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Carbohydrate-deficient glycoprotein syndrome type 1A: expression and characterisation of wild type and mutant PMM2 in *E. coli*

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We have identified the *PMM2* genotypes of 22 unrelated Danish patients with carbohydrate-deficient glycoprotein syndrome type 1A: R141H/F119L (18), R141H/C192G (1), F119L/F119L (1), F119L/G117R (1) and D223E/T237R (1). The lack of patients homozygous for R141H is statistically highly significant, but unexplained. In order to investigate the effect of *PMM2* mutations on phosphomannomutase (*PMM2*) activity, *PMM2*-cDNA was cloned into a pET3a vector. Following introduction of mutations into *PMM2*-cDNA by site-specific mutagenesis, wild type and mutant *PMM2*-cDNA were expressed in *E. coli* BL21(DE3) cells, and the activity of *PMM2* was determined by an enzymatic assay using mannose 1-phosphate as substrate. Recombinant R141H, G117R, and T237R *PMM2* had no detectable catalytic activity, and the F119L *PMM2* had 25% of the activity of the wild type. The activity of the C192G and D223E *PMM2* was in the normal range, but the affinity for their substrate was lower, and the proteins were more sensitive to increased temperatures. Each patient has at least one mutation which retains residual *PMM2* activity. Our results support the hypotheses that a genotype conveying residual *PMM2* catalytic activity is required for survival, and that homozygosity for R141H impairs *PMM2* to a degree incompatible with life.

Keywords: carbohydrate-deficient glycoprotein syndrome 1A; *PMM2*; phosphomannomutase; gene expression

Introduction

Carbohydrate-deficient glycoprotein syndrome type IA (CDG1A, OMIM 212065) is an autosomal recessive disorder characterised by defective protein glycosylation due to deficiency of phosphomannomutase (*PMM*). *PMM* converts mannose 6-phosphate to mannose 1-phosphate which is required for synthesis of

GDP-mannose. Two types of human phosphomannomutase, *PMM1* and *PMM2*, have been identified.^{1,2} Mutations in the *PMM2* (OMIM 601785) gene located on chromosome 16p13 are responsible for CDG1A.² Two independent surveys of patients of CDG1A found *PMM2* mutations on 145 of 148 disease chromosomes (98%).^{2–4} All but one of the 30 different mutations detected so far have been of the missense type.^{2–6} Whilst most patients are compound heterozygotes, homozygosity for the F119L mutation has been observed in a few instances, and one patient is known to be homozygous for D65Y.^{3,5,7} In contrast, patients homozygous for the R141H mutation, present in more than 75% of patients of Caucasian origin, have been

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conspicuously absent in results of the genotype analyses.^{3,4} These observations suggest that homozygosity for R141H may not be compatible with life. In order to investigate the effect of *PMM2* mutations on *PMM2* activity we have expressed wild type and mutant *PMM2*-cDNA in *E. coli* and compared specific activities, affinities for mannose 1-phosphate, and temperature stabilities of the corresponding proteins.

Materials and Methods

Patients

The clinical diagnosis of CDG1A in 28 patients from 22 unrelated families of Danish origin was substantiated by isoelectric focusing of transferrin,^{8,9} by documentation of *PMM2* deficiency in cultured fibroblasts,¹⁰ and by genotype analysis.⁴ Subsequently, we have identified a novel *PMM2* mutation, C192G, in one of our patients.

Expression of Recombinant Wild Type and Mutant *PMM2*-cDNA

Total RNA was prepared from cultured fibroblasts derived from a healthy control using RNeasy (Qiagen, Düsseldorf, Germany), cDNA was generated with GeneAmp RNA-PCR kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) using MuLV reverse transcriptase and random hexamers as primers. The coding sequence of *PMM2* was amplified by *Pfu* DNA polymerase using the generated cDNA as a template. Primers introducing one *Nde*I restriction site (5'-CGGCTAGAACTGGGCATATGG-CAGCGCCTGGCCAGC-3') and one *Bam*HI (5'-CTTGTCAGCCGGGATCCCGCCCTCCCGCT-3') restriction site (underlined) were used. The PCR product (794 bp) was isolated by electrophoresis in a 1% agarose gel, purified with the QiaQuick gel extraction kit (Qiagen) and digested with *Nde*I and *Bam*HI. The insert was then cloned into the *Nde*I and *Bam*HI digested expression vector pET3a¹¹ (Novagen, Madison, WI, USA) by the Rapid DNA Ligation Kit (Boehringer Mannheim, Mannheim, Germany). The pET3a-*PMM2* construct was subsequently transformed into *E. coli* NovaBlue and purified using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The mutations R141H, F119L, D223E, C192G, T237R and G117R were introduced into *PMM2*-cDNA by site-specific mutagenesis,¹² purified and cloned as mentioned above. All constructs (inserts) were sequenced in both directions to rule out any PCR errors and to confirm the introduction of mutations, using the BigDye Terminator Cycle sequencing Kit, ABI Prism 310 (Perkin Elmer Applied Biosystems). The purified plasmid constructs were used to transform *E. coli* B121 (DE3) cells, and the transformed cells were grown in LB medium containing 50 µg/ml ampicillin, at 37°C, until OD₆₀₀ reached 0.6. Isopropylthiogalactoside (IPTG) was then added to a final concentration of 0.4 mM, and incubation was continued for 3 h. The cells were harvested by centrifugation and stored at -80°C until further use.

Phosphomannomutase Assay

Cell extracts of *E. coli* were prepared by resuspending the cell pellet in a buffer containing 20 mM Hepes, 25 mM KCl, 1 mM dithiothreitol, and leupeptin and antipain each at a concentration of 10 µg/ml. The suspension was sonicated 2 × 10 seconds and centrifuged at 10 000 g for 10 min. The resulting extracts were used for assaying *PMM* by the method described by Van Schaftingen and Jaeken¹⁰ with the exception that glucose 1,6-diphosphate (Sigma, St. Louis, MO, USA) instead of mannose 1,6-diphosphate was used as cofactor. The rate of NADPH formation was followed for 5 min. One unit equals the formation of 1 nmol NADPH/min.

***K_m* Estimation** The affinity for mannose 1-phosphate was determined for wild type, F119L, C192G and D223E *PMM2*. The rate of NADPH formation was followed for 5 min with increasing concentration of mannose 1-phosphate keeping constant the concentration of glucose 1,6-diphosphate.

Temperature Stability The cell extracts were incubated for 10 min at 37, 39, 41, 43 and 45°C and then kept on ice until determination of *PMM* activity.

Protein was determined using the method of BCA Protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Results

Table 1 shows the spectrum of Danish *PMM2* mutations including one novel mutation in exon 7, 574T → G (C192G). Except for one patient homozygous for F119L, all are compound heterozygotes.

Both wild type and mutant *PMM2* were successfully produced in *E. coli*. After 3 h of IPTG induction more than 90% of the *PMM2* protein was found in the soluble protein fraction, as determined by SDS-PAGE analysis (data not shown). All cDNA constructs seemed to give the same yield of *PMM2* protein.

The specific activities of wild type and mutant *PMM2* are shown in Table 2. The catalytic activity of wild type *PMM2* was 850 U/mg, whilst induction of the vector without insert gave an activity in the range of 33–56 U/mg. The R141H, G117R and T237R *PMM2* gave no detectable additional activity, ie, the activity

Table 1 *PMM2* mutations in 22 unrelated CDG1A patients of Danish origin

<i>PMM2</i> genotype	No. of patients
R141H /F119L	18
R141H /C192G	1
G117R /F119L	1
F119L/F119L	1
T237R /D223E	1

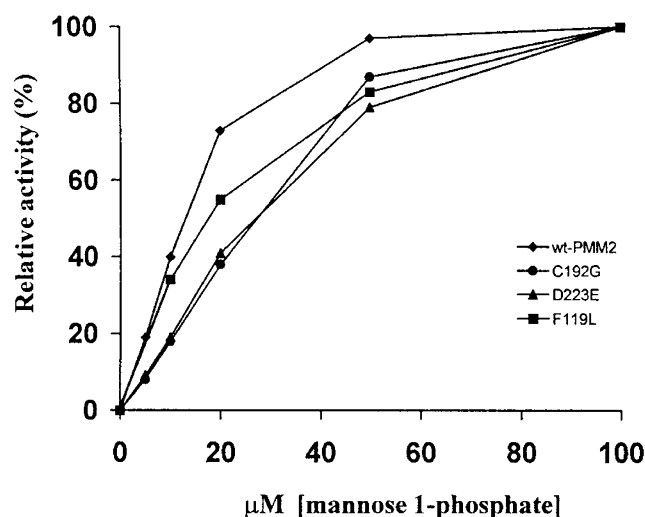
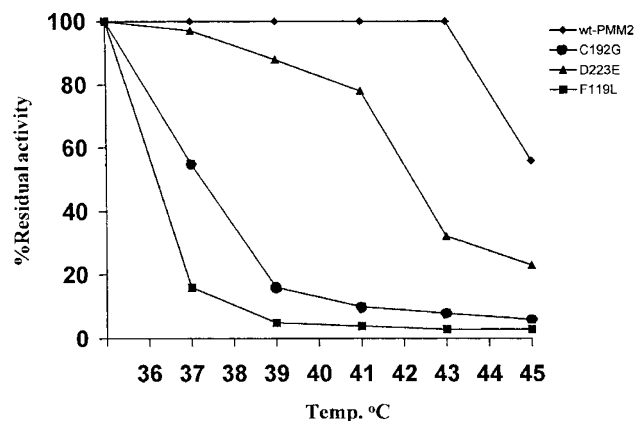
The recombinant proteins corresponding to the mutations in bold have no detectable *PMM2* activity

Table 2 Specific activities of recombinant wild type and mutant *PMM2*

Vector	Specific activity nmol/mean min/mg	Range
pET3a	45	(33-56)
pET3a-wtPMM2	849	(718-1046)
pET3a-F119L	217	(143-289)
pET3a-R141H	47	(31-63)
pET3a-G117R	45	(29-61)
pET3a-D223E	1022	(896-1152)
pET3a-T237R	38	(25-50)
pET3a-C192G	1101	(1009-1193)

was at the same level as if the vector had no insert. The activity of the F119L *PMM2* was 25% of that of the wild type. Surprisingly, the activities of the D223E and C192G *PMM2* were found to be in the high normal range or above.

The D223E and C192G mutations were found in two patients with the genotype T237R/D223E and R141H/C192G, respectively. In order to exclude the presence of other mutations, cDNA from both alleles of the patients were cloned and sequenced. Each patient had the mutations in trans position, and no other mutation was detected in the coding sequences of *PMM2*. Both cDNA alleles were of the correct size, and analysis of cDNA by agarose gel electrophoresis did not show any sign of alternative splicing. To characterise wild type and mutant proteins the apparent K_m values for mannose 1-phosphate (Figure 1) as well as the temperature inactivation patterns were determined (Figure 2). The K_m value of wild type *PMM2* was 17 μM .

**Figure 1** Saturation curves of recombinant wild type and mutant *PMM2* with mannose 1-phosphate. The velocities are expressed as % of the activity at 100 μM mannose 1-phosphate**Figure 2** Temperature inactivation curves of recombinant wild type and mutant *PMM2*. The enzymatic activities are expressed as % of the activity of the protein without temperature inactivation

The apparent K_m of the F119L *PMM2* was close to that of the wild type, whilst the G192C and D223E *PMM2* had overlapping saturation curves indicating a lower affinity for mannose 1-phosphate.

Incubation of crude cell extracts at different temperatures (range 37–45°C) showed that wild type *PMM2* was relatively temperature resistant and that mutant proteins were inactivated rapidly by increasing temperatures. The temperature at which 50% activity remained was much lower for the C192G and the F119L *PMM2* than for the wild type, whilst the activity of the D223E *PMM2* seemed more temperature resistant than that of the other mutant proteins.

Discussion

The six missense mutations identified in the Danish CDG1A patients result in replacement of amino acids which are conserved strictly among phosphomannomutases of yeast (*SEC53*), *Candida albicans* and human phosphomannomutases (*PMM1* and *PMM2*),^{2,13} suggesting that these amino acids are crucial for the catalytic activity of the enzyme.

In order to explore the relationship between the mutant genes and their corresponding enzymes, we introduced all six mutations in *PMM2*-cDNA and expressed the recombinant proteins in *E. coli*. The specific activities measured suggest the presence of two types of mutation. One type results in proteins with zero activity and the other type results in proteins with decreased activity and/or altered characteristics such as

decreased affinity for the substrate and less thermostability.

R141H is the prevalent *PMM2* mutation in the European population.^{3,4} So far R141H has not been observed in the homozygous form, although an incidence of approximately 1:20 000 would be expected on the basis of the frequency of the R141H allele in the European population (E Schollen *et al* 1999, submitted). At present there is no basis for assuming that homozygosity for R141H conveys a clinical phenotype different from classical CDG1A. F119L has been found in approximately 50% of Scandinavian patients, usually in the compound heterozygous form, but a few homozygous patients have been observed.^{4,5,7} So far, T237R, G117R, D223E and C192G have been seen only in compound heterozygous patients, and the latter three mutations only in Danish patients.^{3,4}

Expression of the R141H protein as well as the G117R and the T237R proteins showed repeatedly no measurable residual *PMM2* activity. The mutant proteins were present in the soluble cellular fraction in quantities comparable to that of the wild type protein. When observed in patients, each of these three mutations was found in combination with a mutation with detectable *PMM2* activity, F119L, C192G or D223E (Table 1). Taken together, these results support the notion that lack of *PMM2* activity is incompatible with life. The fate of the zygote with the R141/R141H genotype is unknown. Due to the fundamental importance of *PMM2* in normal cellular functions, it is most likely that the zygote is lost in early pregnancy.

Surprisingly, expression of the C192G and the D223E *PMM2* showed activities in the normal range or higher. Sequencing of the coding region of *PMM2* of these alleles excluded the presence of other mutations which could account for the clinical phenotype. Subsequent studies of the affinity of the mutant proteins for mannose 1-phosphate and their temperature stability did in fact reveal altered properties of the recombinant C192G and D223E *PMM2*. Although we used crude *E. coli* extracts, our results suggest that the mutations interfere also *in vivo* with the properties of the *PMM2* thereby accounting for the clinical phenotype of the patients.

Provided that mannose 1-phosphate is required for formation of GDP-mannose and that no alternative pathway exists for synthesis of mannose 1-phosphate, residual activity would be expected in mutant *PMM2* since the majority of N-linked carbohydrate chains is present on serum proteins of CDG1A patients. Thus

far, alternative pathways to generate mannose 1-phosphate have not been described although the role of *PMM1* is still a puzzle. Recent studies in the rat have shown that *PMM2* appears to be practically the sole *PMM* present in most tissues.¹⁴ The lack of rescue by *PMM1* in man might explain the multi-organ dysfunction of patients with CDG1A. Each of our patients has at least one mutation which retains *PMM2* activity when expressed in *E. coli* (Table 1). This residual activity, however, is not reflected in the specific activities found in cultured fibroblasts derived from the patients.⁴ Even the patient homozygous for F119L has undetectable *PMM* activity in extracts of cultured fibroblasts (data not shown). *PMM* of man is presumed to be a dimer, as in yeast *SEC53*,¹⁵ and three different subunit combinations should therefore be present in compound heterozygous patients. The very low, or lack of, measurable *PMM* activity in fibroblasts could indicate a dominant-negative effect of the null activity polypeptides, resulting in a final catalytic activity less than expected from the genotype. However, due to the very low activity of *PMM* even in control fibroblasts, it is not possible to correlate the residual *PMM* activities with the genotypes.

Our results support the hypotheses that homozygosity for R141H impairs *PMM2* activity to a degree incompatible with life, whilst F119L or other 'mild' mutations convey a phenotype compatible with survival due to residual *PMM2* activity.

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