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Molecular Identification of Mammalian Phosphopentomutase and Glucose-1,6-bisphosphate Synthase, Two Members of the α-D-Phosphohexomutase Family*

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Pushpa Maliekal[1], **Tatiana Sokolova, Didier Vertommen**[2], **Maria Veiga-da-Cunha**[3], **and Emile Van Schaftingen**[4]
From the de Duve Institute, Université Catholique de Louvain, B-1200 Brussels, Belgium

The molecular identity of mammalian phosphopentomutase has not yet been established unequivocally. That of glucose-1,6-bisphosphate synthase, the enzyme that synthesizes a cofactor for phosphomutases and putative regulator of glycolysis, is completely unknown. In the present work, we have purified phosphopentomutase from human erythrocytes and found it to copurify with a 68-kDa polypeptide that was identified by mass spectrometry as phosphoglucomutase 2 (PGM2), a protein of the α-D-phosphohexomutase family and sharing about 20% identity with mammalian phosphoglucomutase 1. Data base searches indicated that vertebrate genomes contained, in addition to PGM2, a homologue (PGM2L1, for PGM2-like 1) sharing about 60% sequence identity with this protein. Both PGM2 and PGM2L1 were overexpressed in *Escherichia coli*, purified, and their properties were studied. Using catalytic efficiency as a criterion, PGM2 acted more than 10-fold better as a phosphopentomutase (both on deoxyribose 1-phosphate and on ribose 1-phosphate) than as a phosphoglucomutase. PGM2L1 showed only low (<5%) phosphopentomutase and phosphoglucomutase activities compared with PGM2, but was about 5–20-fold better than the latter enzyme in catalyzing the 1,3-bisphosphoglycerate-dependent synthesis of glucose 1,6-bisphosphate and other aldose-bisphosphates. Furthermore, quantitative real-time PCR analysis indicated that PGM2L1 was mainly expressed in brain where glucose-1,6-bisphosphate synthase activity was previously shown to be particularly high. We conclude that mammalian phosphopentomutase and glucose-1,6-bisphosphate synthase correspond to two closely related proteins, PGM2 and PGM2L1, encoded by two genes that separated early in vertebrate evolution.

Phosphopentomutase catalyzes the conversion of the nucleoside breakdown products ribose 1-phosphate and deoxyribose 1-phosphate to the corresponding 5-phosphopentoses. Most bacterial phosphopentomutases characterized so far belong to the same protein family as alkaline phosphatases, sulfatases, and cofactor-independent bisphosphoglycerate mutases (1), though the enzyme from *Thermococcus kodakaraensis* belongs to the same protein family as mammalian phosphoglucomutase 1 (PGM1),[5] the enzyme that catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate (2).

The molecular identity of the mammalian phosphopentomutase is still not ascertained. Biochemical characterization of phosphoglucomutase (PGM) isozymes indicated that one of them, designated PGM2 in man (PGM1 in mouse) was more active as a phosphopentomutase than as a phosphoglucomutase, whereas mammalian PGM1 (equivalent to PGM2 in mouse) has a phosphopentomutase activity representing only about 0.2% of its phosphoglucomutase activity (3). Phosphopentomutase has been purified from rat liver to near homogeneity and shown to coelute with a polypeptide of 32.5 kDa (4), the sequence of which was not determined. Analysis of cell hybrids indicated that the PGM2 locus is on human chromosome 4p14-q12 (5). A putative protein sharing ≈20% identity with PGM1 is encoded by a gene present on human chromosome 4p14. It is designated PGM2 in the databases presumably because its chromosomal localization fits with the PGM2 locus but it has never been shown to act as a phosphopentomutase. Furthermore, its size (612 amino acid residues) is about twice that reported for rat liver phosphopentomutase (4). In view of these contradictory findings, we decided to reinvestigate the identity of human phosphopentomutase, which we formally identify in this report as the protein designated PGM2 in the data bases.

We also show that a closely related protein, PGM2-like 1 (PGM2L1), which shares ≈ 60% sequence identity with PGM2, actually corresponds to glucose-1,6-bisphosphate synthase. This enzyme, which had not yet been molecularly identified, catalyzes the 1,3-bisphosphoglycerate-dependent synthesis of glucose 1,6-bisphosphate and other aldose-bisphosphates that serve as cofactors for several sugar phosphomutases and possibly also as regulators of glycolytic enzymes.

EXPERIMENTAL PROCEDURES

Materials—Reagents, of analytical grade whenever possible, were from Sigma, Acros (Geel, Belgium), Roche Applied Sci-

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[1] Fellow of the Fonds pour l'Encouragement à la Recherche dans l'Industrie et dans l'Agriculture.
[2] Collaborateur logistique F.R.S.-FNRS.
[3] Chercheur qualifié of the Fonds National de la Recherche Scientifique.
[4] To whom correspondence should be addressed: UCL 7539, Ave. Hippocrate 75, B-1200 Brussels, Belgium. Tel.: 3227647564; Fax: 327647598; E-mail: emile.vanschaftingen@uclouvain.be.

[5] The abbreviations used are: PGM, phosphoglucomutase; DEAE, diethylaminoethyl; SP, sulfopropyl; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol.

ences (Mannheim, Germany), or Merck (Darmstadt, Germany). DEAE-Sepharose, SP-Sepharose, Q-Sepharose, Superdex-200 10/300 GL, HisTrap, and PD-10 column were purchased from GE Healthcare (Diegem, Belgium). Vivaspin-2 centrifugal concentrator was from Vivascience (Göttingen, Germany). Enzymes and restriction enzymes were purchased from Sigma, Roche Applied Sciences, or Fermentas (St Leon-Rot, Germany). TriPure reagent was from Roche Applied Sciences and the cDNA synthesis kit was from Fermentas.

Purification of Phosphopentomutase—Phosphopentomutase was purified from human erythrocytes. 80 ml of packed erythrocytes were washed three times with 150 mM NaCl and diluted in 400 ml of a buffer containing 10 mM Tris, pH 8, 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, and 1 μg/ml antipain. The hemolysate was centrifuged for 20 min at 11,000 × *g*. The supernatant (300 ml) was diluted twice in buffer A (20 mM Tris, pH 8, 1 mM DTT, 1 μg/ml leupeptin, and 1 μg/ml antipain) and applied to a DEAE-Sepharose column (200 cm^3) equilibrated with the same buffer. The column was washed with 400 ml of buffer A, and protein was eluted with a 0–0.25 M NaCl gradient in 1000 ml of buffer A. The most active fractions were pooled (75 ml), brought to 300 ml with buffer B (25 mM MES, pH 6, 1 mM DTT, 25 mM KCl, 5 μg/ml leupeptin, and 5 μg/ml antipain), and applied to a SP-Sepharose column (25 cm^3) equilibrated with buffer B. The column was washed with 100 ml of equilibration buffer. Phosphopentomutase was recovered in the flow-through and washing fractions, which were concentrated to 37.5 ml by ultrafiltration in a 200-ml Amicon cell equipped with a PM-10 membrane. This sample was brought to 180 ml with buffer C (25 mM glycine, pH 9, 1 mM DTT, 25 mM KCl, 5 μg/ml leupeptin, and 5 μg/ml antipain) and applied to a Q-Sepharose column (20 cm^3) equilibrated with the same buffer. The column was washed with 80 ml of buffer C, and the retained proteins were eluted with a 0–0.75 M NaCl gradient in 300 ml of buffer C. The most active fractions (6.6 ml) were concentrated to 0.5 ml (with Vivaspin-2) and gel-filtered on a Superdex-200 10/300 GL column equilibrated with buffer D (25 mM Hepes, pH 7.4, 1 mM DTT, 5 μg/ml leupeptin, 5 μg/ml antipain, and 100 mM NaCl). Fractions of 0.5 ml were collected. Protein was assayed as described (6) using bovine serum albumin as a standard. The bands co-eluting with the phosphopentomutase activity were cut out from a 12% (w/v) polyacrylamide-SDS gel and digested with trypsin. Peptides were analyzed by nanoelectrospray-ionization tandem mass spectrometry (7) in a LCQ Deca XP Plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with a nanoelectrospray probe. The data were analyzed with the X-calibur software (ThermoFinnigan), and the proteins were identified with TurboSEQUEST in the BioWorks software suite (ThermoFinnigan).

Overexpression and Purification of Human PGM2 and PGM2L1—The open reading frame of human PGM2 and PGM2L1 (GenBank™ accession numbers NP_060760 and NP_775853) were PCR-amplified using a mixture of *Taq* and *Pfu* DNA polymerases and human white blood cells cDNA as a template. For PGM2, a 5′ primer containing the initiator codon (CAAGCTCA**CATATG**GCGGCTCCAGAAGGCAG) in an NdeI site (in bold) and a 3′-primer containing the putative stop codon (GGCTGG**AGATCT**TTAGTCTGCTTTTGGCTGCAGAT) flanked by a BglII site (in bold) were used. The 1840-bp PCR-product was digested with NdeI-BglII and cloned into pET-15b expression vector. The insert was checked by sequencing. This expression vector was used to transform *E. coli* BL21(DE3). For PGM2L1, a 5′ primer containing the initiator codon (C**CATATG**GCTGAAAACACAGAGGGGG) in an NdeI site (in bold) and a 3′ primer containing the putative stop codon (T**GGATCC**CTAAACAGAACGCCAGATCA) flanked by a BamHI site (in bold) were used. A 1870-bp product was obtained, which was subcloned in pBlueScript and checked by sequencing. A NdeI-BamHI fragment was removed from the pBlueScript plasmid and ligated in pET-15b expression vector. This vector was used to transform *E. coli* BL21(DE3) pLysS. Protein expression and preparation of bacterial extracts were performed as described previously (8). Both His-tagged proteins were purified on HisTrap columns (Ni^{2+} form) as previously described (9). Both proteins were eluted with ≈150 mM imidazole (as indicated by SDS-PAGE analysis). Both proteins were desalted on PD-10 columns equilibrated with 25 mM Hepes pH 7.4 and 50 mM KCl. Protein concentration was estimated by measuring A_{280} assuming extinction coefficients of 83,810 and 97,260 M^{-1} cm^{-1} for PGM2 and PGM2L1, respectively; 22 mg of pure PGM2 and 3 mg of pure PGM2L1 were obtained per liter of culture. The purified proteins were supplemented with 10% (w/v) glycerol and stored at −70 °C.

Measurement of Enzymatic Activities—The enzymatic activities were assayed spectrophotometrically at 30 °C. All assay media (reaction mixture of 600 μl) contained 25 mM Hepes pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 100 μM EGTA (assay buffer). Phosphoribomutase was assayed in assay buffer containing 1 mM $MnCl_2$, 50 μM CTP-Mg, 0.15 mM NADPH, 0.5 μM glucose 1,6-bisphosphate, different concentrations of ribose 1-phosphate, ribose-5-phosphate isomerase from spinach (1.7 units/ml), *Haemophilus influenzae* ribulose-5-phosphate reductase (0.3 units/ml) (10) and PGM2 (0.03 μg/ml) or PGM2L1 (2 μg/ml). One unit of enzyme is the amount that converts 1 μmol of substrate per min under these conditions. Phosphodeoxyribomutase was assayed in assay buffer containing 0.15 mM NADH, 5 μM glucose 1,6-bisphosphate, different concentrations of deoxyribose 1-phosphate, deoxyribose-5-phosphate aldolase (6 μg/ml, from *Thermus thermophilus*, overexpressed in *E. coli* and purified to homogeneity),[6] rabbit muscle triose phosphate isomerase (3.5 units/ml) and glycerol-3-phosphate dehydrogenase (1 units/ml), as well as PGM2 (0.03 μg/ml) or PGM2L1 (5 μg/ml). Phosphoglucomutase was assayed in assay buffer containing 0.25 mM NADP, 5 μM glucose 1,6-bisphosphate, different concentrations of glucose 1-phosphate, yeast glucose-6-phosphate dehydrogenase (3 units/ml) and PGM2 (0.15 μg/ml) or PGM2L1 (2.4 μg/ml).

The glucose-1,6-bisphosphate synthase and aldose-bisphosphate synthase activities were determined in assay buffer containing 500 μM inorganic phosphate, 20 μM glyceraldehyde 3-phosphate, 500 μM NAD, 100 μg/ml bovine serum albumin, different concentrations of aldose monophosphate, rabbit mus-

[6] T. Sokolova and M. Veiga-da-Cunha, unpublished results.

TABLE 1
Primer sequences for PGM2, PGM2L1, and internal control genes

Symbol	Name	Forward primer	Reverse primer
PGM2	Phosphoglucomutase 2	GCTGTTATTTGTGCAGAGCTG	CTTCCCGTCATAGTTTCTCAG
PGM2L1	Phosphoglucomutase 2-like 1	GAAGGTAACACTGATGGAGCA	TGGTTATGTCCCGTACATGCA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGATGTTCT
HPRT	Hypoxanthine phosphoribosyl-transferase	GTTAAGCAGTACAGCCCCAAAATG	AAATCCAACAAAGTCTGGCCTGTA

cle glyceraldehyde-3-phosphate dehydrogenase (1 unit/ml) and PGM2 (1.5 μg/ml) or PGM2L1 (0.8 to 2.8 μg/ml).

RNA Extraction and Quantitative Real Time PCR Assays—Total RNA was extracted from fresh tissues from 3 male fed mice with TriPure reagent according to the manufacturer's protocol. RNA integrity was verified by loading RNA onto a 1% agarose gel and evaluating the 28 S and 18 S ribosomal RNA bands, and its purity was assessed by checking A_{260}/A_{280}. Approximately 1 μg of RNA from each sample was converted to cDNA by RT-PCR using random hexamers and Superscript II reverse transcriptase. Gene-specific primers (Table 1) were designed to generate PCR products of about 150 bp from PGM2, PGM2L1 and two reference genes: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine guanine phosphoribosyltransferase). Intron-spanning primers were selected to verify, by agarose gel electrophoresis, that amplification of contaminating genomic DNA did not take place. Real-time PCR was performed using an iCycler IQ™ system (Bio-Rad). The rate of accumulation of amplified DNA was measured by continuous monitoring of SYBR green I fluorescence.

Tree Construction—The sequence of homologues of human PGM2 and PGM2L1, identified with the BLAST program (11), were aligned using Clustal X with the default parameters (12). A neighbor-joining tree was constructed using the tree option of Clustal X after exclusion of positions with gaps and with correction for multiple substitutions. For the bootstrap analysis, 1000 samplings were carried out.

RESULTS

Purification of Human Erythrocyte Phosphopentomutase—During its purification, phosphopentomutase was assayed by measuring the conversion of ribose 1-phosphate to ribose 5-phosphate with a coupled assay using ribose-5-phosphate isomerase and ribulose-5-phosphate reductase. Phosphopentomutase was purified from human erythrocytes about 100-fold by chromatography on DEAE-Sepharose, SP-Sepharose, Q-Sepharose and Superdex with an overall yield of 0.4%. This low yield was due to the fact that after each purification step, only the most purified fractions were used for the next step. SDS-PAGE analysis of the fractions of the last purification step showed that the enzymatic activity co-purified with a 68-kDa band (Fig. 1), which was cut out from the gel and digested with trypsin. Mass spectrometry analysis indicated that the 68-kDa protein corresponded ($p < 5.5 \times 10^{-6}$ in the Bioworks software) to a protein designated PGM2 (GenBank™ accession number NP_060760) in the databases. Nine matching peptides (underlined in Fig. 2) were found, which covered 96 amino acids. As a previous report had suggested that phosphopentomutase corresponded to a 32.5-kDa polypeptide chain, we also analyzed the ≈30-kDa polypeptide band that coeluted with phosphopentomutase activity

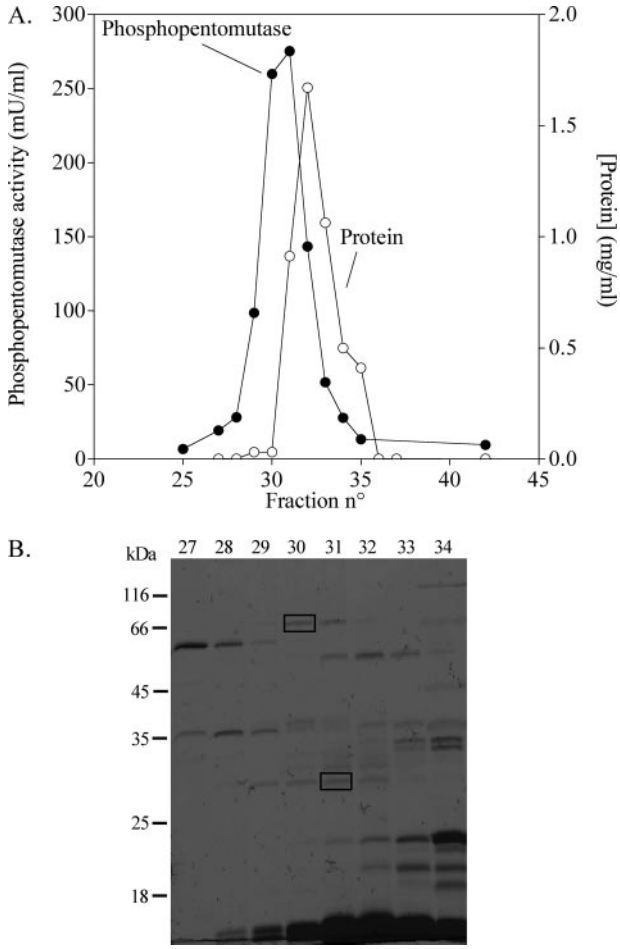

FIGURE 1. **Co-elution of human erythrocyte phosphopentomutase with a ≈68-kDa polypeptide from a Superdex-200 column.** *A*, a preparation of phosphopentomutase (540 milliunits in 0.4 ml) purified by chromatography on DEAE-Sepharose, SP-Sepharose, and Q-Sepharose was applied on a Superdex-200 column. Fractions of 0.5 ml were collected. Phosphopentomutase activity and protein concentration (6) were determined. *B*, the indicated fractions were analyzed by SDS-PAGE (15 μl/lane) and the gel stained with Coomassie Blue. The indicated bands were cut out from the gel, submitted to trypsin digestion, and analyzed by mass spectrometry.

(though less ideally than the 68-kDa band). This protein, which was identified ($p < 2.2 \times 10^{-10}$) as triose phosphate isomerase (GenBank™ accession number NP_000356), is unrelated to phosphopentomutase.

Occurrence of PGM2 Homologues—BLAST searches with human PGM2 indicated that homologues of this protein were found in eukaryotes including vertebrates (>70% identity with the human sequence), insects and *Caenorhabditis elegans* (both ≈50% identity) and fungi (≈40% identity). No orthologue was detected in plant genomes. Vertebrate genomes also encode a closely related protein, designated PGM2-like 1 (PGM2L1), which shares about 60% sequence identity with PGM2. As shown in the sequence alignment of Fig. 2, a remarkable feature

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PGM2     MAAPEGSGLG----------EDARLDQETAQWLRWDKNSLTLEAVKRLIA 40
PGM2L1   MAENTEGDLNSNLLHAPYHTGDPQLDTAIGQWLRWDKNPKTKEQIENLLR 50
         **      *            *  **    ********  * *    *

PGM2     EGNKEELRKCFGARMEFGTAGLRAAMGPGISRMNDLTIIQTTQGFCRYLE 90
PGM2L1   NGMNKELRDRLCCRMTFGTAGLRSAMGAGFCYINDLTVIQSTQGMYKYLE 100
          *   ***     ** ******* *** *    **** ** ***   ***

PGM2     KQFSDLKQKGIVISFDARAHPSSGGSSRRFARLAATTFISQGIPVYLFSD 140
PGM2L1   RCFSDFKQRGFVVGYDTRGQVTSSCSSQRLAKLTAAVLLAKDVPVYLFSR 150
           *** ** * *   * *    *  ** * * * *        ******

PGM2     ITPTPFVPFTVSHLKLCAGIMITASHNPKQDNGYKVYWDNGAQIISPHDK 190
PGM2L1   YVPTPFVPYAVQKLKAVAGVMITASHNRKEDNGYKVYWETGAQITSPHDK 200
           ******  *  **  ** ******* * ********  **** *****

PGM2     GISQAIEENLEPWPQAWDDSLIDSSPLLHNPSASINNDYFEDLKKYCFHR 240
PGM2L1   EILKCIEECVEPWNGSWNDNLVDTSPLKRDPLQDICRRYMEDLKKICFYR 250
          *   ***  ***   * * * * ***   *   *   * ***** ** *

PGM2     SVNRETKVKFVHTSVHGVGHSFVQSAFKAFDLVPPEAVPEQKDPDPEFPT 290
PGM2L1   ELNSKTTLKFVHTSFHGVGHDYVQLAFKVFGFKPPIPVPEQKDPDPDFST 300
           *  *  ****** *****  ** *** *   **  ********* * *

PGM2     VKYPNPEEGKGVLTLSFALADKTKARIVLANDPDADRLAVAEKQDSGEWR 340
PGM2L1   VKCPNPEEGESVLELSLRLAEKENARVVLATDPDADRLAAAELQENGCWK 350
         ** ******  ** **  ** *  ** *** ******** ** *  * *

PGM2     VFSGNELGALLGWWLFTSWKEKNQDRSALKDTYMLSSTVSSKILRAIALK 390
PGM2L1   VFTGNELAALFGWWMFDCWKKNKSRNADVKNVYMLATTVSSKILKAIALK 400
         ** **** ** *** *  **         *  ***  ******* *****

PGM2     EGFHFEETLTGFKWMGNRAKQLIDQGKTVLFAFEEAIGYMCCPFVLDKDG 440
PGM2L1   EGFHFEETLPGFKWIGSRIIDLLENGKEVLFAFEESIGFLCGTSVLDKDG 450
         ********* **** * *   *   ** ******* **  *   ******

PGM2     VSAAVISAELASFLATKNLSLSQQLKAIYVEYGYHITKASYFICHDQETI 490
PGM2L1   VSAAVVVAEMASYLETMNITLKQQLVKVYEKYGYHISKTSYFLCYEPPTI 500
         *****  ** ** * * *  * ***   *  ***** * *** *    **

PGM2     KKLFENLRNYDGKNNYPKACGKFEISAIRDLTTGYDDSQPDKKAVLPTSK 540
PGM2L1   KSIFERLRNFDSPKEYPKFCGTFAILHVRDITTGYDSSQPNKKSVLPVSK 550
         *  ** *** *    *** ** * *   ** ***** *** ** *** **

PGM2     SSQMITFTFANGGVATMRTSGTEPKIKYYAELCAPPGNSDPEQLKKELNE 590
PGM2L1   NSQMITFTFQNGCVATLRTSGTEPKIKYYAEMCASPDQSDTALLEEELKK 600
          ******** ** *** ************** ** *  **   *  **

PGM2     LVSAIEEHFFQPQKYNLQPKAD 612
PGM2L1   LIDALIENFLQPSKNGLIWRSV 622
         *  *  * * ** *  *
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FIGURE 2. **Alignment of human PGM2 and PGM2L1.** Conserved residues are indicated by an *asterisk* below the alignment. The highly conserved TASHNP motif from the α-D-phosphohexomutase family is in *bold*. The peptides identified by mass spectrometry in the protein purified from human erythrocytes are *underlined* in the PGM2 sequence. The following sequences are shown: human PGM2 (GenBank™ accession number gi-63055049) and PGM2L1 (GenBank™ accession number gi-31377548).

of the PGM2L1 proteins was that they do not contain a proline but an arginine in the highly conserved TASHNP motif that surrounds the phosphorylatable serine in the phosphoglucomutase family (13, 14). The evolutionary tree illustrated in Fig. 3 shows that PGM2L1 and PGM2 separated early on during vertebrate evolution, as indicated by the presence of orthologues of both PGM2 and PGM2L1 in *Danio rerio*.

PGM2 and PGM2L1 are distantly related to PGM1, sharing only about 20% sequence identity with this enzyme (not shown). They are also similar to phosphoacetylglucosamine mutase (PGM3) in a region (TASHNP) that surrounds the serine residue that is phosphorylated during catalysis (not shown).

Analysis with PSORT (15) and Target P (16) indicated that the sequences of both human PGM2 and PGM2L1 do not contain a signal peptide, a mitochondrial propeptide, or a nuclear localization signal, suggesting that both proteins are cytosolic.

Tissue Distribution of PGM2 and PGM2L1—The tissue distribution of PGM2 and PGM2L1 mRNAs was determined in mice by quantitative real-time PCR using both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) transcripts as references. Fig. 4*A* shows that for PGM2, the highest expression levels were observed in lung, spleen, and thymus. Other tissues contained at least 20% of the levels found in lung or spleen if HPRT was used as a reference. The lower levels observed in skeletal muscle and heart when GAPDH was used as a reference are presumably because of the high expression of this glycolytic enzyme in muscle. As for PGM2L1 (Fig. 4*B*), the highest levels of expression were observed in brain and testis, intermediate levels were found in thymus, spleen, lung and skeletal muscle, and the lowest levels (about 2–3% of the maximal ratio) were recorded in kidney, liver, and heart.

Overexpression and Characterization of Human PGM2 and PGM2L1—Both proteins were overexpressed in *E. coli* as fusion proteins with a polyHis tag at their N terminus and purified on HisTrap to near homogeneity. The ability of the two enzymes to convert ribose 1-phosphate, deoxyribose 1-phosphate, and glucose 1-phosphate to the corresponding aldose 5- or 6-phosphates was determined with specific spectrophotometric assays. As shown in Table 2, PGM2 displayed significant phosphomutase activity on all three substrates. In terms of catalytic efficiency the best substrates were deoxyribose 1-phosphate and ribose 1-phosphate, which were 27 and 12 times better than glucose 1-phosphate, respectively. The highest V_{max} was observed with ribose 1-phosphate and the lowest K_m, with deoxyribose 1-phosphate. PGM2L1 showed much lower phosphomutase activity on all three substrates, the V_{max} being in all cases less than 5% of that observed with PGM2. All phosphomutase activities mentioned above were stimulated by glucose 1,6-bisphosphate. In the case of the phosphoglucomutase activity of PGM2, half-maximal stimulation was observed at 0.16 and 0.18 μM glucose 1,6-bisphosphate in the presence of 100 and 500 μM glucose 1-phosphate, respectively. In the case of PGM2L1, half-maximal stimulation was observed at much higher glucose 1,6-bisphosphate concentrations (1.15 μM in the presence of both 20 and 200 μM glucose 1-phosphate).

Glucose-1,6-bisphosphate Synthase Activity—The low phosphopentomutase and phosphoglucomutase activities of PGM2L1 suggested that it played a different role. As a screen to identify molecules that could potentially serve as substrates for PGM2L1, we tested the ability of a series of phosphate esters to

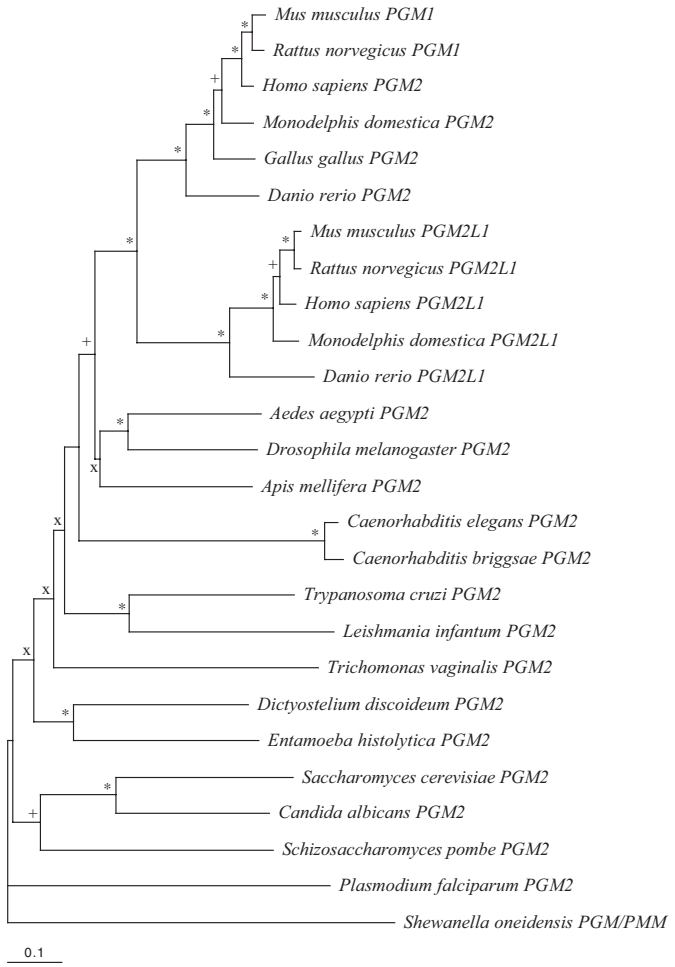

FIGURE 3. **Evolutionary tree of PGM2 homologues in eukaryotes showing the separation of PGM2 and PGM2L1 in the vertebrate lineage.** *Homo sapiens* PGM2 was aligned with the indicated sequences and a neighbor-joining tree was constructed with Clustal X program. The *Shewanella oneidensis* phosphoglucomutase/phosphomannomutase (PGM/PMM) was used as an outgroup. *Symbols* at the nodes represent the support for each node as obtained with 1000 bootstrap samplings: (*), >95%; (+), 70–95%; and (×), 50–70%. Nodes with no symbols were found in <50% of the bootstrap samplings. The branch lengths are proportional to the number of substitutions per site. The *horizontal bar* represents 0.1 substitution per site.

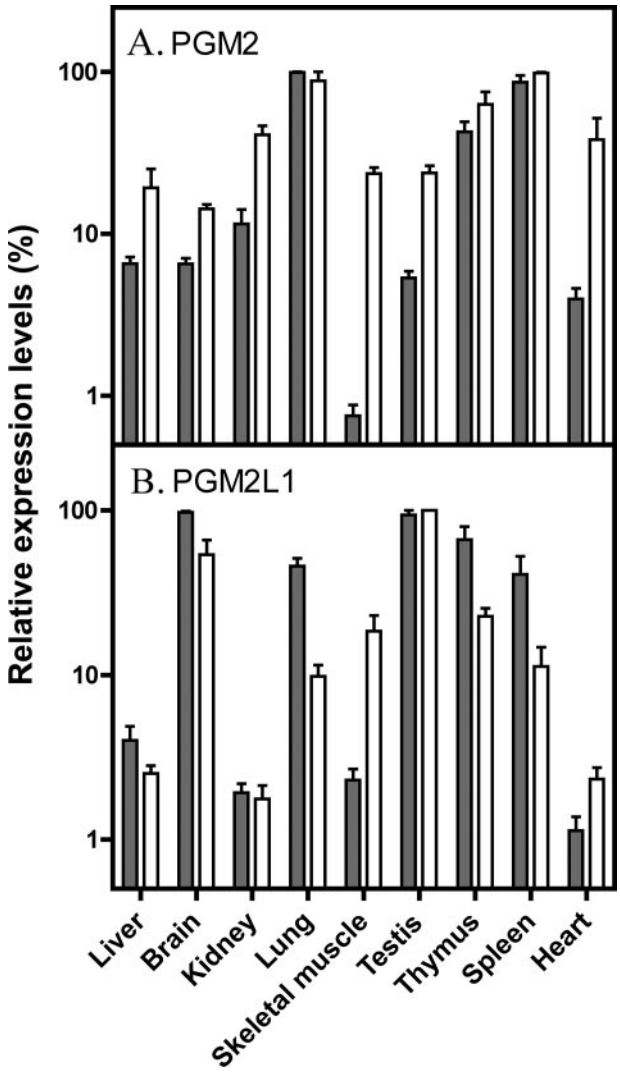

FIGURE 4. **Tissue distribution of PGM2 (*A*) and PGM2L1 (*B*) mRNAs as determined by quantitative real time PCR.** Ct (cycle threshold) values were determined for PGM2, PGM2L1, GAPDH, and HPRT. The expression of a target gene (PGM2, PGM2L1) relative to that of a reference gene (*gray bars* for GAPDH and *white bars* for HPRT) was calculated by determining the $2^{\Delta Ct}$ values (where $\Delta Ct = Ct$ (reference gene) $-$ Ct (gene of interest)) for each gene pair. These values are expressed in the figure as the percentage of the value found in the richest tissue (lung or spleen for PGM2; brain or testis for PGM2L1). Means ± S.E. $n = 3$ different mice.

inhibit the phosphoribomutase activity of this enzyme. Interestingly, we found that glycerol 3-phosphate and 2,3-bisphosphoglycerate were strong inhibitors of PGM2L1 though not of PGM2. At a concentration of 200 μM, glycerol 3-phosphate and 2,3-bisphosphoglycerate inhibited the phosphoribomutase activity of PGM2L1 (tested with 20 μM ribose 1-phosphate) by 95 and 99%, respectively, but that of PGM2 by only ≈50%. This finding suggested that the phosphate ester of a C3 compound could be a substrate for PGM2L1.

Previous work has disclosed the existence of a glucose-1,6-bisphosphate synthase, which uses the high energy compound 1,3-bisphosphoglycerate to convert glucose 1-phosphate or glucose 6-phosphate to glucose 1,6-bisphosphate (17, 18). This enzyme was shown to be particularly active in brain. It was purified from this tissue and identified as a 70-kDa protein, which acted also with low activity, as a phosphoglucomutase, all properties that are similar to those of PGM2L1. To test the ability of the latter to synthesize glucose 1,6-bisphosphate from 1,3-bisphosphoglycerate and glucose 1- or 6-phosphate, we designed a spectrophotometric assay in which the activity of glyceraldehyde-3-phosphate dehydrogenase (monitored by the appearance of NADH) is coupled to the utilization of 1,3-bisphosphoglycerate by the synthase. This coupling is made possible by the fact that the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (Fig. 5*A*) readily reaches its thermodynamic equilibrium in the absence of an enzyme that consumes 1,3-bisphosphoglycerate. However, as illustrated in Fig. 5*B*, the progression of the reaction (and therefore NADH formation) can be made to resume by adding a 1,3-bisphosphoglycerate consuming system, *e.g.* 3-phosphoglycerate kinase and ADP. Fig. 5*B* also shows that NADH formation resumes upon addition of glucose 1-phosphate and PGM2L1 (although not if each of them is added separately; not shown), which indicates that PGM2L1 utilizes 1,3-bisphosphoglycerate to catalyze

TABLE 2

Phosphomutase activities of PGM2 and PGM2L1

The results are the means of three determinations ± S.E. The first three columns indicate the V_{max}, K_m, and k_{cat} (catalytic efficiency) values for the indicated substrates. The last column shows ratios of the catalytic efficiencies of PGM2 and PGM2L1 for each of the substrate.

	V_{max}	K_m	k_{cat}/K_m	Catalytic efficiency ratio (PGM2/PGM2L1)
	μmol min^{-1} *mg*$^{-1}$	*μM*	s^{-1} μM^{-1}	
PGM2				
Ribose-1-P	104.3 ± 3.7	45.7 ± 3.7	2.7 ± 0.2	16.5 ± 2.5
Deoxyribose-1-P	20.8 ± 1.0	4.1 ± 1.3	6.2 ± 0.7	407 ± 74
Glucose-1-P	22.8 ± 0.6	114 ± 3.1	0.23 ± 0.01	4.7 ± 0.5
PGM2L1				
Ribose-1-P	0.71 ± 0.04	5.2 ± 0.8	0.17 ± 0.02	
Deoxyribose-1-P	0.09 ± 0.01	6.7 ± 0.7	0.015 ± 0.001	
Glucose-1-P	0.87 ± 0.09	20.7 ± 1.3	0.051 ± 0.003	

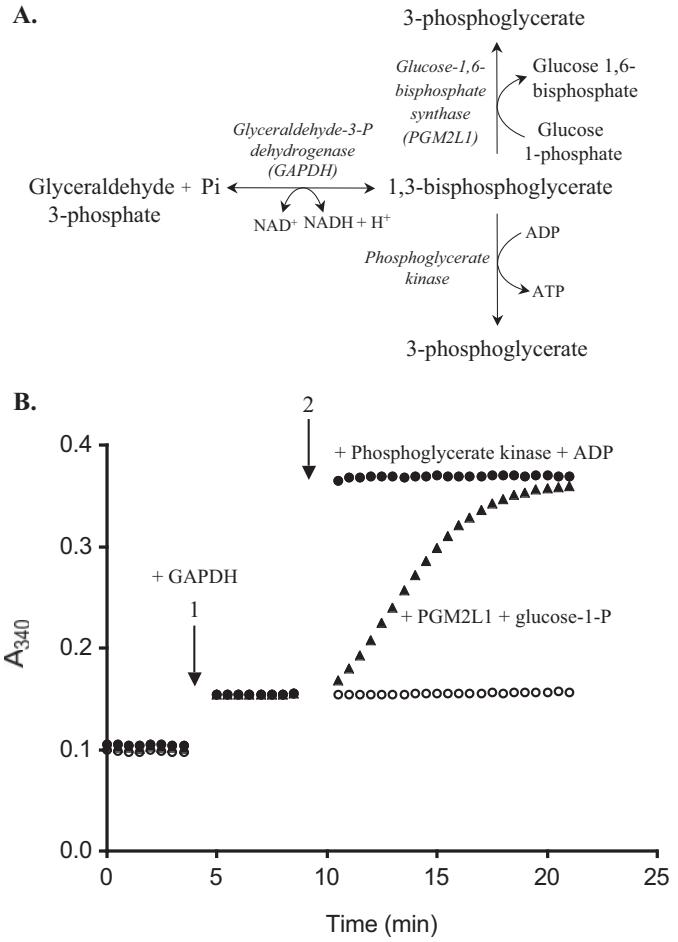

FIGURE 5. **Assay of 1,3-bisphosphoglycerate-dependent glucose-1,6-bisphosphate synthase through displacement of the equilibrium of glyceraldehyde-3-phosphate dehydrogenase.** *A*, scheme illustrating the principle of the assay is shown. *B*, assay mixture contained assay buffer as well as 5 mM inorganic phosphate, 1 mM NAD, and 50 μM glyceraldehyde 3-phosphate. *Arrow 1* indicates when rabbit muscle GAPDH (1 unit/ml) was added and *arrow 2* when either 15 μg/ml yeast phosphoglycerate kinase and 0.5 mM ADP (●) or 5 μg/ml PGM2L1 and 1 mM glucose 1-phosphate (▲) were added. (○): no further addition.

the formation of glucose 1,6-bisphosphate. The rate of NADH formation in this phase was proportional to the concentration of PGM2L1.

Using this assay, we found that PGM2L1 showed an aldose bisphosphate synthase activity with glucose 1-phosphate, deoxyribose 1-phosphate, ribose 1-phosphate, glucose 6-phosphate, and mannose 1-phosphate as substrates, but little or no activity on mannose 6-phosphate, ribose 5-phosphate, and deoxyribose 5-phosphate (Table 3). These findings are in good agreement with previous results obtained with glucose-1,6-bisphosphate synthase purified from mouse brain (17, 19). The homologous enzyme PGM2 showed some ability to synthesize aldose bisphosphates but was about 5–20-fold less active than PGM2L1 in this respect. The synthase activity was more potently inhibited by glucose 1,6-bisphosphate, the product of the reaction, in the case of PGM2 than of PGM2L1: when tested with 1 mM glucose 1-phosphate, 50% inhibition was reached with 10 μM glucose 1,6-bisphosphate in the case of PGM2 as compared with 50 μM in the case of PGM2L1.

DISCUSSION

Identification of Phosphopentomutase as PGM2—Our work demonstrates that phosphopentomutase activity in human and presumably also in other vertebrates is contributed by the protein designated PGM2 in the protein data bases (except in mouse where PGM2 is the orthologue of human PGM1). This conclusion is based on the finding that human erythrocyte phosphopentomutase copurifies with PGM2. Furthermore, recombinant PGM2 displays a higher phosphopentomutase activity than its phosphoglucomutase activity, having therefore properties similar to those of the phosphopentomutase (PGM2) previously characterized in mammalian tissues (20, 21). These findings are at variance with the results of Barksy and Hoffee (4), who purified phosphopentomutase from rat liver to near homogeneity and concluded that it was a dimer consisting of two subunits of 32.5 kDa. However, the SDS-PAGE gel shown by these authors indicates that their most purified fractions also contained a faint ≈70-kDa band in addition to the 32.5-kDa band. Our presumption is that the ≈70-kDa band rather than the 32.5-kDa band corresponds to rat phosphopentomutase. Accordingly the rat genome also encodes a PGM2 orthologue sharing 90% sequence identity with human PGM2.

Our findings indicate that PGM2 comprises the TASHNP motif common to members of the α-D-phosphohexomutase family, which comprises eukaryotic phosphoglucomutases, bacterial phosphoglucomutases and phosphomannomutases, eukaryotic phosphoacetylglucosaminemutase and bacterial phosphoglucosamine mutase (14). The reaction mechanism of phosphoglucomutase has been thoroughly studied (14, 22, 23). To be active, phosphoglucomutase needs to be phosphorylated on the serine present in the TASHNP motif, and this phosphorylation is achieved by transferring one of the phospho-groups

TABLE 3

Glucose 1,6-bisphosphate synthase and other aldose-bisphosphate synthase activities of PGM2 and PGM2L1

For the best substrates of PGM2L1, the activity was determined at a series of substrate concentrations to calculate V_{max} (indicated by an asterisk in the table) and K_m values. For other substrates of PGM2L1 and for PGM2, the activity was measured at 1 mM substrate. Means ± S.E. ($n = 3$).

Substrate	PGM2	PGM2L1		
	Activity at 1 mM substrate	V_{max}* or activity at 1 mM substrate	K_m	k_{cat}/K_m
	$\mu mol\ min^{-1}\ mg^{-1}$	$\mu mol\ min^{-1}\ mg^{-1}$	mM	$s^{-1}\ \mu M^{-1}$
Glucose-1-P	0.22 ± 0.01	1.45 ± 0.03*	0.32 ± 0.23	5.5 ± 0.4
Ribose-1-P	0.14 ± 0.01	1.93 ± 0.10*	0.44 ± 0.01	5.4 ± 0.1
Deoxyribose-1-P	0.05 ± 0.00	1.36 ± 0.03*	0.30 ± 0.01	5.6 ± 0.2
Mannose-1-P	0.15 ± 0.01	1.38 ± 0.11*	0.79 ± 0.04	2.1 ± 0.1
Glucose-6-P	0.08 ± 0.01	0.77 ± 0.05*	0.36 ± 0.02	2.6 ± 0.1
Ribose-5-P	0.07 ± 0.00	0.14 ± 0.01	ND[a]	ND[a]
Deoxyribose-5-P	Not detectable	0.04 ± 0.01	ND[a]	ND[a]
Mannose-6-P	Not detectable	Not detectable	ND[a]	ND[a]

[a] ND, not determined.

of glucose 1,6-bisphosphate. To catalyze the net conversion of glucose 1-phosphate to glucose 6-phosphate, the phosphorylated enzyme reacts with glucose 1-phosphate, thus re-forming glucose 1,6-bisphosphate. The latter flips inside the catalytic site and rephosphorylates the enzyme by transferring its 1-phospho group, thus forming glucose 6-phosphate. Because of the conservation of the TASHNP motif in PGM2 and of the fact that this enzyme also requires glucose 1,6-bisphosphate as a cofactor, it is likely that the conserved serine also serves as a transient phosphate acceptor.

The role of phosphopentomutase is to utilize ribose 1-phosphate and deoxyribose 1-phosphate, which are formed by purine nucleoside phosphorylase and uridine phosphorylase. The molecular identification of phosphopentomutase might help to solve unexplained cases of congenital immunodeficiency. Both adenosine deaminase and nucleoside phosphorylase deficiencies are indeed known to be causes of immunodeficiency (24, 25). The absence of phosphopentomutase should result in the accumulation of ribose 1-phosphate and deoxyribose 1-phosphate and therefore in a functional block of purine nucleoside phosphorylase.

Identification of PGM2L1 as Glucose-1,6-bisphosphate Synthase—The presence of a human protein sharing about 60% sequence identity with human PGM2 was very intriguing. Our first hypothesis was that this enzyme was a second form of phosphopentomutase/phosphoglucomutase with slightly different kinetic properties. However, this enzyme proved to have a very low phosphomutase activity on the three tested substrates (glucose 1-phosphate, ribose 1-phosphate, and deoxyribose 1-phosphate). The finding that PGM2L1 is expressed at a high level in brain, its molecular mass of 72 kDa and its (low) phosphomutase activity are all properties that PGM2L1 shares with glucose-1,6-bisphosphate synthase (17, 18, 26), an enzyme whose molecular identity had not yet been established.

That PGM2L1 corresponds to glucose 1,6-bisphosphate synthase is indicated by the fact that recombinant PGM2L1 used 1,3-bisphosphoglycerate as a phosphate donor and a series of sugar-monophosphate as acceptors. As for brain glucose-1,6-bisphosphate synthase (17, 19), 1-phosphosugars (glucose 1-phosphate, mannose 1-phosphate, ribose 1-phosphate, and deoxyribose 1-phosphate) were good substrates, whereas 5 or 6-phosphosugars (to the exception of glucose 6-phosphate) were not. This broad substrate specificity makes that PGM2L1 is able to synthesize cofactors for various phosphomutases.

The fact that PGM2L1 also comprises a potentially phosphorylatable serine in a similar motif as found in PGM1-related enzymes agrees with previous findings indicating that glucose-1,6-bisphosphate synthase is phosphorylated on a serine residue by transfer of the 1-phosphogroup of 1,3-bisphosphoglycerate (18). As previously shown, this reaction is the first step of aldose-bisphosphate synthesis. The second step is the transfer of the phosphate group from the phosphorylated serine onto a suitable sugar-phosphate acceptor (18, 26).

As previously reported by others for the enzyme purified from human erythrocytes (21), recombinant PGM2 also displays glucose-1,6-bisphosphate synthase activity. However, as shown in the present study, the synthase activity of PGM2 is lower than that of PGM2L1 and it is more powerfully inhibited by glucose 1,6-bisphosphate. This means that PGM2L1 is more prone to form elevated concentrations of glucose 1,6-bisphosphate than PGM2. This agrees with the finding that the glucose 1,6-bisphosphate concentration is particularly elevated in brain, where values of the order of 75 nmol/g have been reported (27, 28). These concentrations are far above the levels needed to saturate phosphomutases ($K_a \approx 0.1\ \mu M$) and it is therefore thought that glucose 1,6-bisphosphate plays another role, possibly a regulatory one.

Glucose 1,6-bisphosphate is, at least *in vitro*, an inhibitor of low K_m hexokinases (29, 30), of 6-phosphogluconate dehydrogenase (31) and of fructose-1,6-bisphosphatase (32), and a stimulator of phosphofructokinase (33), though a much poorer one than fructose 2,6-bisphosphate (34), and of liver pyruvate kinase (35). The idea that glucose 1,6-bisphosphate plays a regulatory role is supported by the finding that brain is also rich in glucose-1,6-bisphosphatase, the enzyme that degrades glucose 1,6-bisphosphate. The activity of this enzyme is nearly dependent on inosine-monophosphate, a nucleotide that accumulates in anoxia (36) and this effect may at least partly account for the fact that the glucose 1,6-bisphosphate concentration decreases in brain during ischemia. The decrease in the concentration of glucose 1,6-bisphosphate is likely to deinhibit low K_m hexokinases and favor glycolysis. A complementary role is presumably played by ribose 1,5-bisphosphate, which is also formed by PGM2L1 and is a potent stimulator of phosphofructokinase (37). The concentration of ribose 1,5-bisphosphate was shown to increase in macrophages during hypoxia (38). The molecular identification of the enzyme that synthesizes glucose 1,6-bisphosphate and ribose 1,5-bisphosphate will facilitate inves-

tigations aimed at testing the regulatory role of these molecules in intact cells.

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