sequences. Note that the sequence of the antisense strand is given. *C*, Effect of the deletion on the translational frame. The frameshift results in a stop 19 codons downstream of the deletion site (not shown).

casian) one. The D188G mutation seems to be restricted to Belgian (Flemish) and Dutch patients. The V44A mutation is probably of Spanish origin, whereas the D65Y mutation, homozygous in a Portuguese patient, is also found in a French patient with Portuguese ancestors. The V129M mutation might well be of Italian origin. We have not tried to link the most frequently observed mutations to a common haplotype of the flanking polymorphic markers, but it seems that some mutations are old mutations that have been present in the different populations for centuries. The R141H mutation is caused by a CGC→CAC transition, but the equally likely CGC→TGC transition (R141C, which is found in a processed pseudogene, derived from *PMM2* and located on chromosome 18p; see Schollen et al. 1998) has not been observed in patients. Thus, the R141H mutation may be an old mutation, like the frequent ΔF508 in cystic fibrosis (Morral et al. 1993). On the other hand, some mutations must have occurred independently on different chromosomes in the different populations. For instance, the R123G mutation, shared by two Spanish and two Dutch patients, is syntenic with a polymorphism at nucleotide 324 in the coding region and also has been identified in a Japanese patient and in a French patient, but without the polymorphism. The T237R and T237M mutations are caused by C→T transitions on opposite strands in the same CpG-dinucleotide. It is known that CpG-dinucleotides are hot spots for mutations. An interesting observation is that the corresponding CpGs have also been mutated in the processed pseudogene (Schollen et al. 1998).

A complex genotype has been observed in two families. DNA from the parents has been used to establish the phase of these mutations. The A233T mutation is syntenic with the T237R and T237M mutations in, respectively, patient pair SN and SJ and patient LS, whereas the T237R is present on the other allele in the

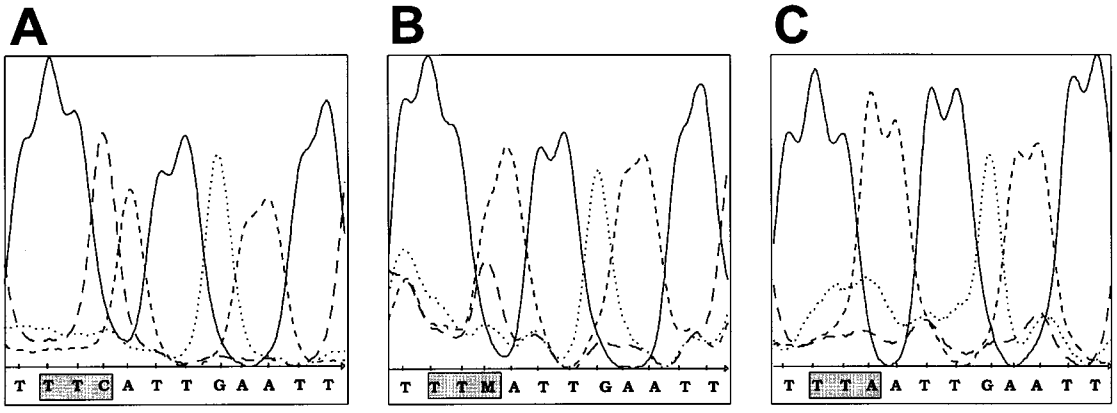

Figure 3 Sequence analysis revealing the occurrence of the F119L mutation in the homozygous state. The results of the direct sequencing are shown for (*A*) a normal control (homozygous for the normal sequence), (*B*) the father of patient SN (heterozygous), and (*C*) patient PG (homozygous for the mutation). Codon 119 is boxed.

family of the latter patient. Taken together, the alternative combinations of the T237R and T237M mutations with the A233T mutation are remarkable, and the occurrence of two different mutations in the same codon on the two chromosomes in patient LS is intriguing.

Relationship between the Mutations and the Phenotype

Very limited inferences can be made from the genotype-phenotype comparison. There is no clear correlation between the PMM activities and the genotype in 6 and 14 patients with the R141H/P113L and R141H/F119L combinations, respectively. It has previously been remarked that a great variability exists in the clinical expression among affected siblings (Jaeken et al. 1997*b*). One significant observation is the high mortality in the patients with the D188G/R141H genotype: four of five patients died before the age of 2 years,whereas the fifth patient, now age 10 years, is severely affected. On the other hand, the twin patients, described originally by Jaeken et al. (1980), are now age 21 years, are relatively well, and have the R141H/P113L genotype. The R123G/P113L genotype is observed in a Spanish patient with pubertal development, which is not normally seen in females with CDG1.

Even in the most severe cases, the bulk of N-linked carbohydrate chains is present on serum glycoproteins. This would mean that some residual activity is required. The fact that mainly missense mutations are found suggests that a total lack of PMM2 activity is incompatible with life. The lack of homozygotes for R141H supports this idea (see below). Thus far, alternative pathways to bypass PMM activity and to generate mannose 1-phosphate have not been described.

Given the low frequency of CDG1, one would expect this recessive disorder to be most frequently encountered in consanguineous families. In our previous haplotype analysis of 15 patients, we have not encountered homozygosity for a disease-associated haplotype of linked markers (Matthijs et al. 1996). The molecular analysis revealed the significant lack of homozygotes for R141H. Thus, Hardy-Weinberg equilibrium is not reached, probably because of selection against this genotype. Probably, R141H is a severe mutation that is deleterious in the homozygous state, leading to fetal wastage, miscarriage, or early death. It might also give rise to a different phenotype. In view of the fundamental role of PMM2 in the normal functioning of the cell, we hypothesize that it is lethal soon after conception. There are only two ways to address this question. One is to try to find families in which both parents are carriers of R141H, to prove that the R141H/R141H genotype is absent in their children, and to look for an association with miscarriages. Given that the gene frequency is very low, this is practically impossible. The other way is to await the availability of a transgenic mouse model, to find out whether the homozygous knockout is (embryonic) lethal or results in a different phenotype.

Most other mutations must be relatively mild, because they are found in association with R141H. In the recent report on haplotype data from the Swedish group, Bjursell et al. (1997) report at least two homozygous cases in their population. On the basis of the complete lack of R141H-homozygous patients in the present series, we speculate that the underlying mutation will be a milder mutation. In our series, patients were homozygous for the D65Y and F119L mutations. The D65Y mutation is rare, but the F119L mutation is frequent, and the occurrence of one F119L/F119L patient in a series of 56 reflects Hardy-Weinberg equilibrium. This observation suggests that these are mild mutations. This might be reflected by the relatively high value of the residual PMM activity in fibroblasts of the D65Y/D65Y patient. However, the same D65Y mutation, in combination with the R141H mutation, was found in a patient (patient 2) with a severe phenotype, who died at a very young age. The F119L/R141H genotype is especially frequent; thus, the combination of the two most frequent disease mutations is not lethal.

It remains to be investigated how these mutations affect the function of the protein. Nothing is currently known about the functional domains of PMMs. The availability of a plethora of functional mutants will be of great value in the interpretation of structural data.

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