MAMMALIAN PHOSPHOMANNOMUTASE 1 (PMM1) IS BRAIN, IMP-SENSITIVE GLUTOSE-1,6-BISPHOSPHATASE

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Glucose 1,6-bisphosphate (Glc16P2) concentration in brain is much higher than what is required for the functioning of phosphoglucomutase, suggesting that this compound has (an)other role(s) to play than to be a cofactor of phosphomutases. In cell-free systems, Glc16P2 is formed from 1,3-bisphosphoglycerate and glucose 6-phosphate by two related enzymes, PGM2L1 (phosphoglucomutase 2-like 1) and to a lesser extent, PGM2 (phosphoglucomutase 2). It is hydrolyzed by the inosine-monophosphate (IMP)-stimulated brain glucose-1,6-bisphosphatase of still unknown identity. Our aim was to test if glucose-1,6-bisphosphatase corresponded to phosphomannomutase 1 (PMM1), an enzyme of mysterious physiological function sharing several properties with glucose-1,6-bisphosphatase. We show that IMP, but not other nucleotides, stimulated by more than 100-fold (Ka ≈ 20 µM) the intrinsic glucose-1,6-bisphosphatase activity of recombinant PMM1, while inhibiting its phosphoglucomutase activity. No such effects were observed with PMM2, the enzyme paralogous to PMM1 that physiologically acts as a phosphomannomutase in mammals. Transfection of HEK293T cells with PGM2L1, though not with the related enzyme PGM2, caused a ≈ 20-fold increase in the concentration of Glc16P2. Transfection with PMM1 caused a profound decrease (more than 5-fold) in Glc16P2, both in cells that were or were not co-transfected with PGM2L1. Furthermore, the concentration of Glc16P2 in brain from wild-type mice decreased with time after ischemia while it did not change in brain from PMM1-deficient mice. Taken together, these data show that PMM1 corresponds to the IMP-stimulated glucose-1,6-bisphosphatase and that this enzyme is responsible for the degradation of Glc16P2 in brain. In addition, the role of PGM2L1 as the enzyme responsible for the synthesis of the elevated concentrations of Glc16P2 in brain is established.

Glucose 1,6-bisphosphate (Glc1,6P2) a well-known cofactor for phosphoglucomutase and other sugar phosphomutases (1), is ubiquitously present in tissues. Its concentration is particularly elevated in brain, where it reaches values > 100 µM (2), i.e. more than 1000-fold higher than the concentrations required to stimulate phosphoglucomutase. Glc16P2 has been proposed to be an effector for several enzymes. Phosphofructokinase (3, 4) and liver pyruvate kinase (5) are both stimulated by this compound, whereas low-Km hexokinases (6, 7, 8), 6-phosphogluconate dehydrogenase (9) and fructose-1,6-bisphosphatase (10) are inhibited. These effects have been demonstrated in vitro, but under conditions that are not necessarily physiologically relevant. In addition, the occurrence of this regulation in intact cells has not been demonstrated.

Glc16P2 is synthesized from 1,3-bisphosphoglycerate and glucose 1-phosphate or glucose 6-phosphate by Glc16P2 synthase, an enzyme particularly abundant in brain (11) and recently identified as PGM2L1 (phosphoglucomutase 2-like 1) (12). In vitro, the related enzyme phosphoglucomutase 2 (PGM2), which shares about 60 % identity with PGM2L1 and mainly acts as a phosphopentomutase, also catalyzes the synthesis of Glc16P2, though with a lower Vmax than PGM2L1 and a much stronger
inhibition by the reaction product Glc16P₂. In comparison to PGM2, PGM2L1 is therefore better suited to provide cells with elevated concentrations of Glc16P₂, though this still deserves to be demonstrated in intact cells.

Glc16P₂ is degraded by glucose-1,6-bisphosphatase. The brain enzyme, which was best characterized (13, 14), is dependent for its activity on the presence of inosine-monophosphate (IMP), the concentration of which increases in anoxia. This effect is presumably responsible for the decrease in Glc16P₂ concentration in brain during anoxia (2).

Brain glucose-1,6-bisphosphatase, although not yet molecularly identified, has several characteristics (13, 14) that may help to identify its sequence. It catalyzes an exchange reaction between glucose 6-phosphate and Glc16P₂, indicating that the reaction mechanism involves the formation of a phosphoenzyme. It also acts as a mannose-1,6-bisphosphatase and displays some phosphoglucomutase activity. Finally, the molecular mass of this enzyme, as determined by gel filtration, is ≈87 kDa.

These four properties are reminiscent of those of phosphomannomutase 1 (PMM1), an enzyme belonging to the Haloacid Dehalogenase (HAD) family of phosphatases/phosphomutases, with a reaction mechanism involving a phosphoenzyme intermediate (15). Interestingly, PMM1 shares 66% sequence identity with phosphomannomutase 2 (PMM2), a specific phosphomannomutase (16) that is deficient in the most frequent form of Congenital Disorders of Glycosylation (CDG Ia) (17, 18). In contrast to PMM2, PMM1 is less specific: it has nearly equal phosphomannomutase and phosphoglucomutase activities as well as a modest glucose-1,6-bisphosphatase activity corresponding to about 3% of its phosphomannomutase activity (16). However, the effect of IMP on PMM1 activity has never been tested. PMM1, just like PMM2, was once thought to be involved in the in vivo formation of mannose-1-phosphate needed for glycoprotein biosynthesis. However, gene knock-out studies in mice have shown that PMM2 deficiency is early-lethal (19), whereas PMM1 deficiency does not lead to any pathological findings (20). As PMM1 is often present in the same cell types as PMM2 (21), these findings indicated that despite its phosphomannomutase activity, PMM1 cannot substitute for PMM2 in PMM2-deficient mice, suggesting that PMM1 has another physiological role.

The purpose of the present work was to establish whether the enzyme that catalyses the hydrolysis of Glc16P₂ in brain indeed corresponds to PMM1, and to identify which of the two enzymes, PGM2L1 and PGM2 is able to make elevated concentrations of Glc16P₂ in intact cells.

**Experimental Procedures**

**Overexpression and purification of mouse PMM1 and PMM2** - To produce recombinant mouse PMM1, 1 l of E. coli BL21(DE3) pLysS harbouring pET-3d containing the full-length mouse PMM1 cDNA was grown and PMM1 overexpressed and purified as described in (21, 22). Upon DEAE Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) chromatography, we used the most purified fraction (90% purity as indicated by SDS-PAGE analysis) eluted with approximately 150 mM NaCl for the characterization of the kinetic properties of PMM1. For the production of recombinant mouse PMM2, the coding region of PMM2 cDNA was cloned in pET-15b using an NdeI (containing the initial ATG site) and a BamHI (placed after the STOP codon) restriction sites. After confirmation of the sequence by sequencing, the plasmid was used to transform E. coli BL21(DE3) pLysS. The culture was grown in LB rich medium at 30°C and PMM2 with a N-terminal (His)₆ tag was overexpressed for 18h after addition of 0.4 mM isopropylthiogalactoside (IPTG). Preparation of the bacterial extract and purification of the (His)₆ tagged PMM2 on a 1 ml HisTrap column (Ni²⁺ form) (GE-Healthcare Life Sciences, UK) was done as previously described (23). PMM2 was eluted (as indicated by SDS-PAGE analysis) with 20 ml of 150 mM imidazole, concentrated 4-fold on two 15 ml ultrafiltration Vivaspin columns (Sartorius, Stockport, UK) (10 ml/column) and finally desalted on two PD-10 columns (GE-Healthcare Life Sciences, UK) equilibrated with 25 mM Hepes, pH 7.2, 30 mM NaCl, 1 mM dithiothreitol (DTT) and 2 μg/ml leupeptin and antipain. Protein concentration was estimated by measuring A₂₈₀ assuming a molar absorptivity of 20,775 M⁻¹ cm⁻¹.
1 cm⁻¹. Purified PMM2 (30 mg of pure protein/l of culture) was supplemented with 10% glycerol and stored at -70°C.

Expression of human PGM2 and PGM2L1 and of mouse PMM1 and PMM2 in HEK293T cells - The open reading frames encoding human PGM2 or PGM2L1 and mouse PMM1 or PMM2 where PCR-amplified (primer sequence available upon request: maria.veiga@uclouvain.be). The template DNAs for the polymerase reactions were the corresponding bacterial expression plasmids described above (PMM1 and PMM2) and in (12) for PGM2 and PGM2L1. After cloning the PCR-amplified fragments in pBluescript and excluding any PCR errors by sequencing, PGM2 and PGM2L1 inserts were ligated into the eucaryotic expression vector pEF6/Myc-His A (Gibco/Invitrogen, Merelbeke, Belgium) using EcoRV and NotI (PGM2) or EcoRI and EcoRV (PGM2L1) restriction sites, allowing the production of a protein with a C-terminal (His)₆ tag. PMM1 and PMM2 inserts were ligated into the pEF6/His A (Gibco/Invitrogen, Merelbeke, Belgium) expression vector using KpnI and NotI restriction sites, allowing the production of proteins with an N-terminal (His)₆ tag. HEK293T cells were transfected (8 µg of DNA) or co-transfected (2 x 4 µg of DNA) using the jetPEI transfection kit (Polyplus transfection, Illkirch, France) as previously described (24). We used 4 culture dishes per condition tested: triplicates to measure Glc16P₂ concentration and one dish to confirm protein expression. After 48 h incubation at 37°C, the medium was completely removed and the proteins were immediately denatured in 3 dishes by addition of 400 µl of ice-cold 5% HClO₄. The cells of the fourth dish were washed with phosphate buffer saline and harvested in 500 µl of 20 mM Hepes (pH 7.1) containing 5 µg/ml leupeptin and antipain. The HClO₄ extracts were prepared by recovering the suspension from the dish and centrifuging at 4°C for 5 min at 16 000 x g. To quantify the proteins, the pellets were re-solubilized in 200 µl of 0.2 M NaOH and protein concentration measured using γ-globulin as a standard (25). The supernatant was recovered, neutralized with 3M K₂CO₃ and used to assay Glc16P₂ after elimination of the salt precipitate by centrifugation. In the fourth dish, proteins were extracted as described in (24) and expression of all four (His)₆ tagged proteins was quantified by western blot analysis using Penta-His (Qiagen GmbH, Hilden) monoclonal antibody.

Assay of Glc16P₂ from mouse tissues - Tissues were removed from wild-type or PMM1-deficient mice-anesthetized by inhalation with sevoflurane and immediately frozen-clamped. When various tissues were taken from the same animal, brain was always the first one to be removed, followed by kidney, liver, lung and muscle. When blood was collected, we used a different set of mice. Neutralized HClO₄ extracts were prepared by homogenizing the frozen tissues (or 300 µl of freshly collected whole blood) in 3 vol (w/v) of ice-cold 5% HClO₄, centrifuging (10 min at 16 000 x g at 4°C), neutralizing the supernatant with 3M K₂CO₃ and eliminating the salt precipitate by centrifugation. PMM1-deficient mice were obtained as described previously (20).

Assay of Glc16P₂ in neutralized HClO₄ extracts from mouse tissues or transfected HEK293T cells - Glc16P₂ was assayed through the stimulation of the activity of muscle phosphoglucomutase. Phosphoglucomutase activity was measured spectrophotometrically at 30°C in a cuvette containing 50 mM Tris pH 7.1, 0.1 mM EGTA, 5 mM MgCl₂, 0.5 mM NADP⁺, 0.5 mM glucose 1-phosphate free from Glc16P₂ (Merck), 1.75 U.ml⁻¹ of yeast glucose-6-phosphate dehydrogenase (Roche) and 0 to 0.5 µM Glc16P₂ (Roche; used as a standard) or 5 to 20 µl of sample neutralized HClO₄ extracts. The reaction was initiated by addition of 0.05 U.ml⁻¹ desalted rabbit muscle phosphoglucomutase (Roche). The concentration of Glc16P₂ in the unknown samples was calculated from a standard curve obtained under the same conditions.

Enzymatic assays - The phosphoglucomutase and phosphomannomutase activities of PMM1 and PMM2 were assayed in 25 mM Mes pH 6.5, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.5 mg.ml⁻¹ bovine serum albumin, 0.5 mM NADP⁺, 1 µM Glc16P₂, 1.75 U.ml⁻¹ desalted yeast glucose-6-phosphate dehydrogenase and 25 µM of glucose 1-phosphate (phosphoglucomutase activity) or mannose 1-phosphate (phosphomannomutase activity). When the substrate was mannose 1-phosphate (Sigma), we added 2 U.ml⁻¹ yeast phosphomannose isomerase (Sigma) and 3 U.ml⁻¹ desalted yeast phosphoglucose isomerase (Roche) to convert the product of the reaction, mannose 6-phosphate to glucose.
The reaction was started by addition of purified PMM1 (0.1 µg.ml-1) or PMM2 (1.8 µg.ml-1) to assay phosphoglucomutase activity and 0.18 µg.ml-1 to assay phosphomannomutase activity). Glucose-1,6-bisphosphatase activity was measured in 50 mM Tris pH 7.1, 0.1 mM EGTA, 2.5 mM MgCl₂, 1 mM DTT, 0.5 mg.ml⁻¹ bovine serum albumin, 0.5 mM NADP⁺, 0.15 mM Glc16P₂, with or without IMP or other nucleotides, 0.2 U.ml⁻¹ rabbit muscle phosphoglucomutase and 1.75 U.ml⁻¹ yeast glucose-6-phosphate dehydrogenase. The reaction was started by addition of purified PMM1 (0.02 µg.ml⁻¹) or PMM2 (18 µg.ml⁻¹).

In the assay conditions specified, one unit is the amount of enzyme that catalyzes the conversion of 1 µmol of substrate that is coupled to the reduction of 1 µmol of NADP⁺ per min.

RESULTS

IMP and GMP stimulate PMM1 but not PMM2 glucose-1,6-bisphosphatase activity - In order to investigate whether PMM1 was the glucose-1,6-bisphosphatase described in mouse brain by Guha and Rose (13, 14), we overexpressed recombinant mouse PMM1 in E. coli and partially purified it on an anion exchange column. The most purified fraction (approximately 90% purity) was used to investigate the glucose-1,6-bisphosphatase activity of PMM1. This activity amounted to 2.1 ± 0.25 µmol.min⁻¹.mg protein⁻¹ in the presence of 150 µM Glc16P₂. Both IMP and GMP (Fig.1), but not AMP (not shown), increased the glucose-1,6-bisphosphatase activity of PMM1 up to 110-fold and with a Ka of 3 µM in the case of IMP and up to 30-fold and with a Ka of 80 µM in the case of GMP. The Km of PMM1 for Glc16P₂ was 17 and 40 µM in the presence of 1 and 20 µM IMP respectively. In contrast to PMM1, the glucose-1,6-bisphosphatase activity of PMM2 was extremely low (0.01 ± 0.0005 µmol.min⁻¹.mg protein⁻¹), i.e. 0.05 % of the basal glucose-1,6-bisphosphatase activity of PMM1) and was insensitive to IMP and GMP (both tested at up to 1 mM). Furthermore, other nucleotides (AMP, ADP, ATP, UMP, UDP, UTP, CTP, GMP, GTP; all tested at 100 µM) neither stimulated the glucose-1,6-bisphosphatase activity of PMM1 nor of PMM2 (not shown).

Since IMP activated the glucose-1,6-bisphosphatase activity of PMM1, we were interested to find out whether IMP inhibited the phosphoglucomutase and phosphomannomutase activities of this enzyme. Figure 2 shows that both mutase activities, measured with glucose-1-phosphate (Fig. 2a) or mannose-1-phosphate (Fig. 2b) as substrates, were inhibited by increasing IMP concentrations. In contrast, neither the phosphoglucomutase (0.85 ± 0.006 µmol.min⁻¹.mg protein⁻¹) nor the phosphomannomutase activity (16 ± 0.15 µmol.min⁻¹.mg protein⁻¹) of PMM2 decreased by more than 5% in the presence of 1 mM IMP (not shown).

Intracellular Glc16P₂ in HEK293T cells transfected with human PGM2, PGM2L1 and mouse PMM1 and PMM2 – Even though PGM2L1 is particularly suited to synthesize elevated concentrations of Glc16P₂, PGM2 is also able, at least in vitro, to form Glc16P₂ from the same substrates (12). Furthermore, human erythrocyte Glc16P₂ synthase was shown to copurify with phosphopentomutase, i.e. PGM2 (26, 27). On the other hand, PMM1 was never shown to hydrolyze Glc16P₂ in vivo. In order to investigate the synthesis and the degradation of Glc16P₂ under more physiological conditions, we transfected HEK293T cells with plasmids driving the expression of human PGM2 or PGM2L1 and/or mouse PMM1 or PMM2 (Fig. 3). Glc16P₂ concentration was determined in cell extracts with a highly sensitive assay based on the activation of rabbit muscle phosphoglucomutase by Glc16P₂. Neutralized HClO₄ extracts from cell cultures transfected with PGM2L1 or PGM2 showed either a 20-fold (p<0.0001; n = 3) or a non-significant 1.5-fold (p = 0.076; n = 3) increase in the concentration of Glc16P₂ when compared to control cultures transfected with the empty plasmid (51.7 ± 5.2 nmoles.g protein⁻¹), confirming that PGM2L1 is indeed the enzyme responsible for Glc16P₂ synthesis. Remarkably, transfection of PMM1 alone or in cells co-transfected with PGM2L1 almost depleted the cells in Glc16P₂ (3.7 ± 3.7 nmoles.g protein⁻¹), i.e., 14-fold lower than control, p = 0.0016; n = 3) in the first case or lowered its concentration to levels close to control values in the second case. When on the other hand PMM2 was co-transfected with PGM2L1, intracellular Glc16P₂ levels were only decreased by 25% (p = 0.0025; n = 3)
despite the fact that PMM2 was 6-fold better expressed than PMM1 (see below). Similarly, overexpression of PMM2 alone did not significantly lower the intracellular concentration of Glc16P$_2$ ($22.3 \pm 11.2$ nmoles.g protein$^{-1}$) compared to control cells, confirming that the role of PMM1 in intact cells is indeed to hydrolyze Glc16P$_2$.

As all constructs encoded (His)$_6$ tagged proteins, we could verify the expression of the transfected enzymes by western blot (results not shown). Interestingly, PGM2 was more than 2-fold better expressed than PGM2L1, and PMM2 was 6- to 7-fold better expressed than PMM1. When proteins were co-transfected, expression of PGM2L1 and of each of the PMMs was lowered by 2 to 4-fold (not shown).

The deletion of PMM1 blocks Glc16P$_2$ hydrolysis in the brain of PMM1-deficient mice - To confirm that PMM1 is the physiological IMP-activated glucose-1,6-bisphosphatase described in mouse brain (13, 14) and is indeed responsible for the Glc16P$_2$ hydrolysis observed in ischemic brain (2), we measured Glc16P$_2$ concentration in neutralized HClO$_4$ extracts from brain of wild-type and PMM1-deficient mice. For this purpose, the brain of deeply anesthetized mice was removed. One hemisphere was frozen as fast as possible ($\approx 30$ sec) after removal from the mouse skull ($t = 0$ min), while the other was frozen after 5 min at room temperature ($t = 5$ min). Figure 4a shows that the Glc16P$_2$ concentration in ($t = 0$ min) brains was about two times higher in the brain of PMM1-deficient mice in comparison to wild-type. In addition, we observed that the Glc16P$_2$ concentration decreased with time in brain of wild-type and PMM1-deficient mice. For this purpose, the brain of deeply anesthetized mice was removed. One hemisphere was frozen as fast as possible ($\approx 30$ sec) after removal from the mouse skull ($t = 0$ min), while the other was frozen after 5 min at room temperature ($t = 5$ min). Figure 4a shows that the Glc16P$_2$ concentration in ($t = 0$ min) brains was about two times higher in the brain of PMM1-deficient mice in comparison to wild-type. In addition, we observed that the Glc16P$_2$ concentration decreased with time in brain of wild-type mice, though not in PMM1-deficient ones.

Although PMM1 is highly expressed in brain of adult mice, some authors also reported low PMM1 expression in other adult tissues such as lung and liver (21). Furthermore, Passonneau et al. (2) reported Glc16P$_2$ concentrations in muscle and red blood cells that are similar to the approximate 100 µM measured in brain. In view of these results we decided to investigate Glc16P$_2$ in neutralized HClO$_4$ extracts of various tissues from wild-type and PMM1-deficient mice. Figure 4b shows that the absence of PMM1 failed to affect significantly the concentration of Glc16P$_2$ in liver, lung, blood and skeletal muscle, suggesting that PMM1 is absent from these tissues and/or that the concentration of IMP is too low to stimulate its activity.

**DISCUSSION**

**PMM1 is the IMP-sensitive glucose-1,6-bisphosphatase** - Our results show that PMM1 actually corresponds to the enzyme described by Guha and Rose as the glucose-1,6-bisphosphatase (13, 14). PMM1 and glucose-1,6-bisphosphatase have similar molecular masses (87 kDa by gel filtration for glucose-1,6-bisphosphatase; PMM1 is known to be a 2 x 30 kDa dimer, 28). Furthermore, they both have the ability of catalyzing phosphomutase reactions in the absence of IMP, they both are Mg$^{2+}$-dependent and act predominantly as glucose-1,6-bisphosphatases in the presence of IMP. Both PMM1 and brain glucose-1,6-bisphosphatase are exquisitely sensitive to this nucleotide, with $K_a$ values in the micromolar range. In both cases, GMP is the only nucleotide that mimics the effect of IMP. Finally, both enzymes show a particularly high level of expression in brain. The identity of PMM1 as brain glucose-1,6-bisphosphatase is therefore warranted.

The present work leads moreover to the conclusion that PMM1 is the enzyme responsible for Glc16P$_2$ degradation in ischemic brain (2). This role was initially suggested by the finding that glucose-1,6-bisphosphatase is stimulated by a metabolite (IMP) whose concentration is elevated in anoxia (29). We now show that the decrease in the concentration of Glc16P$_2$ observed in ischemic brain does not occur in PMM1-deficient mice.

That PMM1 acts as a glucose-1,6-bisphosphatase in the physiological environment of the cytosol is further indicated by transfection studies. The level of Glc16P$_2$ was markedly decreased by PMM1 overexpression, both in cells that had control levels of Glc16P$_2$ to start with and in cells in which this level had been raised by overexpression of PGM2L1. On the contrary, PMM2 had only a minor effect on the Glc16P$_2$ level, despite its higher levels of overexpression in HEK293T cells compared to PMM1. Thus, in spite of their close structural similarity, PMM1 and PMM2 have distinctly different functions. This accounts for the apparent inability of PMM1 to ensure
sufficient phosphomannomutase activity in PMM2-deficient mice (20).

**Reaction mechanism of glucose-1,6-bisphosphatase** - To account for the multiple activities of glucose-1,6-bisphosphatase, Guha and Rose (13, 14) proposed that this enzyme used Glc16P2 to form a phosphoenzyme, thus releasing glucose 1-phosphate (or glucose 6-phosphate). PMM1 has indeed been shown to form an aspartylphosphate on Asp19 (15). The phosphomutase activity results from subsequent binding of glucose 6-phosphate (or glucose 1-phosphate) to the phosphoenzyme followed by the transfer of the phosphoryl group to re-form Glc16P2 (30, 31). The phosphomannomutase activity of this enzyme can be explained in a similar manner. The phosphatase activity involves IMP, which presumably binds to the same site as hexose monophosphates. This prevents the reformation of the bisphosphate cofactor (thereby inhibiting the phosphomutase activity), but more importantly stimulates the phosphatase activity of glucose-1,6-bisphosphatase/PMM1, which exceeds the mutase activity by more than 5-fold. The observation that this type of effect takes place with IMP, and to a lesser extent with GMP, but not with AMP, underlines the importance of the presence of an oxygen atom bound to C6 and/or of a hydrogen atom bound to N1 on the purine base. Furthermore, PMM2 does not become a phosphatase in the presence of IMP (or any other nucleotide that we tested) and its mutase activity is unaffected by IMP, despite its close structural similarity to PMM1. This indicates that IMP is unable to bind to PMM2.

Multiple alignments of vertebrate PMM1 and PMM2, and PMMs from fungi and plants indicate that all residues that putatively contact the substrate are totally conserved in the two proteins. However, a few residues that are highly conserved in vertebrate PMM2s as well as in PMMs from primitive organisms (which are most likely all phosphomannomutases) are replaced in eukaryotic PMM1s by residues that appear to be strictly conserved in the PMM1 subfamily. This is particularly the case of Glu219 in mouse PMM1. The carboxylic oxygen of this residue, which is at a distance of about 10 Å from the pyranose ring of mannose 1-phosphate in the structure of this enzyme, possibly makes a hydrogen-bond with the purine base N1 hydrogen in IMP and in GMP, if these nucleotides bind where mannose 1-phosphate does. No such bond would be made with AMP, explaining the specificity of the stimulatory effect of IMP and GMP. Another residue that potentially plays a role is Met186, which replaces a highly polar residue, glutamine, in PMM2s and PMMs from fungi and plants. The methyl group in Met186 is at a distance of 6 Å from mannose 1-phosphate in the crystal structure of PMM1. Its hydrophobic character could help PMM1 to bind the purine ring of IMP.

**Respective roles of PGM2 and PGM2L1 in Glc16P2 synthesis** - Our results show also that PGM2L1 is able to induce a large increase in the concentration of Glc16P2, whereas this is not the case for PGM2. This does not mean that PGM2 cannot make Glc16P2 in vitro. The explanation for the lack of rise in intracellular Glc16P2 following PGM2 overexpression in HEK293T cells could rather be the intrinsically lower Glc16P2 synthase activity of PGM2 in comparison to PGM2L1 and its stronger inhibition by the reaction product Glc16P2 (12). Thus PGM2L1 is tailored to raise the concentration of Glc16P2 to high values.

This conclusion may apparently contradict the findings that erythrocytes contain elevated concentrations of Glc16P2 (2, 32) and that Glc16P2 synthase copurifies with PGM2 in human erythrocyte extracts (26). An explanation for this discrepancy could be that PGM2L1 was lost in the purification reported by Accorsi et al. (26), due for instance to denaturation or proteolysis. Alternatively, free Glc16P2 may represent only a small fraction of total Glc16P2 in erythrocytes due to binding to hemoglobin. Hemoglobin is indeed known to bind avidly multiply charged phosphate esters, such as 2,3-bisphosphoglycerate and inositolpentakis-phosphate (33). It is therefore likely that it will also bind with great affinity bisphosphate esters such as Glc16P2. If so, the free concentration of Glc16P2 may be extremely low, leading to insignificant feedback inhibition of the Glc16P2 synthase activity of PGM2.

**Physiological role of Glc16P2** - The existence of specialized enzymes able to make high concentrations of Glc16P2 and to degrade it in a controlled manner, indicates that Glc16P2 plays a role other than being a cofactor of phosphomutases. As mentioned in the introduction, Glc16P2 is in vitro an effector of
several enzymes that are either inhibited (hexokinase I and II; fructose-1,6-bisphosphatase; 6-phosphogluconate dehydrogenase) or activated (liver pyruvate kinase; phosphofructokinase). Several of these are not relevant for the regulation of brain intermediary metabolism. This is the case for liver type pyruvate kinase (which is not expressed in brain) and probably also for fructose-1,6-bisphosphatase (whose activity in brain is negligible compared to that of phosphofructokinase).

The availability of the sequences of the enzymes that make and degrade Glc16P2 will allow one to study the effect of this compound in intact cells. Preliminary data indicate that changing the levels of Glc16P2 over a 50-fold range in HEK293T cells (by overexpressing PMM1 or PGM2L1) does not significantly affect the rate of [2-3H]glucose detritiation (which measures glucose phosphorylation) and of lactate formation. These negative results have to be taken with caution, since the effect of Glc16P2 on hexokinase may be masked by compensatory changes in the concentration of glucose 6-phosphate, an even more powerful regulator of hexokinase activity than Glc16P2.

The fact that Glc1,6P2 degradation is (almost) specifically and markedly stimulated by IMP suggests a link between the role of this compound and the energy state of the cell. PMM1-deficient mice have apparently no phenotype, which suggests that an increase in the concentration of brain Glc1,6P2 does not appear to have a detrimental effect. The approximate 2-fold difference that we found in the concentration of Glc16P2 in brain between wild-type and PMM1-deficient mice (Fig.4a), probably overestimates the true difference found in basal physiological conditions. In fact, it is very likely that there is already a fall in the concentration of Glc16P2 during the ≈30 sec period that it takes to freeze the brain after removal from the skull (t = 0 in Fig.4a). In this respect, it would be interesting to test the effect of anoxic episodes (and the recovery thereafter) in PMM1-deficient mice to see whether the absence of PMM1 is detrimental under these conditions. The identification of the role of Glc16P2 would also very much benefit from a mouse model of Glc16P2 synthase (PGM2L1)-deficiency.
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The abbreviations used are: PMM1, phosphomannomutase 1; PMM2, phosphomannomutase 2; PGM2, phosphoglucomutase 2 (phosphopentomutase); PGM2L1, phosphoglucomutase 2-like 1; Glc16P2, glucose 1,6-bisphosphate

FIGURE LEGENDS

Figure 1: Stimulation of the glucose-1,6-bisphosphatase activity of PMM1 by IMP and GMP. The enzymatic activities (µmol.min⁻¹.mg of protein⁻¹) were assayed as described in materials and methods with 150 µM Glc16P₂ and the indicated concentrations of IMP or GMP. The results shown are means ± S.E.M. of 3 determinations with the same enzyme preparation.

Figure 2: Inhibition by IMP of phosphoglucomutase (a) and phosphomannomutase (b) activities of PMM1. Both enzymatic activities were assayed as described in materials and methods with 1 µM Glc16P₂, increasing concentrations of IMP and 25 µM (a) glucose 1-phosphate or (b) mannose 1-phosphate. The results shown are means ± S.E.M. of 3 determinations with the same enzyme preparation. PGM: phosphoglucomutase; PMM: phosphomannomutase.

Figure 3: Effect of overexpression of human PGM2L1 or PGM2 and of mouse PMM1 or PMM2 on the Glc16P₂ level in HEK293T cells. HEK293T cells were plated in 10 cm diameter dishes and transfected with the indicated quantities of plasmids encoding mouse PMM1 (mPMM1), mouse PMM2 (mPMM2), human PGM2 (hPGM2) or human PMG2L1 (hPGM2L1). Perchloric acid extracts were prepared 48 hrs after transfection for the assay of Glc16P₂. The results shown represent one example of at least four similar experiments. In the experiment shown, the concentrations of Glc16P₂ are means ± S.E.M. of 3 independent transfections. Glc16P₂ values were compared using a Student’s t test and the difference was considered non-significant (n.s.) when p>0.05.

Figure 4: Concentration of Glc16P₂ in tissues of control and PMM1-deficient mice. Mice were deeply anesthetized before organs were removed. Glc16P₂ was measured in neutralized HClO₄ extracts from (a) brain hemispheres that were frozen as fast as possible after removal from the mouse skull (t = 0 min) or after 5 min at room temperature (t = 5 min), or from (b) other tissues that were frozen as soon as they were removed from the animal. The results shown are means ± S.E.M. of determinations made in 4 to 7 different mice. Glc16P₂ values were compared using a Student’s t test. When a p value is not given (p>0.05) the difference was considered non-significant (n.s.).
Figure 1 (Veiga-da-Cunha et al.)

![Graph showing PMM1 glucose 1,6-bisphosphatase activity in response to IMP or GMP concentrations.](http://www.jbc.org/)

**Figure 1**

Effect IMP PMM1/PMM2.
**Figure 2 (Veiga-da-Cunha et al.)**

![Graph](image)

**PGM activity of mPMM1**

- Activity in µmol.min⁻¹.mg⁻¹ as a function of [IMP] (µM).

**PMM activity of mPMM1**

- Activity in µmol.min⁻¹.mg⁻¹ as a function of [IMP] (µM).
Figure 3 (Veiga-da-Cunha et al.)

![Graph showing glucose 1,6-bisphosphate levels](image)

- Empty plasmid
- mPMM1
- mPMM2
- hPGM2
- hPGM2L1 + Empty plasmid
- hPGM2L1 + mPMM1
- hPGM2L1 + mPMM2

**Glucose 1,6-bisphosphate (nmoles/g protein)**

- p=0.0025
- p<0.0001
- n.s.
- n.s.
- p=0.0016

**Legend:**
- p<0.0001
- n.s.
- p=0.0016
Figure 4 (Veiga-da-Cunha et al.)

**A**

![Graph A](image)

**B**

![Graph B](image)
Mammalian phosphomannomutase 1 (PMM1) is brain, IMP-sensitive glucose 1,6-bisphosphatase

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