Identification and characterisation of a new human glucose-6-phosphatase isoform

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Abstract **The liver endoplasmic reticulum glucose-6-phosphatase catalytic subunit (G6PC1) catalyses glucose 6-phosphate hydrolysis during gluconeogenesis and glycogenolysis. The highest glucose-6-phosphatase activities are found in the liver and the kidney; there have been many reports of glucose 6-phosphate hydrolysis in other tissues. We cloned a new G6Pase isoform (G6PC3) from human brain encoded by a six-exon gene (chromosome 17q21). G6PC3 protein was able to hydrolyse glucose 6-phosphate in transfected Chinese hamster ovary cells. The optimal pH for glucose 6-phosphate hydrolysis was lower and the K_m higher relative to G6PC1. G6PC3 preferentially hydrolyzed other substrates including pNPP and 2-deoxy-glucose-6-phosphate compared to the liver enzyme.**

Key words: Brain; Liver; Glucose-6-phosphatase; Enzymatic activity

1. Introduction

The liver glucose-6-phosphatase catalytic subunit (G6PC1; EC 3.1.3.9) is part of a multi-component system, located mainly with its catalytic site oriented towards the lumen of the endoplasmic reticulum in liver and kidney cells [1–3]. Two proteins of the liver system have been cloned: the catalytic subunit [4] and a glucose-6-phosphate transporter [5,6]. Genetic deficiencies of any of the protein components of the glucose-6-phosphatase system are classified as glycogen storage disease type 1 (GSD) [3]. In liver, G6PC1 system catalyses hydrolysis of glucose-6-phosphate into glucose and phosphate and plays a critical role in blood glucose homeostasis [1–3,7,8]. Glucose-6-phosphatase activity has also been detected in the nuclear envelope [9], and in tissues other than liver [1,2,10–12], this would suggest that its enzymatic activity might also be used for other purposes such as locally regenerating glucose stocks or ATP levels [13].

Kinetic analysis of G6P transport in brain and liver suggested that a different isoform of glucose-6-phosphate translocase (G6PT1) transport protein might be present in brain [14]. It was subsequently shown that a larger isoform of the protein was present in brain arising from differential splicing of the G6PT1 gene. The K_m of G6Pase catalytic subunit activity is also not the same in all tissues raising the possibility that there could be a different isoform of G6PC in non-hepatic tissues [15].

An islet-specific cDNA [16] (termed here G6PC2) was isolated in 1999, it was a different gene product [17,18] which potentially encodes a similar protein to G6PC1. The significance of the G6PC2 protein remains unclear as it was reported to be inactive [16–18].

In the present work we cloned and expressed a third cDNA (G6PC3) from brain RNA with sequence similarity to G6PC1. We also characterised its enzymatic activity and compared it to that of the liver G6PC1.

2. Materials and methods

2.1. Materials

Primers were synthesised by Genosys Biotech. The M-MLV reverse transcriptase, oligod(T) primer, RNasin and pGEM-T cloning kit were from Promega. Human brain RNA was from Ambion Inc.; pcDNA 3.1+ vector was from Invitrogen. 2-Deoxy-glucose-6-phosphate (sodium salt, 2-deoxy-G6P), Mannose-6-phosphate (sodium salt), Histone IIAS and cacodylic acid were from Sigma. Para-nitrophenyl phosphate (sodium salt, pNPP) was from ICN. Glucose-6-phosphate (sodium salt) and all other chemicals were from Merck. Human G6PC3 clone was kindly provided by the IMAGE consortium (Cambridge, UK).

2.2. Methods

2.2.1. Reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated by the TRIZOL procedure (Gibco) or SV total RNA isolation (Promega). Total human brain RNA (1 μg) or Chinese hamster ovary (CHO) cell RNA (6 μg) were reverse transcribed according to the manufacturer's instructions (oligod$(T)_{15}$ primer for human RNA, random hexamers for CHO cells RNA) using M-MLV reverse transcriptase, RNase H minus. Sense (5′-ACTCTGGTTTCCGCCCTGGAGCAA-3′) and antisense (5′-CTGGAGTGTGGTGGTCACAGAGT-3′) primers were used for human RNA and sense (5′-AGATATCCAGCACAGTGGCGGC-3′) and antisense (5′-TGTGGGAGGGAAAGGAAAGGGA-3′) primers were used for CHO cell RNA. PCR products were purified using Wizard PCR preparation kit (Promega) then incubated with 10 mM dATP and 2.5 U Taq polymerase (MBI Fermentas) at 72°C for 1 h. The DNA was repurified, ligated into pGEM-T vector and used to transform XL1 Blue cells. Colonies were grown in LB-Amp and plasmid DNA purified using a Wizard miniprep kit (Promega).

2.2.2. Construction of expression vector. RT-PCR using adult human brain RNA was prepared as described above. The 1160 bp cDNA was reamplified using sense (5′-ATTACTCTCGAGTTTCCGCCCTGGA-3′) and antisense (5′-TGATTCTAGAGTGTGGTGGTCACA-3′) primers containing Xho*I* and Xba*I* restriction sites

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Abbreviations: CHO, Chinese hamster ovary cell line; G6PC, catalytic subunit of glucose-6-phosphatase; G6PT, glucose-6-phosphate translocase; pNPP, para-nitrophenyl phosphate

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respectively (underlined sequences). PCR was prepared in a final volume of 25 μl. PCR programme was performed as follows: 1 cycle: 95°C (5 min); 30 cycles: 95°C (1 min), 63°C (1 min), 72°C (2 min) and last cycle: 72°C (8 min). The PCR product was analysed on a 1% agarose gel, extracted and digested with Xho*I* and Xba*I* restriction enzymes, as pcDNA 3.1+ vector. After dephosphorylation of the opened vector, the digested PCR product was ligated into it. XL1 Blue cells were transformed by electroporation. Colonies were grown up and plasmid DNA was purified using a QIAgen plasmid maxi kit (QIAgen). DNA was sequenced on an Applied Biosystem automated DNA sequencer.

2.2.3. Cell culture and stable transfection. The CHO-K1 cells, epithelial cells from Chinese hamster ovary, were transfected with G6PC3-pcDNA 3.1 using the lipofectin reagent (GibcoBRL) according to the manufacturer's instructions. Forty-eight transfected colonies were obtained.

2.2.4. Northern blots. Total RNA (16 μg) was separated on a 1.0% formaldehyde-agarose gel and transferred to nitrocellulose membranes (Sigma). The membranes or the Clontech 'Multiple Human Tissue Northern Blot' were hybridised overnight with appropriate [^{32}P]-labelled probes.

2.2.5. Preparation of rat liver microsomes. Microsomes were isolated from Sprague Dawley rat livers as described elsewhere [19]. Microsomes were resuspended in a 0.25 M sucrose, 5 mM HEPES buffer, pH 7.4, quickly frozen, and kept at −80°C until use.

2.2.6. Preparation of cells for enzymatic assay. Confluent cells grown in 75 cm^3 flasks were harvested by scraping and resuspended in 5 mM HEPES, pH 7.4, centrifuged at 10 000×*g* for 10 s and resuspended in 600 μl of ice-cold HEPES buffer. The suspension was homogenised mechanically by hand with a glass/glass homogeniser and used immediately.

2.2.7. Assays. Glucose-6-phosphatase activity was measured using a 96-well microplate assay derived from the colorimetric technique previously described [20] using various concentrations of G6P and other substrates. In some assays 1 mg/ml histone IIA [21] was used to permeabilise microsomal vesicles [22]. Microsomal intactness was measured and corrected for using mannose-6-phosphate [20,23]. Intactness was greater than 94% in all rat liver microsomal preparations. K_m and V_{max} were determined by non-linear regression analysis [24]. All experiments were repeated at least three times. Protein concentrations were determined as in [25]. All statistical calculations were carried out using an unpaired *t*-test with two-tail *P* value (Prism computer programme for Macintosh).

3. Results and discussion

Glucose-6-phosphatase activity has been reported in many tissues especially during foetal development [1,2]. Kinetic parameters measured in some of these tissues, e.g. [16], differ from those in liver and indicate that another G6PC protein might exist in a number of non-hepatic tissues. Patients with type 1a GSD (G6PC1 deficiency) have glycogen accumulation in liver and kidney and symptoms associated with lack of glucose production by the liver (and to a lesser extent the kidney) [3]. Patients suffer from episodes of fasting hypoglycaemia after birth [3] but there is no evidence that there are problems in utero [3] raising the possibility that there might be another G6PC gene. More recently G6PC2 cDNA was isolated from islets, it encodes a similar protein to G6PC1 [16]. However, G6PC2 mRNA seems to be islet specific [16–18] and therefore cannot account for glucose-6-phosphatase activity in other tissues. We therefore looked for the presence of another G6PC isoform in non-hepatic tissues.

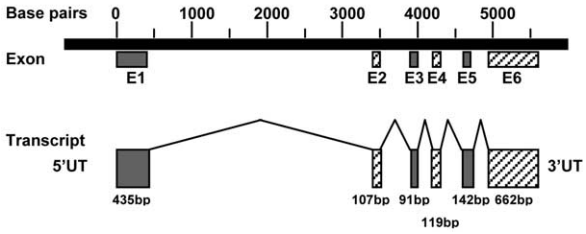

Fig. 1. Organisation of the G6PC3 gene and splicing of its transcript.

3.1. Cloning and analysis of the new phosphatase sequence

The GenBank EST database contains a cDNA clone (accession number AA232171) isolated from human brain RNA that has a length of about 1.3 kbp. This sequence contains an ORF and we named it G6PC3 because of its similarity to both liver G6PC1 [4] and G6PC2 [16]. Oligonucleotides designed from the EST sequence were used to perform RT-PCR on human brain mRNA. A single PCR product was obtained with an apparent size of 1.1 kbp, it was cloned into a pGEM-T vector, subcloned into pcDNA 3.1, and sequenced in both directions. The nucleotide sequence was strictly identical to the AA232171 sequence. A BLAST search of the NCBI nr database using the obtained sequence identified two further clones: accession numbers BC002494 and BC021574. These two clones and our PCR product showed that the predicted G6PC3 initiation codon is located in the context of a strong Kozak concensus sequence [26]. A BLAT search of the UCSC Genome Bioinformatics database (http://genome.ucsc.edu) allowed us to compare the G6PC3 sequence and human genomic sequences in order to determine the number of exons/introns and their boundaries (Fig. 1). The

Table 1
Comparison of the kinetic parameters of the glucose-6-phosphatase activity in rat liver microsomes and in G6PC3-transfected CHO cells

	K_m (mM)			V_{max} (nmol/min/mg)		
	pH 5.5	pH 6.5	*P*	pH 5.5	pH 6.5	*P*
Not disrupted						
Liver	5.1 ± 0.7	1.3 ± 0.1*	0.0046	284 ± 19	294 ± 15	NS
G6PC3	4.1 ± 0.5	4.3 ± 0.4	NS	15.6 ± 1.6	18.3 ± 2.3	NS
Disrupted						
Liver	0.5 ± 0.04**	0.3 ± 0.01***	0.0054	495 ± 15	545 ± 3.8	0.0318
G6PC3	1.0 ± 0.06	2.0 ± 0.1	< 0.0001	36.5 ± 3.9	31.5 ± 2.4	NS

Each assay mixture contained 125 mM cacodylate pH 5.5 or 6.5, 2.5 mM EDTA, and various concentrations of G6P (1–10 mM). *P* values in the table are pH 5.5 compared to respective values at pH 6.5. NS, not significant. *$P = 0.0012$ compared to G6PC3 (not disrupted) K_m at pH 6.5; **$P = 0.0035$ compared to G6PC3 (disrupted) K_m at pH 5.5; ***$P < 0.0001$ compared to G6PC3 (disrupted) K_m at pH 6.5. G6PC3 activity values were calculated removing the basal glucose-6-phosphate hydrolysis found in CHO cells from the values obtained using G6PC3-transfected CHO cells. Control CHO cells transfected with empty pcDNA 3.1 vector had basal levels of glucose-6-phosphate hydrolysis which were not significantly different from hydrolysis levels in normal CHO cells (data not shown).

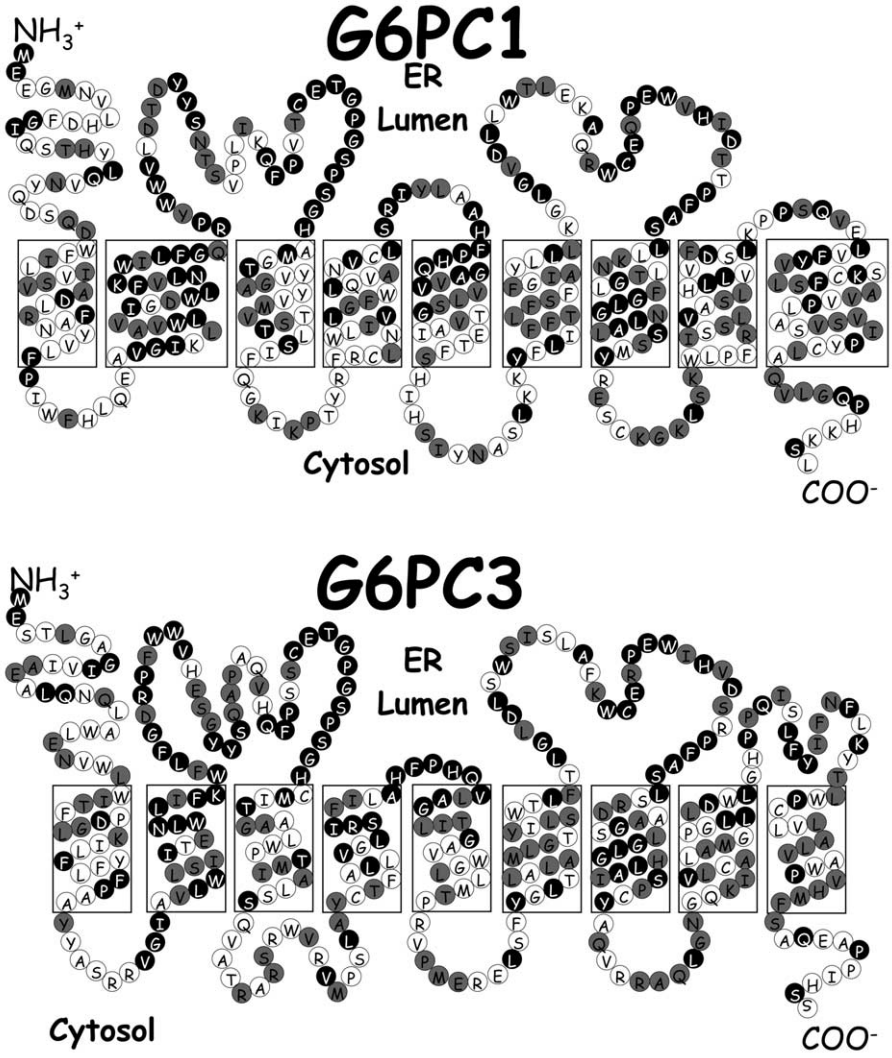

Fig. 2. The predicted membrane topology of both human G6PC1 and G6PC3 proteins. Structural predictions for both human G6PC1 and G6PC3 were performed using internet resources (http://www.ch.embnet.org/software/TMPRED_form.html). Strictly conserved amino acids are in black and partially conserved residues in grey.

G6PC3 cDNA maps chromosome 17q21, as does G6PC1 [27]. It contains six exons (Fig. 1) and the cDNA (1041 bp) encodes a hydrophobic protein of 346 amino acids (Fig. 2) with predicted M_w and pI of 38 734 and 8.47, respectively.

3.2. Comparison of expression of G6PC1 and G6PC3 mRNA in human tissues

It has been known for many years that G6PC1 protein is predominately expressed in gluconeogenic tissues, i.e. the liver and to a lesser extent the kidney. Here we confirm that human G6PC1 mRNA has the same expression pattern (Fig. 3). In complete contrast G6PC3 mRNA levels are low in liver, and relatively high in brain, heart, skeletal muscle, placenta and kidney (Fig. 3). The tissues which express high levels of G6PC3, with the exception of kidney (which also has high levels of G6PC1), are not normally considered to be gluconeogenic tissues. These patterns of mRNA expression strongly suggest that the glucose-6-phosphate hydrolysis activities previously found in a variety of non-hepatic tissues [1,2,10–12,14,15], including brain and skeletal muscle, may be catalysed by G6PC3 rather than G6PC1.

3.3. Structure predictions of G6PC3

Structure predictions were performed using Internet resources (http://www.ch.embnet.org/software/TMPRED_form.html) for both human G6PC1 and G6PC3. The structure prediction of the G6PC1 (Fig. 2) corresponds to the one previously described [28]. The predicted structure of the two types of proteins (Fig. 2) is very similar with nine membrane spanning segments. It must be stressed that in contrast to G6PC1 and G6PC2, G6PC3 does not contain a C-terminus (KK) endoplasmic reticulum transmembrane retention signal [29] although not all endoplasmic reticulum transmembrane proteins have the KK retention signal [29]. The regions of the proteins that are thought to be part of the active sites [28,30,31] are very conserved between the two sequences: a conserved phosphatase sequence motif KXXXXXXRP-(X_{12-54})-PSGH-(X_{31-54})-SRXXXXXHXXXQ/D is present in the G6PC3 sequence (Fig. 4). The three most important catalytic residues correspond to those of G6PC1, with Arg^{79} of G6PC3 aligned to Arg^{83} of G6PC1, His^{114} to His^{119}, and His^{167} to His^{176}. Furthermore, these predictions suggest that these amino acid residues are located on the same side of the

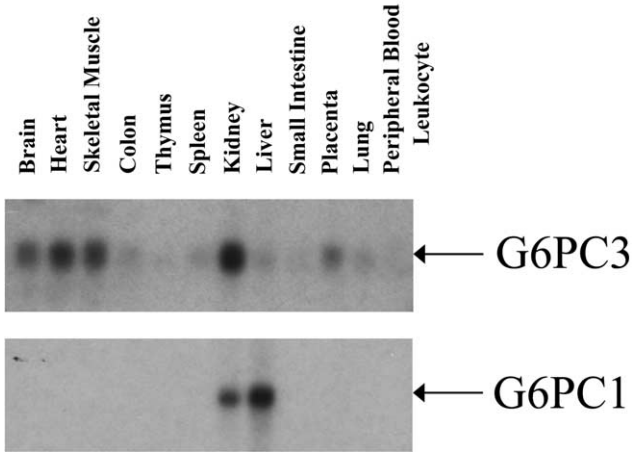

Fig. 3. Comparison of G6PC1 and G6PC3 expression in human tissues RNA blotting analysis was performed using Clontech MTN with 1 μg of poly(A) mRNA in each lane of the blot. The hybridisation was carried out sequentially on the same membrane using an internal G6PC1 cDNA sequence of 646 bp covering exon 2 to exon 5 and an internal G6PC3 cDNA from exon 2 to exon 5 (677 bp) as [^{32}P]-labelled probes.

membrane (Fig. 2), indicating the presence of a putative functional active site. All 48 amino acids changes reported in G6PC1 in cases of GSD type 1a [32] are shown in Fig. 4: 31 are strictly conserved in G6PC3, 10 are partially conserved in G6PC3 and only seven of these amino acids differ in G6PC3 (Q54 > S50, G68 > T64, G122 > I117, W156 > S147, G222 > I213, L265 > S255, S298 > C286). This conservation of key amino acids suggests G6PC3 might be catalytically active.

3.4. Enzymatic activities

CHO cells were therefore stably transfected with a plasmid containing G6PC3. A clone in which Northern blot analysis revealed relatively high levels of G6PC3 was chosen for the study of G6PC3 function (Fig. 5). Glucose-6-phosphate hydrolysis occurred in the transfected cells in both untreated (Fig. 6A) and disrupted vesicles (Fig. 6B). In disrupted vesicles the values are a direct measurement of G6PC3 activity [21,22]. In intact vesicles there is latency (Table 1) due to the need to transport glucose-6-phosphate across a membrane [1,3,19,20]. The presence of some activity with glucose-6-phosphate as substrate in intact vesicles together with the relative differences in substrate specificity between untreated and disrupted vesicles indicates that a glucose-6-phosphate transporter and possibly other substrate transporter are present in

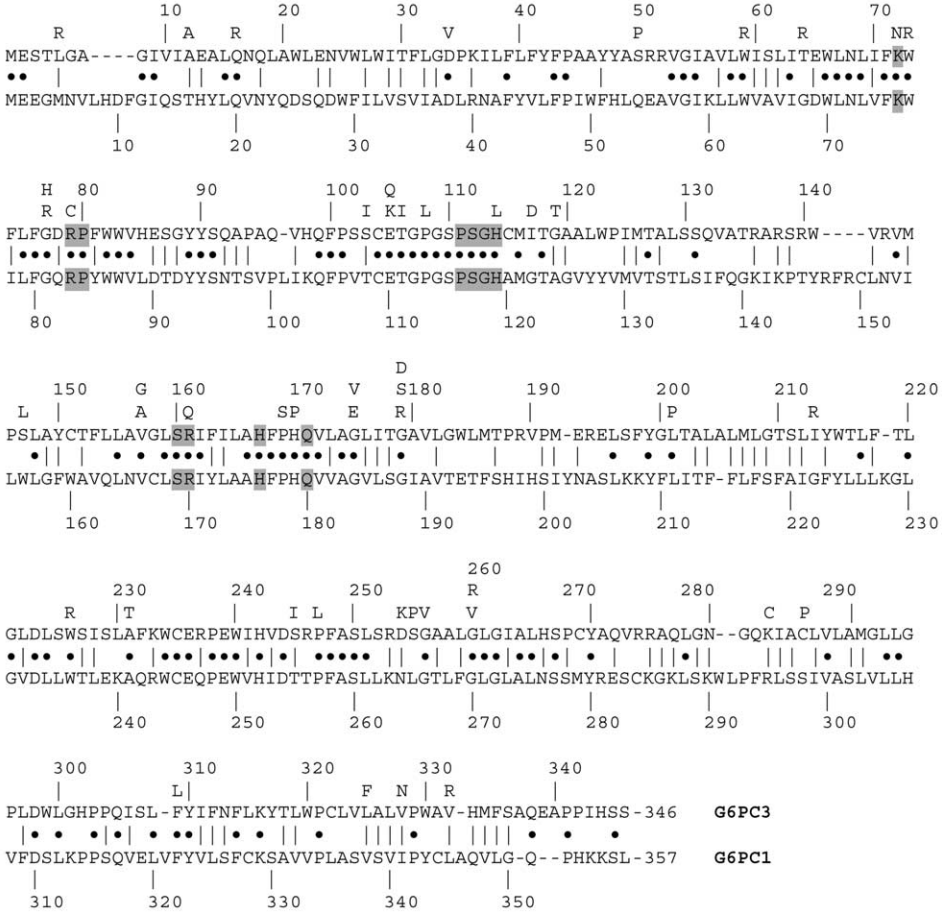

Fig. 4. Alignment of the deduced peptides sequences of the G6PC3 and G6PC1. Identities are indicated by black dots and similarities by vertical bars. Residues defined as being of key catalytic importance in phosphatases are shaded grey. Inactivating point mutations that abolish or reduce G6Pase catalytic subunit and give rise to GSD type 1a are shown in single letter code above the G6PC3 sequence.

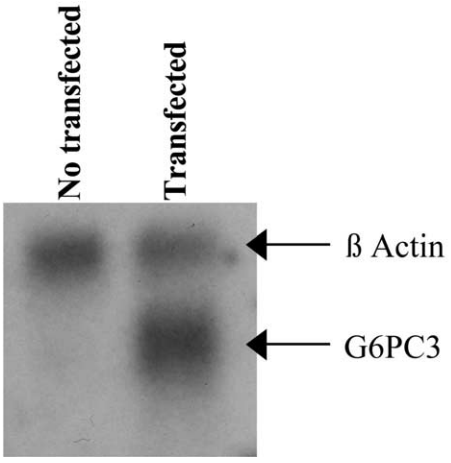

Fig. 5. Analysis of human G6PC3 presence by RNA blotting. Both lanes were loaded with 16 μg total RNA from transfected or non-transfected CHO cells. The hybridisation was carried out using an internal β actin cDNA (424 bp) and an internal G6PC3 cDNA from exon 2 to exon 5 (677 bp) as [^{32}P]-labelled probes.

CHO cell membrane vesicles. The possibility that low levels of G6PT1 are present cannot be ruled out but it seems more likely that the transport of glucose-6-phosphate is via the recently described less specific hexose phosphate transporter

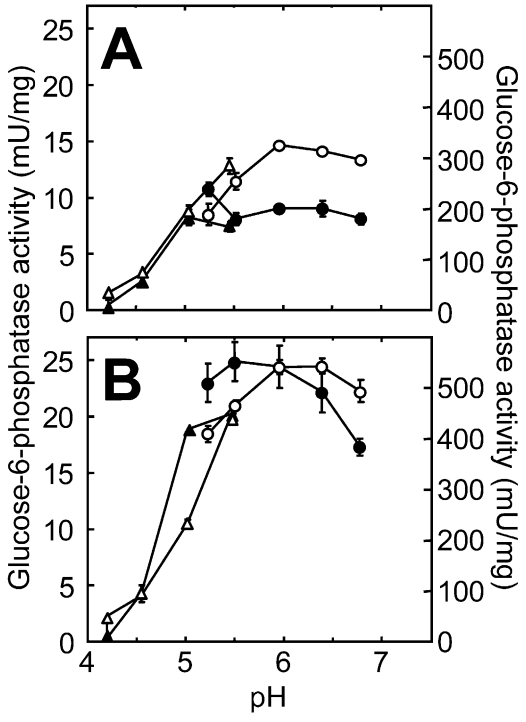

Fig. 6. pH profiles of glucose-6-phosphatase activities in absence (A) or presence of histone (B). Assays mixtures contained 125 mM histidine (△, ▲) or cacodylate (○, ●), 2.5 mM EDTA, 10 mM glucose-6-phosphate. The actual pH of the mixtures was determined under assay conditions. The enzymatic activities of G6PC3 (▲, ●, left *Y* axis) were calculated by removing the basal enzymatic activities found in untransfected CHO cells from the values obtained using G6PC3-transfected CHO cells. CHO cells transfected with empty pcDNA 3.1 vector had basal levels of glucose-6-phosphate hydrolysis which were not significantly different from levels in normal CHO cells (data not shown). The activity scale of rat liver microsomes (△, ○) is represented on the right *Y* axis. The results represent the mean of at least three different assays ± S.E.M.

recently found in cells lines and fibroblasts from G6PT1 deficient patients with GSD 1b [33].

Levels of enzymatic activity in transfected CHO cells were lower than in rat liver microsomes. This obviously reflects the levels of transfection but it is also consistent with the levels of glucose-6-phosphatase activity in human non-hepatic and foetal tissues that can be 10 times lower than in liver [12,15]. Rat liver microsomes were chosen as a source of G6PC1 activity rather than human liver microsomes for ethical reasons. Previously G6PC1 activity in adult rat and human liver microsomes have been shown to have the same K_m and substrate specificity, e.g. [3,20]. The pH optima for G6PC3 enzymatic activity in disrupted vesicles is lower than that of G6PC1 (Fig. 6B), (approx. pH_{opt} 6.5 as previously shown [34]). K_m values of glucose-6-phosphate hydrolysis by G6PC3 and liver G6PC1 in conditions where vesicles are not disrupted are similar at pH 5.5 but different at pH 6.5 (Table 1). In disrupted conditions, all K_m values are lower for both isoforms at both

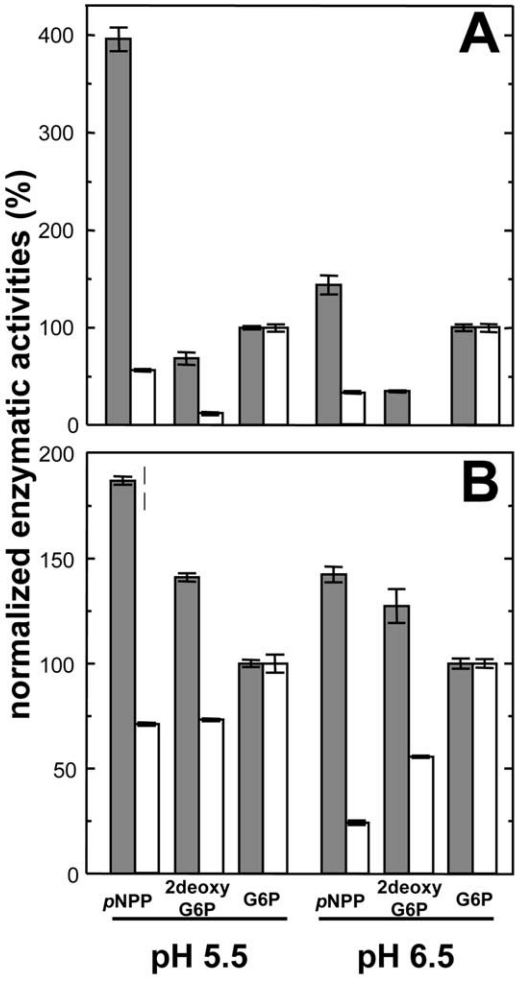

Fig. 7. Enzymatic activities using different substrates in absence (A) or presence of histone (B). Liver microsomal activity (open bars). G6PC3 activity values (shaded bars) were calculated removing the basal enzymatic activities found in CHO cells from the values obtained using G6PC3-transfected CHO cell solution. Each mixture contained 125 mM cacodylate pH 5.5 or 6.5, 2.5 mM EDTA, and 10 mM of substrate (pNPP, 2-deoxy-G6P, G6P) or 1 mM of M6P (for intactness calculation). Each rate of substrate hydrolysis is relative to G6P hydrolysis (100%) for a given condition. The results represent the mean of at least three different assays ± S.E.M.

pH's (Table 1). The K_m values for disrupted liver microsomes (Table 1) indicate a better affinity for glucose-6-phosphate at pH 6.5 for G6PC1 and pH 5.5 for G6PC3. This confirms that G6PC3 is a glucose-6-phosphatase enzyme, with a different K_m to G6PC1 in our experimental conditions. While our work was in progress, another similar sequence to G6PC1 and G6PC2 was identified and cloned from human melanotic melanoma [35]. It has an identical sequence to G6PC3. It was termed ubiquitously expressed G6Pase catalytic subunit-related protein as the authors failed to detect any enzymatic activity of transiently expressed constructs in COS cells. It is not clear why the authors [35] failed to detect any enzymatic activity. The obvious differences are (a) that a different cell line was used and (b) that transient transfection experiments were done in [35] and in contrast here we cloned cells that stably express relatively high levels of G6PC3.

3.5. Substrate specificity

Liver G6PC1 is a relatively non-specific phosphohydrolase. We therefore used two other potential G6PC3 substrates to determine if the two isoforms have the same relative substrate specificities. Assays were performed at pH 5.5 and 6.5 and normalised to respective rates of glucose-6-phosphate hydrolysis (Fig. 7). pNPP, a classical substrate for many phosphatases, is rapidly hydrolysed by G6PC3 compared to glucose-6-phosphate in both disrupted and non-disrupted conditions (Fig. 7). In contrast to G6PC1, 2-deoxy-G6P is also a better substrate than glucose-6-phosphate for G6PC3.

The fact that G6PC3 hydrolyses glucose-6-phosphate is logical based on its sequence similarities to G6PC1 and its conserved consensus phosphatase sequences. However, glucose 6-phosphate is not its preferred substrate in vitro (Fig. 7) raising the possibility that glucose-6-phosphate might not be the only substrate of G6PC3 in vivo. The role of G6PC3 in brain and other tissues is not yet clear but one logical possibility is that it supplies glucose for local use, i.e. for other cell types in the same tissue.

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