A different isoform of the transport protein mutated in the glycogen storage disease 1b is expressed in brain

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Abstract There are differences in the kinetic properties of the liver and brain microsomal glucose-6-phosphate transport systems suggesting the possibility of tissue specific isoforms. The availability of a human liver cDNA sequence which is mutated in patients with deficiencies of liver microsomal glucose-6-phosphate transport (glycogen storage disease 1b) made it possible to determine if a brain isoform exists. Northern blots of liver and brain RNA revealed that the mRNA of the brain form is slightly longer than the liver one. Isolation and sequencing of the respective human brain cDNA revealed that the brain protein has an additional 22 amino acid sequence.

Key words: Glucose-6-phosphatase; Glucose-6-phosphate translocase; Glycogen storage disease; Endoplasmic reticulum

1. Introduction

Liver glucose-6-phosphatase (EC 3.1.3.9) plays a key role in blood glucose homeostasis, catalysing the terminal step of both gluconeogenesis and glycogenolysis [1,2]. The principal sites of expression of the enzyme are liver and kidney [3], but it also present in a variety of other human tissues such as brain, pancreas, the adrenal foetal zone and intestinal mucosa [4–8]. The glucose-6-phosphatase enzyme is the catalytic subunit of a multi-protein system situated with its active site inside in the endoplasmic reticulum membrane of the cells [2,8]. This system (Fig. 1) also involves at least three different transport functions termed T1 (glucose-6-phosphate), T2 (phosphate) and T3 (glucose) [2]. The carriers allow the transport of substrate molecules through the membrane into the endoplasmic reticulum lumen and then the elimination of the reaction products from the endoplasmic reticulum cisternae (Fig. 1) (for a review see [2]). The genetic deficiencies of the catalytic subunit, T1, T2, and T3 are termed glycogen storage disease 1a, 1b, 1c and 1d respectively [8]. These are severe metabolic disorders that usually present with fasting hypoglycaemia [8]. Glycogen storage disease 1b is often more problematical than the other subtypes as the patients have additional symptoms e.g. neutropenia [8].

In liver the glucose-6-phosphate transport protein is the rate limiting step in glucose-6-phosphate hydrolysis in intact microsomal vesicles [9] and the K_M for glucose-6-phosphatase activity with glucose-6-phosphate as substrate in normal intact microsomes isolated from a variety of species is usually less than 5 mM e.g. [10–12]. It was therefore rather surprising that the K_M reported in intact microsomes with glucose-6-phosphate as substrate in brain was much higher, >20 mM [13]. This difference in K_M raised the possibility that the glucose-6-phosphate transport proteins in brain and liver might be different.

A liver EST (expressed sequence tag) with some sequence homology to bacterial sugar phosphoester transporters was recently found to be mutated in patients with defective microsomal glucose-6-phosphate transport (glycogen storage disease 1b) [14]. We have used the published human liver sequence as a probe to show that the brain and liver mRNA are different sizes and also to clone the human brain isoform.

2. Materials and methods

2.1. Materials

The M-MLV reverse transcriptase and pGEM-T cloning kit were both from Promega; *Taq* polymerase/*Pwo* mix was from Hybaid. Radioactive compounds were from Amersham.

2.2. Human and animal tissues

Adult human biopsy tissue and rat tissues were rapidly frozen in liquid nitrogen and stored at −80°C prior to use. Adult Wistar rats were either allowed free access to food and water, or were denied food but not water for 48 h. Diabetes was induced by a single tail vein injection of 75 mg/kg body weight streptozotocin in citrate buffer pH 4.5 [15]. Only animals in which the development of diabetes was confirmed 48 h later by blood glucose measurements >12 mM were used. Ethical approval was given by the Tayside Health Board Ethical Committee.

2.3. RNA preparations

The RNA was isolated according to the TRIZOL procedure (Gibco). The amounts of RNA were estimated by the spectrophotometric method at 260 nm.

2.4. Northern blot

Total RNA (5 to 20 μg, see figure legends) was separated on a 1.3% formaldehyde-agarose gel, transferred to GeneScreen membranes (Dupont-NEN) and fixed with UV. The membranes were rehydrated in 5×SSC, prehybridised for 4 h at 42°C in 1 M NaCl, 50% formamide, 1% SDS, 10% dextran sulphate, 10 mg/ml sheared denatured herring sperm DNA and hybridised overnight with a [^{32}P]-labelled probe (full length human liver cDNA mutated in glycogen storage disease 1b) in the same buffer. The blot was washed twice for 30 min at 65°C in 2×SSC, 1% SDS, twice for 30 min at 65°C in 1×SSC, 1% SDS and twice for 30 min at 65°C in 0.2×SSC, 1% sodium dodecyl sulphate. The membranes were then exposed overnight to X-ray film (GRI) at −80°C.

2.5. RT-PCR

One μg of total RNA was reverse transcribed at 42°C for 50 min in a volume of 25 μl with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 800 μM dNTPs, 2–4 U RNasin ribonuclease inhibitor, 0.5 μg oligodT primer and 100 U M-MLV reverse transcriptase, RNase H minus. The enzyme was inactivated at 94°C for 5 min. Ten μl of the cDNA was then combined with 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 μM dNTPs, 1.5 mM $MgCl_2$, 25 pmoles sense (5′CCATGGCAGCCCAGGGCTATG3′) and antisense (5′CTTCACTCAGCGTTGTTGGACACTCGGCC-

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C3′) primers and 2.5 U of a mixture of *Taq*/*Pwo* polymerases. PCR was carried out as follows using a Perkin-Elmer Cetus thermo-cycler: cycle 1: 94°C (5 min), 65°C (2 min), 72°C (3 min), cycles 2–29: 94°C (1 min), 65°C (2 min), 72°C (3 min) and last cycle: 94°C (1 min), 65°C (2 min), 72°C (10 min). PCR products were analysed on a 1% agarose gel containing ethidium bromide.

2.6. DNA sequencing

PCR products were purified from low melting point agarose using Wizard PCR preps 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 re-purified and cloned into pGEM-T vector. DNA sequences were determined both manually from double stranded plasmids by the dideoxynucleotide-chain termination method using the T7 Sequenase version 2.0 sequencing kit from Amersham and automatically on an applied Biosystems automated DNA sequencer.

3. Results and discussion

The different characteristics of the microsomal glucose-6-phosphate in liver and brain suggested the possibility that different isoforms of T1 might exist [10,13]. The existence of a liver EST, exhibiting sequence similarities with a family of bacterial proteins involved in phosphoester transport [16] together with the finding that the EST was mutated in patients suffering from liver endoplasmic reticulum glucose-6-phosphate transport defects (glycogen storage disease 1b) [14], made it feasible to investigate whether or not a brain isoform exists. The EST was most like UhpC, a membrane protein involved in the regulation of UhpT (the sugar phosphate carrier of *Escherichia coli*) [16]; UhpT is itself very similar to UhpC (32% strict homology or 58% similarity) [16]. We do not yet know if, like in bacteria, the mammalian endoplasmic reticulum glucose-6-phosphate transport system is comprised of more than one protein. However for simplicity, in this paper we have termed both the published EST liver cDNA and the protein G6PT1, because in patients this protein is

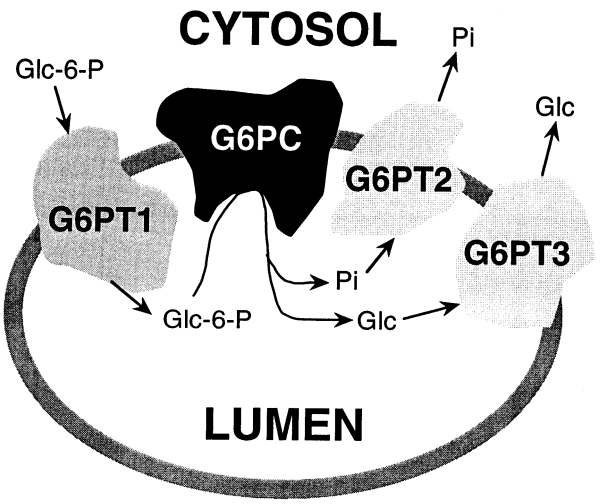

Fig. 1. Schematic representation of the microsomal glucose-6-phosphatase system. Glucose-6-phosphate is transported in the endoplasmic reticulum by a glucose-6-phosphate transport system (G6PT1) and dephosphorylated by the glucose-6-phosphatase catalytic subunit (G6PC). The phosphate and glucose produced are leaving the endoplasmic reticulum lumen via the phosphate and glucose transport systems (G6PT2 and G6PT3 respectively). The term system is used for each function to denote that normal function of each may require more than one polypeptide chain.

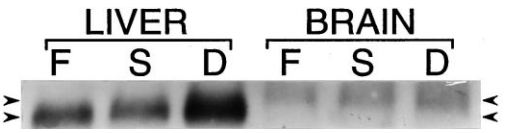

Fig. 2. Northern blot of rat liver and brain RNA from fed (F), starved (S) or diabetic (D) animals. The three first lanes were loaded with 5 μg total RNA from rat liver. The three other lanes were loaded with 20 μg total RNA from rat brain. The hybridisation was carried out using the full length human liver cDNA that is mutated in glycogen storage disease 1b as a [^{32}P]-labelled probe.

essential for glucose-6-phosphate transport ([14] and Marcolongo et al., submitted for publication).

3.1. A comparison of the mRNAs in liver and brain

Northern blots of RNA prepared from fed, starved and diabetic rat liver and brain tissue were visualised using as a probe the human liver cDNA that is mutated in glycogen storage disease 1b (Fig. 2). The size of the mRNA visualised was larger in brain than in liver strongly suggesting that tissue specific isoforms exist. The amount of the mRNA in liver is increased as expected in the starved and diabetic liver (Fig. 2) where it is well known that the glucose-6-phosphatase system is upregulated in these conditions e.g. [9,10,15]. In contrast, in brain no significant change in levels was observed between the different metabolic states, which may indicate differential regulation of the two isoforms. Previously tissue specific differences in the regulation of the glucose-6-phosphatase catalytic subunit have been demonstrated and this presumably reflects the different roles of the glucose-6-phosphatase system in the individual tissues [2–4].

3.2. Cloning and sequencing of the T1 translocase in liver and brain

RT-PCR was performed with human brain and liver RNA using specific oligonucleotides designed against the liver sequence. Two differently sized products were obtained, the liver PCR product had the expected size with regard to oligo binding position (1.3 kbp) whereas the brain one was slightly larger (not shown). The sequences of both products were identical except that the brain sequence contained an extra 66 bp (Fig. 3). This extra sequence does not interrupt the reading frame and 22 additional amino acids are then included in the protein sequence (Figs. 3 and 4). The human liver cDNA encodes a protein of 429 amino acids [14] and the brain isoform has 451 amino acids (Fig. 4).

There is evidence for the existence of two different endoplasmic reticulum Pi transport proteins with different characteristics [12,17]. These have been termed T2α and T2β or G6PT2α and G6PT2β. To be consistent we therefore suggest that the G6PT1 isoforms be termed α and β respectively in the order in which they have been described.

The two different forms of human G6PT1 are strictly homologous except for the extra 22 amino acids in G6PT1β indicating that G6PT1α and G6PT1β are products of a single gene. Recently, the gene for glycogen storage disease 1b has been mapped to chromosome 11q23 by linkage analysis in nine affected families [18]. The isoforms are due to a differential splicing of a single 9 exon gene on chromosome 11 and G6PT1α, the liver type mRNA does not contain the 66 bp exon 7 (Marcolongo et al., submitted for publication).

The bacterial phosphoester transporter UhpT has been pre-

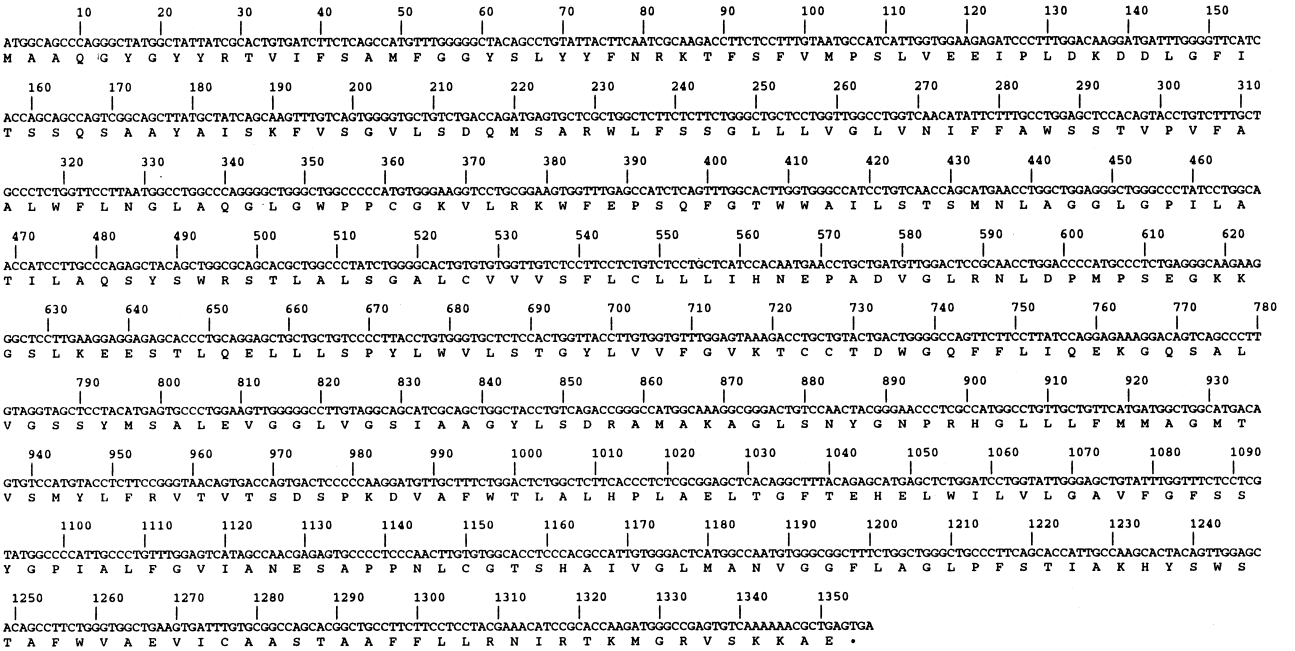

Fig. 3. Sequence of the human brain cDNA (G6PT1β) encoding the brain isoform of the protein mutated in glycogen storage disease 1b and deduced amino acid sequence.

dicted to have 12 membrane spanning regions [19]. Alignment of G6PT1α with UhpT also led to the suggestion of 12 membrane spanning regions [14]. The C terminal KK motif of membrane spanning endoplasmic reticulum proteins is an endoplasmic reticulum retention signal [20,21], which is on the cytoplasmic side of the endoplasmic reticulum membrane. Adding this information to the predicted topology produces a model (Fig. 5) showing that the extra 22 amino acids are in

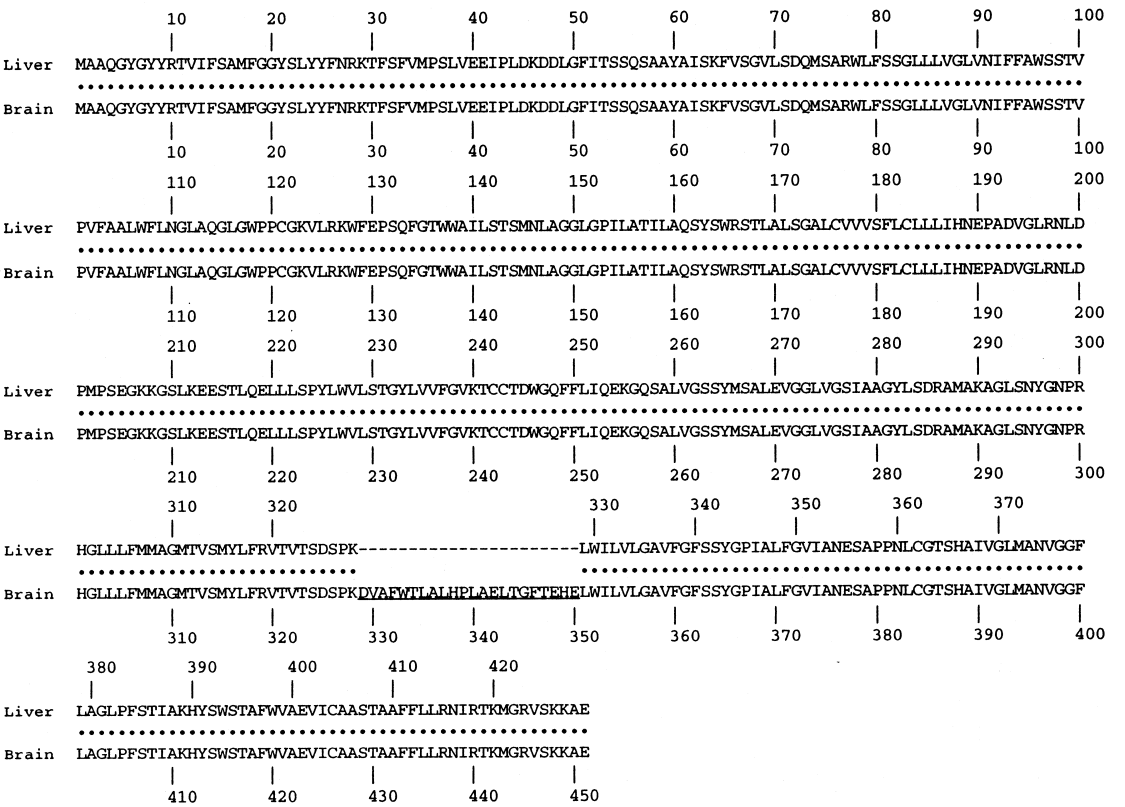

Fig. 4. Sequence alignment of the human liver (G6PT1α) and brain (G6PT1β) forms of the protein mutated in glycogen storage disease 1b. The extra 22 amino acid sequence found in brain is underlined. All the other residues are strictly conserved between the two forms of the protein.

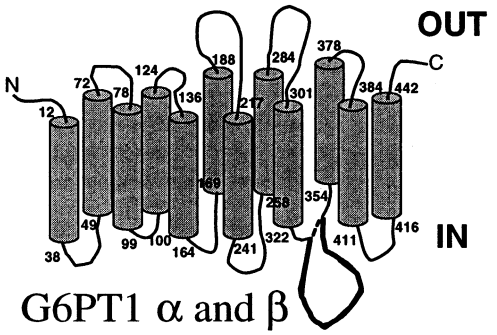

Fig. 5. Schematic model of the G6PT1 protein transmembrane structure predicted in comparison to those made on the homologous bacterial UhpC protein [19]. The loop containing extra 22 amino acids of G6PT1β is shown in bold.

a loop region. Until the topology of G6PT1 is unequivocally established it will not be certain which side of the endoplasmic reticulum is the loop containing the extra amino acids resides. In bacteria the UhpT transporter and a second protein regulatory protein termed UhpC are both involved in phosphoester transport. We do not yet know if mammalian endoplasmic reticulum glucose-6-phosphate transport also requires a second protein for normal functioning in vivo as no mammalian equivalent of the UhpC regulatory protein has been cloned. It is interesting that the 22 extra amino acids described here in G6PT1β have similarity to a region of UhpC between amino acids 387–410 (48% similarity and 31% strict homology) which suggests that they may have the potential to provide part of the regulatory function supplied by UhpC in bacteria.

The existence of a further exon of 66 bp in brain G6PT1β has implications for glycogen storage disease 1b as it seems possible that there will be patients with mutations in that exon where liver glucose-6-phosphate transport would not be affected.

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