Letters to the Editor

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Phosphomannose Isomerase Deficiency: A Carbohydrate-Deficient Glycoprotein Syndrome with Hepatic-Intestinal Presentation

To the Editor:

Carbohydrate-deficient glycoprotein (CDG) syndromes are genetic diseases that are due to defects in the glycosylation of glycoproteins (Jaeken et al. 1993, 1997*b*). With one exception (Billette de Villemeur et al. 1995), all reported patients presented moderate to severe brain disease (Jaeken and Casaer 1997). The diagnosis is usually made by isoelectrofocusing of serum transferrin showing different patterns of cathodal shift due to the deficiency of the terminal sialic acid (Jaeken et al. 1984; Stibler and Jaeken 1990). The majority of patients show the so-called type I pattern characterized by an increase of di- and asialotransferrin. About 70% of patients with the type I pattern have a deficiency of phosphomannomutase (PMM) (type IA) (Van Schaftingen and Jaeken 1995; Jaeken et al. 1997*a*), the enzyme catalyzing the second step in the conversion of fructose 6-phosphate to GDP-mannose (fig. 1), and mutations in the PMM2 gene have been found in all patients with a PMM deficiency (Matthijs et al. 1997*a,* 1998).

Among the patients from which we analyzed the fibroblasts (Van Schaftingen and Jaeken 1995; Jaeken et al. 1997*a;* Matthijs et al. 1997*b*), ~30 did not show PMM deficiency. On the rationale that type I glycosylation pattern may be due to other enzymatic defects in the pathway leading to GDP-mannose, the activity of phosphomannose isomerase, which catalyzes its first step, was systematically analyzed in the samples from the latter patients. As shown in table 1, three of them showed a marked deficiency in this enzyme, whereas PMM activity was normal. Experiments in which extracts were mixed indicated that the deficiency was not due to the presence of an inhibitor. As reported elsewhere (Van Schaftingen and Jaeken 1995), PMI activity was normal in PMM-deficient patients. For patient C, the PMI deficiency was independently found at about the same time by other investigators (Niehues et al., 1998).This patient, who had a similar liver disease associated with protein-losing enteropathy (Freeze et al. 1997; Niehues et al., 1998) as patient A (see below) was not further investigated in the present study. The parents of patient B, but not of patient A, were available for testing; surprisingly, normal activities of PMI were found

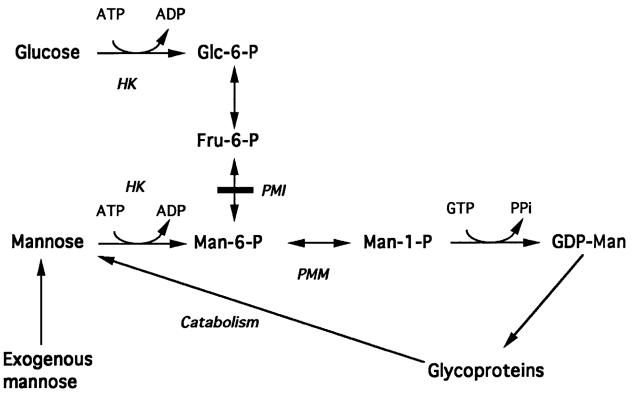

Figure 1 Scheme of mannose metabolism. Phosphomannose isomerase = PMI; phosphomannomutase = PMM; hexokinase = HK. The horizontal bar indicates the defect in phosphomannose isomerase.

Table 1

Enzyme Activities in Fibroblasts (in mU/mg protein) of Controls, Patients with Phosphomannomutase Deficiency, and Three Patients with Phosphomannose Isomerase Deficiency

	PMI Activities	PMM Activities
Controls	6.8 ±2.4 (7)	3.8 ± .9 (8)
PMM deficient	8.0 ± 4.4 (13)	.5 ± .3 (13)
Patient A	.2, .3	6.3
Patient B	.2, .8	3.2
Patient C	.2, .2	3.5

NOTE.—Phosphomannomutase and protein were measured as described elsewhere (Van Schaftingen and Jaeken 1995; Jaeken et al. 1997*a*). Phosphomannose isomerase was assayed at 30°C in a reaction mixture (1 ml) containing 50 mM Hepes, pH 7.1, 5 mM $MgCl_2$, 25 mM KCl, 1 mM dithiothreitol, 0.6 mM NAD^+, 0.5 mM mannose 6-phosphate, 2.5 U/ml glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 10 μg/ml phosphoglucose isomerase with 10 μl of an extract containing 5–20 mg protein/ml. Control and PMM deficient measures are mean values ± SD. Where two data are given, the values were obtained on two different subcultures.

in their leukocytes (mother 5.5 mU/mg protein; father 6.7 mU/mg protein; controls 5.4 ± 0.9 for $n = 6$).

On the basis of the available sequence of the PMI cDNA (Proudfoot et al. 1994), primers were designed for the amplification and analysis of PMI. Primers PMI-Biot (5′-Biotin-CGA GCA TGG CCG CTC CGC-3′) and PMI-R2 (5′-CCA GCT CTT CCA GGG TAT AC-3′) were used to amplify the PMI cDNA from total RNA isolated from fibroblasts according to established procedures (Sambrook et al. 1989; Matthijs et al. 1997*b*). The amplified fragment was sequenced using three fluorescently labeled primers: PMI-F1: 5′-FITC-CCA ATC AGG AAC TGA AAC TC-3′, PMI-F2: 5′-FITC-ACA GGG TTG GCA CTT CAA TG-3′, and PMI-F3: 5′-FITC-GAA TTT AGG GTG GCT GGC AG-3′. The cDNA fragment generated using primer PMI-R2 and PMI-F1 (identical to PMI-Biot but lacking the biotin) was cloned in pCR.2 by use of the TA-cloning kit (Invitrogen). The mutations were retrieved by sequencing with PMI-F1. To confirm the phase of the mutations, two clones representing the two alleles of the patients were entirely sequenced using the previously described primers and the M13-reverse and universal primers.

Patient A was found to be compound heterozygous for a C→T mutation at position 304, causing a Ser→Leu substitution at codon 102, and a T→C mutation at position 413, replacing Met at codon 138 by Thr (fig. 2). Both mutations concern positions that are conserved from *Saccharomyces cerevisiae* and *Candida albicans* to human PMI (Proudfoot et al. 1994). The corresponding amino acids are situated near the active site, as determined by X-ray crystallography of the *C. albicans* enzyme (Cleasby et al. 1996). The Glu residue adjacent to Met138 (Glu138 in *C. albicans* corresponding to Glu137 in human PMI) is one of the four residues involved in binding the catalytic Zn^{2+} ion. The Lys residue at position 100 in *C. albicans* (corresponding to Lys99 in human PMI) points inward into the active site, and the mutation at Ser102 may well affect this orientation. Thus, the mutations in the PMI gene are likely to explain the lack of activity of this enzyme in patient A.

Except for a polymorphism at codon 377 (GTA/GTG, both coding for Val), no sequence variation was detected in PCR-amplified cDNA from patient B. The presence of the polymorphism allowed us to select and fully sequence two clones derived from different alleles. Since there is no mutation, the low activity in fibroblasts of patient B could be due to a decrease in the transcription of the gene because of mutations either in the promoter or in a transcription factor required for the expression of the enzyme. The finding of a normal activity in leukocytes of the parents argues for this second possibility, if at least the same isozyme is expressed in fibroblasts and leukocytes. The fact that the supposed genetic defect in patient B is different from that in patient A probably explains why the enzyme deficiency is not as pronounced in the former.

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canal  95 LPFLFKVLSIEKVLSIQAH 113
sacce  93 LPFLFKVLSIEKVLSIQAH 111
emeni  91 LPFLFKVLSIRKALSIQAH 109
caeel  83 LSFLFKVLSVLGPLSIQIH 101
human  94 LPFLFKVLSVETPLSIQAH 112
                L       * *

canal 129  NYPDDNHKPEMAIAVT 145
sacce 127  NYPDDNHKPEMAIAVT 143
emeni 125  NYPDDNHKPEMTIAIT 141
caeel 117  NYPDDNHKPEIAIALT 133
human 128  HYPDANHKPEMAIALT 144
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Figure 2 Sequence alignment showing the position of the mutations in patient A. The sequences of the enzymes from *Candida albicans* (canal), *Saccharomyces cerevisiae* (sacce), *Emericella nidulans* (emeni), *Caenorhabditis elegans* (caeel), and man (human) are shown. Numbering of the residues starts from the initiator methionine. Conserved residues are in bold. Three of the four residues liganding Zn^{2+} in the *C. albicans* enzyme are indicated by an asterisk (*). The mutated residues are shown below the alignment.

The clinical description of patient A has been briefly reported elsewhere (Billette de Villemeur et al. 1995). He was the third child of unrelated Lebanese parents. His siblings were healthy. He was admitted at the age of 5.5 mo for chronic diarrhea since the age of 3 mo and hypoglycemia with convulsions, coma, and apnea. There was no dysmorphy. Liver was 6 cm below the costal margin and firm. Stool culture showed *Pseudomonas aeruginosa*. Hypoglycemia tended to recur particularly after 4 h following feeding. Liver biopsy revealed fibrosis of the portal spaces and microvesicular steatosis. At the age of 10 mo, he was readmitted with generalized edema secondary to hypoalbuminemia. Weight was 6.7 kg ($< P_3 = 7.8$), length 65 cm ($< P_3 = 69$), and head circumference 43 cm ($< P_3 = 44.2$). Gross motor development was moderately retarded. Liver was unchanged. Liver function testing showed slightly increased bilirubin (50 mM) and glutamate-oxaloacetate transaminase (82 U/liter). IgG (2.6 g/liter) and IgA (0.32 g/liter) were decreased. Prothrombin time was 63%. Several clotting and anticlotting factors were decreased: factor XI (20%), antithrombin III (45%), protein C (29%) and total protein S (51%). There was intermittent slight proteinuria and moderate hyperaminoaciduria (particularly of glycine). Further extensive metabolic screening did not reveal the cause of the hypoglycemias. Serum transferrin isoelectrofocusing, however, showed the typical CDG syndrome type I pattern (decreased hexa-, penta- and tetrasialotransferrin, and increased di- and asialotransferrin) but phosphomannomutase activity in fibroblasts was normal. Radiological examination of the intestine suggested malabsorption, with fragmentation

and flocculation of the contrast substance. Sweat test was normal as well as intestinal disaccharidase activities. On endoscopy, fragility of the intestinal mucosa with easy bleeding was noted. Histology of duodenal biopsies showed partial villus atrophy with hypercellularity and only rare and discrete lymphangiectasias. Repeat liver biopsy at the age of one year showed a more pronounced picture of congenital hepatic fibrosis with stellate fibrosis of the portal spaces and proliferation of numerous biliary canaliculi without any inflammation and without notable alteration of the hepatocytes. Renal hyperechogenicity was found on ultrasound examination. Treatment consisted of Diazoxide, which normalized glycemia, regular intravenous administration of immunoglobulins and albumin, and nasogastric tube feeding with only a hydrolyzed, MCT-supplemented milk. He suffered from frequent bacterial as well as viral gastroenteritis. At the age of 26 months, weight was 9 kg ($< P_3 = 10.8$) and length 77.5 cm ($< P_3 = 82$). He had a large abdomen with pronounced collateral circulation, numerous disseminated angiomas, and unchanged hepatomegaly. Since oral feeding was still not supported, the nasogastric tube feeding was changed to tube feeding via a gastrostomy. Neurological examination was normal. He was last seen at the age of 2 9/12 years with persisting protein-losing enteropathy. However, psychomotor development was normal. He died at the age of 4 years from an unknown cause.

Patient B had a similar clinical picture consisting mainly of liver disease, which disappeared after the introduction of solid food at 3 mo. The case will be more extensively presented elsewhere.

PMM deficiency results in a multisystem disease with severe neurological involvement and dysmorphy, besides, usually mild, liver, intestinal, and other organ disease (Jaeken et al. 1993, 1997*a,* 1997*b*). PMI deficiency causes the same kind of sialotransferrin electrofocusing pattern as PMM deficiency, but its clinical presentation appears to be quite different in that the disease was enterohepatic in patients A and C and hepatic with mild neurological signs in patient B. Different explanations can be provided for the fact that the clinical manifestations of PMI deficiency are mainly hepatic and intestinal. A first one is the possible existence of different isozymes of PMI, as is the case for PMM (Matthijs et al. 1997*a,* 1997*b;* Pirard et al. 1997). The cDNA that has been identified and sequenced by Proudfoot et al. (1994) appears to be derived from a gene that is only poorly expressed in liver, as shown by northern blot analysis. Still, the fact that mutations in this gene lead to liver involvement in patient A indicates that this form of PMI is expressed in liver. Furthermore, all human expressed sequence tags found in data banks seem to be derived from this gene, except maybe one that could possibly encode an isozyme. However, we have been unable to PCR-amplify human liver and leukocyte cDNA with primers designed on the basis of this sequence.

A second explanation stems from the different position of PMM and PMI in the pathway leading to the formation of GDP-mannose. Both enzyme deficiencies are expected to cause a decrease in the biosynthesis of dolichol-pyrophosphate-oligosaccharide in the endoplasmic reticulum (Powell et al. 1994; Krasnewich et al. 1995), and both defects are indeed known to be lethal in yeast (Kepes and Schekman 1988). There is, however, a major difference between PMM and PMI in that the latter, but not the former, can be by-passed by the low-K_m hexokinases. These enzymes can indeed convert mannose to mannose 6-phosphate, displaying for this substrate a rather high affinity with K_m 5 μM in the case of the brain enzyme hexokinase I (Sols and Crane 1954). In contrast, glucokinase—also known as "hexokinase IV"—the major, if not the only, hexokinase present in hepatocytes (Reyes and Cardenas 1984) has a very low affinity for mannose with a K_m equal to 33 mM according to Weinhouse (1976) and is therefore rather inefficient to phosphorylate this substrate at the concentrations prevailing in plasma (50 μM in controls; Etchison and Freeze 1997). Blood mannose, which derives from glycoprotein degradation and alimentary mannose in unknown proportion, is therefore most likely sufficient to support N-glycosylation in tissues other than the liver. The exception appears also to apply to the intestinal mucosa, as evidenced by the marked enteropathy found in patients A and C (Niehues et al. 1988; Freeze et al. 1997). Remarkably, the specific activity of PMM is more than four times higher in the rat mucosa than in other tissues, which indicates that its GDP-mannose requirements are elevated (M. Pirard and E. Van Schaftingen, unpublished data). Furthermore, it is a tissue in which the glucose concentration is elevated, due to active absorption from the intestinal lumen and, during postabsorptive period, to equilibration with blood glucose through Glut2 (Thorens et al 1988). Glucose can therefore competitively inhibit the phosphorylation of mannose. Difference in tissue involvement of PMI deficiency could also possibly arise from difference in expression of the specific, low K_m mannose transporter (Panneerselvam and Freeze 1996), which appears to be involved in the preferential utilization of radiolabeled mannose over radiolabeled glucose in cultured fibroblasts (Panneerselvam et al. 1997).

A logical consequence of the fact that hexokinase phosphorylates mannose to mannose 6-phosphate is that PMI deficiency, unlike PMM deficiency (Jaeken and Casaer 1997; Marquardt et al. 1997; Mayatepek et al. 1997), should be treatable by administration of mannose supplements. This appears to be the case for patient C (Niehues et al. 1988; Freeze et al. 1997), in which we

have also found PMI deficiency. Unfortunately, patient A died before trials with mannose could be attempted. It would be interesting to know if the improvement of the symptomatology observed in patient B following introduction of solid food is due to the presence of mannose in the latter.

Because of its very different clinical presentation, this new type of CDG syndrome was probably not recognized as a glycosylation defect until now. In 1985, the same clinical picture as that of patient A and C was reported as a new syndrome in four infants from the northeastern part of Quebec. They died between the ages of 4 and 21 mo. They had also antithrombin III deficiency (Pelletier et al. 1985), a typical feature of CDG syndromes. We strongly suspect that these patients had a PMI deficiency. In conclusion, PMI deficiency is a newly recognized, lethal but potentially treatable CDG syndrome that should be searched for in unexplained enteropathy and/or liver disease.

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Temperature-Sensitive Phenotypes of Peroxisome-Assembly Processes Represent the Milder Forms of Human Peroxisome-Biogenesis Disorders

To the Editor:

Peroxisome-biogenesis disorders (PBDs) are lethal hereditary diseases caused by abnormalities in the assembly processes of peroxisomes (Moser et al. 1995). The peroxisome is a ubiquitous organelle involved in vital metabolic functions, such as oxidative processes involving H_2O_2, β-oxidation of fatty acids, and biosynthesis of plasmalogens (Van den Bosch et al. 1992). PBDs are characterized by multiple defects in these functions, as well as by the lack of morphologically normal peroxisomes. They are genetically classified into complementation groups (CGs), the number of which is $\geqslant 11$ (Shimozawa et al. 1993; Moser et al. 1995; Poulos et al. 1995). Each CG contains significantly different clinical phenotypes—for example, Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). ZS patients have severe neurological abnormalities, dysmorphic features, hepatomegaly, and multiple renal cysts, and most die at age <6 mo. NALD patients have similar symptoms, but they survive considerably longer, dying during early childhood. In contrast, IRD patients do not exhibit significant abnormalities in the CNS, and they have the longest average life span among patients with PBDs (Lazarow and Moser 1995; Moser et al. 1995). Although the causal genes (*PEX*s) for several CGs have been cloned and the mutations have been identified at the molecular level (Shimozawa et al. 1992 ; Dodt et al. 1995; Wiemer et al. 1995; Fukuda et al. 1996; Yahraus et al. 1996; Chang et al. 1997; Okumoto and Fujiki 1997; Portsteffen et al. 1997; Reuber et al. 1997), it is unknown why such diverse clinical phenotypes occur in the same CGs although, in all CGs, the phenotypes are very similar. We report that milder forms of PBDs are characterized by temperature-sensitive (TS) phenotypes of peroxisome-assembly processes in the fibroblasts of patients.

In spite of the variations in the clinical features, the fibroblasts from patients of all three PBD phenotypes generally lack peroxisomes. Although the occurrence of a reduced number of peroxisomes occasionally has been noted in several PBD cell lines (Arias et al. 1985; Wiemer et al. 1991; Slawecki et al. 1995), no correlation with clinical features has been apparent. We assumed that limited types of leaky mutations in the *PEX* genes could be the causes of the milder forms of PBDs. As a possible parameter representing such leakiness, we examined temperature sensitivity. Fibroblasts from PBD patients with different CGs were incubated at 30°C and at 37°C and were subjected to immunofluorescence staining with anti-catalase antibody. After 72 h incubation at 30°C, punctate staining of catalase typical of peroxisomes was detected in the fibroblasts of all six patients with IRD and in three of five of those with NALD, belonging to four different CGs (fig. 1*b* and table 1), whereas no peroxisomes appeared in the same cells after incubation at 37°C (fig. 1*a*). Catalase and the 70-kD peroxisomal membrane protein (PMP70) were colocalized in these