Brain Contains a Functional Glucose-6-Phosphatase Complex
Capable of Endogenous Glucose Production

Abhijit Ghosh, Yuk Yin Cheung, Brian C. Mansfield and Janice Yang Chou§

Section on Cellular Differentiation, Heritable Disorders Branch, National Institute of Child Health
and Human Development, National Institutes of Health, Bethesda, Maryland, 20892

Running title: endogenous glucose production in the brain

§ Correspondence should be addressed to J.Y.C.

Building 10, Room 9S241, NIH
9000 Rockville Pike, Bethesda, MD 20892-1830
Tel: 301-496-1094
Fax: 301-402-6035
Email: chouja@mail.nih.gov
ABSTRACT

Glucose is absolutely essential for the survival and function of the brain. In our current understanding, there is no endogenous glucose production in the brain and it is totally dependent upon blood glucose. This glucose is generated, between meals, by the hydrolysis of glucose-6-phosphate (G6P) in the liver and the kidney. Recently we reported a ubiquitously expressed G6P hydrolase, glucose-6-phosphatase-\(\beta\) (G6Pase-\(\beta\)), that can couple with the G6P transporter to hydrolyze G6P to glucose in the terminal stages of glycogenolysis and gluconeogenesis. Here we show that astrocytes, the main reservoir of brain glycogen, express both the G6Pase-\(\beta\) and G6P transporter activities and that these activities can couple to form an active G6Pase complex, suggesting that astrocytes may provide an endogenous source of brain glucose.
INTRODUCTION

Most tissues and organs in the body are not thought to be able to generate endogenous glucose. Therefore, between meals, these tissues depend on glucose, generated predominantly in the liver and kidney, distributed via the blood. The liver and the kidney are the primary organs responsible for interprandial blood glucose homeostasis. This homeostasis is dependent upon the activity of the glucose-6-phosphatase (G6Pase) complex, which is comprised of a glucose-6-phosphate transporter (G6PT) and a G6Pase catalytic unit (reviewed in Ref. 1 and 2). The G6PT is a single copy gene (3-5) that is expressed ubiquitously (6). In contrast, until recently, G6Pase activity was considered restricted solely to the liver, kidney and intestine (1, 2). However, there is evidence that endogenous glucose production does occur outside of these tissues. In the genetic disease glycogen storage disease type Ia (1, 2), where the liver/kidney/intestine G6Pase-α (G6PC) (7, 8) activity is deficient, patients are still capable of producing glucose (9-11), implying there are alternative pathways for endogenous glucose production. This led to the discovery of a second G6Pase activity, now called G6Pase-β (G6PC3 or UGRP) (12-14), which is widely expressed (12).

G6Pase-α (15), G6Pase-β (16), and G6PT (17) are co-localized in the membrane of the endoplasmic reticulum (ER), embedded by multiple transmembrane domains. G6Pase-α is a 357 amino acid phosphohydrolase (7, 8) expressed primarily in the liver, kidney and intestine (18, 19). G6Pase-β is a 346 amino acid phosphohydrolase (13, 14) expressed ubiquitously (12). Both exhibit a similar active site structure, form a similar covalently bound phosphoryl-enzyme intermediates during catalysis (16, 20) and exhibit similar kinetic properties (14). In the active G6Pase complex, G6PT transports cytoplasmic glucose-6-phosphate (G6P) across the ER membrane into the lumen where G6Pase, with its active site inside the lumen, hydrolyses intralumenal G6P to glucose and phosphate (reviewed in Ref. 1 and 2). Alone, neither G6PT nor G6Pase-α have significant microsomal G6P transport activity, but when co-expressed they couple to significantly increase the activity of the G6Pase/G6PT complex (3). We have recently shown that G6Pase-β also has the ability to couple functionally with G6PT (14).
The discovery of G6Pase-β implies that non-hepatic tissues may be capable of endogenous glucose production through the activity of a G6Pase-β/G6PT complex. This is of particular interest, since the liver and kidney are not the sole sites of glycogen storage in the body. Indeed, the muscle is the major reservoir of body glycogen (21, 22) and the brain, which expresses significant levels of both the G6PT (6) and G6Pase-β (12, 13), also stores glycogen. Moreover, glucose export from the brain has been demonstrated in children undergoing elective cardiopulmonary bypass surgery for congenital heart disease (23), suggesting the brain may be capable of endogenous glucose production. In the brain, the primary sites of glycogen storage are the astrocytes. Astrocytes are the most abundant glial cells in the central nervous system, responsible for regulating the external neuronal environment, responding to injury, and modulating neuronal growth and maturation (reviewed in Ref. 24, 25). An acid-labile G6Pase-like activity (26) has been reported in astrocytes. However, the role of astrocyte glycogen in glucose production is controversial because the presence of a functional G6Pase complex has never been demonstrated in the brain. In this study, we show that brain astrocytes possess an active G6Pase-β/G6PT complex that can hydrolyze G6P to glucose, suggesting that astrocyte glycogen can be converted to glucose, and may be a source of alternative energy in the neurons.
EXPERIMENTAL PROCEDURES

Construction of Recombinant Adenoviral Mouse G6Pase-β - Mouse G6Pase-β (mG6Pase-β) containing a C-terminal FLAG peptide, DYKDDDK (pSVL-mG6Pase-β-FLAG) was constructed by PCR from the pSVL-mG6Pase-β template (22), as described previously (14, 22). Recombinant adenoviruses containing mG6Pase-β (Ad-mG6Pase-β) or mG6Pase-β-FLAG (Ad-mG6Pase-β-FLAG) were generated by the Cre-lox recombination system (27). The recombinant virus was plaque purified and amplified to produce viral stocks with titers of approximately 5 to 10 x 10⁹ plaque forming unit (pfu) per ml. Recombinant adenoviruses, containing human G6Pase-β (Ad-hG6Pase-β), human G6Pase-α (Ad-hG6Pase-α) and human G6PT (Ad-hG6PT) have been described (14).

Expression in COS-1 Cells - COS-1 cells in 150-cm² flasks were grown at 37 °C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with 4% fetal bovine serum. The cells were infected with Ad-mG6Pase-β or Ad-hG6Pase-α at various pfu/cell and after incubation at 37 °C for 48 h, either cell lysates, or microsomes isolated from lysates, were used for phosphohydrolase assays and Western-blot analyses. For G6PT expression, COS-1 cells were infected with 50 pfu/cell of Ad-hG6PT. For co-expression of G6PT and G6Pase, COS-1 cells were co-infected with 25 pfu/cell of either Ad-mG6Pase-β or Ad-hG6Pase-β, and 50 pfu/cell of Ad-hG6PT. For G6P uptake analysis, microsomes were isolated from lysates prepared after incubation at 37 °C for 24 h.

Phosphohydrolase and G6P Uptake Analyses - Phosphohydrolase activity was determined essentially as described previously (14). G6Pase-β in brain microsomes was assayed at the optimal temperature of 37 °C and G6Pase-α in hepatic microsomes was assayed at 30 °C (14). Reaction mixtures (100 µl) containing 50 mM cacodylate buffer, pH 6.5, 10 mM G6P and appropriate amounts of microsomal preparations were incubated at either 37 °C or 30 °C for 10 min.
Disrupted microsomal membranes were prepared by incubating intact microsomes in 0.2% deoxycholate for 20 min at 0 °C. Non-specific phosphatase activity was estimated by pre-incubating disrupted microsomal preparations at pH 5, for 10 min, at 37 °C, to inactivate the acid labile G6Pase-α and G6Pase-β.

G6P uptake measurements were performed as described (3, 14). Briefly, microsomes (40 µg) were incubated in a reaction mixture (100 µl) containing 50 mM sodium cacodylate buffer, pH 6.5, 250 mM sucrose, and 0.2 mM [U-14C]G6P (50 µCi/µmol). The reaction was stopped at the appropriate time by filtering immediately through a nitrocellulose membrane (BA85, Schleicher & Schuell, Keene, NH) and washing with an ice-cold solution containing 50 mM Tris-HCl, pH 7.4 and 250 mM sucrose. The radioactivity measured within the microsomes represents both the translocated substrate, [U-14C]G6P, and its hydrolytic product, [U-14C]glucose. Microsomes permeabilized with 0.2% deoxycholate, to abolish G6P uptake, were used as negative controls. Two to three independent experiments were conducted, and at least three G6P uptake studies were performed for each microsomal preparation.

Statistical analysis using the unpaired t test was performed with The Prism Program (GraphPad Software, San Diego, CA). Data are presented as the mean ± SEM.

G6Pase-α⁻/⁻ and G6PT⁻/⁻ Mice - Mice deficient in G6Pase-α (28) and G6PT (29) have been described. All animal studies were conducted under an animal protocol approved by the NICHD Animal Care and Use Committee. To maintain viability of the G6Pase-α⁻/⁻ and G6PT⁻/⁻ mice, glucose therapy consisting of intraperitoneal injection of 25-100 µl of 15% glucose, every 12 h, was initiated on the first post-natal day (29). Weaned mice were also given unrestricted access to Mouse Chow (Zeigler Bros., Inc., Gardners, PA). Microsomes were isolated from the brain and liver of 6- to 7-week-old mice essentially as described (8, 30). Each microsomal preparation represents one individual mouse and at least three independent microsomal preparations were used for each assay.
Northern-blot and Western-blot Analyses - Total RNA was isolated by the guanidinium thiocyanate/CsCl method, fractionated by electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to a Nytran membrane by electroblotting. The filters were hybridized to a uniformly labeled mG6Pase-β, mG6PT, or β-actin riboprobe.

For Western blot analysis, cell lysates or microsomal proteins were separated by electrophoresis through a 12% polyacrylamide-SDS gel, blotted onto polyvinylidene fluoride membranes (Millipore Co., MA). The membranes were incubated either with a monoclonal antibody against the FLAG epitope (Scientific Imaging Systems, Eastman Kodak, CT), a polyclonal antibody against hG6Pase-β (22), or a rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Affinity BioReagents, Inc., Golden, CO). The antigen-antibody complex was visualized as described previously (14, 22).

Mouse Astrocytes in Primary Culture – Mouse astrocytes were prepared using the method of Pousset et al. (31). Briefly, brains were dissected from 2- to 3-day-old wild-type, G6Pase-α−/− or G6PT−/− pups and the meningeal tissues removed. The cortices were suspended in PBS and flushed several times with a fire polished Pasteur pipette. The resulting cell suspension was passed through a sterile nylon sieve (70 µm pore size, Falcon) to remove clumps and centrifuged at 1,200 rpm for 5 min to collect the cells. The cell pellet was resuspended in Dulbecco's modified minimal essential medium (DMEM) containing 20% heat-inactivated fetal bovine serum, then seeded at a density of 9 x 10⁴ cells/6-mm dishes, and incubated at 37 °C in a humidified 5% CO₂/95% air atmosphere. Under these conditions, neurons do not survive the mechanical dissociation and the low plating density prevents oligodendrocytes proliferation (31). After 7 days in culture, the cells were incubated with fresh DMEM containing 10% fetal bovine serum and this medium was changed weekly. After 21 days in culture, one set of cultures was fixed in 4% paraformaldehyde to stain for marker proteins. The second set of cultures was used to prepare cell lysates and microsomes for phosphohydrolase and G6P uptake assays, and Western-blot analyses.
The purity of the astrocytes was determined by staining for GFAP (32). The cells were fixed for 10 min in 4% paraformaldehyde, incubated for 30 min at room temperature in buffer TST (0.05 M Tris-HCl, pH 7.5, 0.15M NaCl, and 0.1% Triton-X-100) containing 1% bovine serum albumin (BSA) and 10% horse serum (TST-BSA), and then incubated overnight at 4 °C with a rabbit anti-GAFP antibody at 4 µg/ml in TST-BSA. Following three washes with TST, the cells were then incubated with a biotinylated goat anti-rabbit IgG for 30 min and the antigen-antibody complex visualized with the Vectastatin Elite ABC Kit (Vector Laboratories, Burlingame, CA). Replicates omitting the primary antibody or substituting the primary antibody with a preimmune rabbit serum were used as controls.

A mouse monoclonal antibody against fibronectin (Sigma) and a rat monoclonal antibody against myelin basic protein (Sigma) were used to measure contamination of the cultures by fibroblasts and oligodendrocytes, respectively.
RESULTS

Mouse G6Pase-β is a Phosphohydrolase and Couples with G6PT to Form a Functional G6Pase Complex

The hG6Pase-β is a functional phosphohydrolase (13, 14) that couples with the G6PT to form an active G6Pase complex (14). However, while hG6Pase-β (12) and mG6Pase-β (33) are very similar, both 346 amino-acid proteins with a conserved active site structure and an overall 84% amino acid sequence, mG6Pase-β is reported to lack activity (33). To investigate whether mG6Pase-β might have a level of activity below the sensitivity of the reported assay, we examined the G6P hydrolytic activity of mG6Pase-β using a sensitive adenoviral-based expression system. Viral stocks of Ad-mG6Pase-β, Ad-mG6Pase-β-3FLAG, Ad-hG6Pase-β (or Ad-hG6Pase-β-3FLAG) (14), and Ad-hG6Pase-α (or Ad-hG6Pase-α-3FLAG) (14) were used to infect monkey kidney COS-1 cells, and the resulting phosphohydrolase activities were assayed at pH 6.5, 37 ºC for G6Pase-β and pH 6.5, 30 ºC for G6Pase-α (14). The Ad-hG6Pase-α-3FLAG-infected COS-1 lysates yield activities of 208.2 ± 7.9 and 347.6 ± 9.9 nmol/mg/min at a multiplicity of infection of 25 and 50 pfu/cell, respectively (Fig. 1A). The Ad-mG6Pase-β-3FLAG-infected COS-1 lysates have a lower, but significant activity, showing a dose-dependent G6P hydrolytic activity ranging from 11.8 ± 0.3 nmol/mg/min, at a multiplicity of infection of 5 pfu/cell, to 82.4 ± 5.1 nmol/mg/min, at multiplicity of infection of 100 pfu/cell (Fig. 1A). In the same assay, the non-tagged protein, Ad-mG6Pase-β, has an activity identical to the tagged protein, Ad-mG6Pase-β-3FLAG (data not shown). The mG6Pase-β activity is similar to the hG6Pase-β activity (14), both being 6-fold lower than that of hG6Pase-α (14) (Fig. 1A). Western analysis shows that the expression of mG6Pase-β and hG6Pase-α proteins correlates with enzymatic activity (Fig. 1A), as was shown previously for hG6Pase-β (14).

Previous studies have shown that both hG6Pase-α and hG6Pase-β are acid labile and latent (14). The mG6Pase-β is similarly acid labile, losing over 98% of G6P hydrolytic activity when Ad-mG6Pase-β-infected COS-1 microsomes are incubated at pH 5.0 for 10 min at 37ºC (Fig. 1B). The mG6Pase-β also exhibits a similar latency to hG6Pase-α and hG6Pase-β (Fig. 1B).
Both human G6Pases also share a common pH optimum of 6.5, although the optimal temperatures for hG6Pase-α and hG6Pase-β differ, being 30 °C and 37 °C, respectively (14). Like hG6Pase-β, the optimal assay condition for mG6Pase-β is pH 6.5 and 37 °C (Fig. 1B).

In the liver and kidney, G6P transport and hydrolysis are tightly coupled (28). The uptake and accumulation of G6P into the lumen of the ER is stimulated dramatically when hG6PT is co-expressed with either hG6Pase-α or hG6Pase-β (3, 14). The mG6Pase-β demonstrates a similar functional coupling to hG6PT (Fig. 1C). COS-1 cells infected with Ad-mG6Pase-β (or Ad-hG6Pase-β) have a very low level of microsomal G6P uptake activity (Fig. 1C) as was shown previously for hG6Pase-α (3). Microsomal G6P transport activity was significantly increased in COS-1 cells infected with Ad-hG6PT alone (Fig. 1C), and the activity was markedly increased in cells co-infected with either Ad-hG6PT and Ad-mG6Pase-β or Ad-hG6PT and Ad-hG6Pase-β (Fig. 1C). The co-infected cultures also have identical time courses for microsomal G6P accumulation.

**Brain Expresses Active G6Pase-β**

G6Pase-α is expressed primarily in the liver, kidney, and intestine (18, 19). Although both G6Pase-β (12, 33) and G6PT (6) are expressed ubiquitously, only brain, heart, skeletal muscle, and kidney express significant levels of both proteins (6, 12, 33). In the wild-type mice, expression of the G6Pase-β transcript is slightly higher in the brain than the kidney, while the expression of the G6PT transcript is substantially more elevated in the kidney than the brain (Fig. 2A). Western-blot analysis confirms the presence of the G6Pase-β protein in the brain of wild-type mice (Fig. 2B). Western-blot analysis of G6Pase-α−/− mouse brain, lacking the G6Pase-α gene (28), shows a similar level of G6Pase-β expression, confirming the specificity of the G6Pase-β detection (Fig. 2B). As expected, the G6PT−/− mouse brain (29) contains a similar level of the G6Pase-β protein as the wild-type mouse brain (Fig. 2B).

In contrast to non-specific phosphohydrolases, G6Pase has a characteristic acid labile profile (34). Therefore, to measure the specific G6P hydrolase activity in the brain, we performed
two measurements; one on disrupted brain microsomes, to measure total phosphatase activity; the other on disrupted microsomes pre-incubated at pH 5.0, 37 °C for 10 min to measure the residual acid-resistant non-specific phosphatases. The difference between these measurements reflects the acid-sensitive G6Pase specific activity. The acid-sensitive G6P hydrolase activity in microsomes isolated from the brain of wild-type mice is $10.07 \pm 0.84$ nmol/mg/min (Table 1), which represents 60% of the total phosphohydrolase activity. The brain G6P hydrolase activity is ~5% of the activity of the liver at $217.2 \pm 6.0$ nmol/mg/min (22). A previous report (26) ascribed 40% of the activity to acid-sensitive G6Pase when assayed at pH 6.5 and 30 °C. Our findings are reasonably consistent with this report, given the initial finding was assayed at a temperature suboptimal for G6Pase-β (Fig. 1B).

To exclude the possibility that a low level of expression of G6Pase-α occurs in brain and is more readily measured at the higher temperature we used to assay G6Pase-β, we examined the G6P hydrolytic activity of brain microsomes from G6Pase-α−/− mice. The G6Pase-α−/− brain has an identical activity to wild-type brain, implying G6Pase-α is not expressed in the brain (Table 1). As expected, G6PT−/− brain microsomes have an identical activity to wild-type brain (Table 1). To further support the identification of the acid-labile brain activity as G6Pase-β, the vanadate sensitivity (Table 1) of the G6Pase in brain was examined. The hepatic hG6Pase-α activity is inhibited 50% at a vanadate concentration of 3.1 mM ([Vanadate]₀.₅ = 3.1 mM) (14), while the hG6Pase-β has a sensitivity of [Vanadate]₀.₅ of 1.4 mM (14). Consistent with this, the mouse brain G6Pase activity is similar to the hG6Pase-β, with [Vanadate]₀.₅ of 1.3 to 1.34 mM (Table 1).

**Brain Possesses an Active G6Pase Complex**

*In vivo*, G6PT must couple functionally with G6Pase to form an efficient G6Pase complex (28). A comparison of G6P uptake in wild-type, G6Pase-α−/− and G6PT−/− mouse brain shows that while the time courses of G6P uptake activity in the intact microsomes of wild-type and G6Pase-α−/− mice are identical, the G6PT−/− mice do show a markedly reduced G6P
accumulation (Fig. 3A), consistent with the absence of G6PT in these mice (29). The brain G6P uptake activity is not attenuated in G6Pase-α/− mice, consistent with the brain G6Pase-β coupling with G6PT. Knockout of G6PT in G6PT−/− mice results in ~82% loss of brain activity (Fig. 3B), confirming the importance of G6PT in both tissues. Since ~18% of wild-type G6P uptake activity does remain in the brain of G6PT-deficient mice, the possibility that there is another minor G6P transport protein in the brain can not be excluded.

**Brain Astrocytes Posses an Active G6Pase-β/G6PT Complex**

In the brain, astrocytes constitute over 50% of the cell mass and are the only cells that contain substantial amounts of glycogen (reviewed in Ref. 35). If astrocytes are a site of expression of G6Pase-β and G6PT, they may be capable of providing an endogenous source of glucose for the brain. To examine this we isolated primary mouse astrocytes from 2- to 3-day-old wild-type, G6Pase-α/− and G6PT−/− pups. After 21 days in culture, nearly all cells from wild-type, G6Pase-α/− or G6PT−/− pups have the characteristics of astrocytes. Immunocytochemical analysis (Fig. 4A) shows that nearly all the cells stained positively for GFAP, a marker for brain astrocytes (32) and negatively for fibronectin and myelin basic protein, which are markers of fibroblasts and oligodendrocytes, respectively (31). Western-blot analysis confirmed that the primary astrocytes from wild-type, G6Pase-α/− or G6PT−/− pups express the GFAP (Fig. 4B) but not fibronectin or myelin basic protein (data not shown). Importantly, primary astrocytes from wild-type, G6Pase-α/− and G6PT−/− pups express the G6Pase-β protein in their microsomes (Fig. 4B), strongly suggesting that brain astrocytes may be capable of hydrolyzing G6P to glucose via a functional G6Pas-β complex. To examine if this complex is functional we assayed the astrocyte microsomes for G6P uptake and hydrolysis.

The acid-sensitive G6P hydrolase activities in microsomes isolated from the astrocyte primary cultures of wild-type, G6Pase-α/− and G6PT−/− mice are similar ranging from 15.0 to 15.7 nmol/mg/min (Fig. 4C), accounting for ~7% of the activity of the liver (22). This is ~50% higher
than the specific activities in microsomes isolated from the whole brain homogenates (Table 1), suggesting, based on cell mass, that the astrocytes are the primary source of the brain activities.

A comparison of G6P uptake in primary astrocytes shows that G6P uptake activities in the intact microsomes of wild-type and G6Pase-α−/− mice are identical (Fig. 4C). In contrast, the G6PT−/− microsomes show a markedly reduced G6P accumulation (Fig. 4C), again consistent with the absence of G6PT in these mice (29).
DISCUSSION

The mammalian brain has no energy reserves and a constant supply of oxygen and glucose is required for its function and survival. Blood glucose homeostasis between meals is maintained by balancing the blood glucose uptake by tissues with the release of G6P-derived glucose to the blood by the liver and kidney. Therefore, the dephosphorylation of G6P to glucose, catalyzed by the G6Pase complex in the terminal step of both gluconeogenesis and glycogenolysis, is a key control point for interprandial glucose homeostasis (1, 2). Until very recently, specific G6Pase activity has not been detected outside of the liver, kidney, and intestine. This has led to the current view that interprandial glucose homeostasis depends solely upon these organs. The recent recognition of G6Pase-β (13, 14) and its universal expression profile (12, 33) has raised the possibility that glucose recycling might also occur to some extent in many tissues. The tissues which immediately become of interest to this hypothesis are those with substantial reserves of glycogen that could be converted to glucose via glycogenolysis, and which also express the higher levels of G6Pase-β and G6PT. Of particular interest are the skeletal muscle (21, 22), brain, and heart.

The G6Pase found in mouse brain in this study, and the in vitro expressed mouse and human G6Pase-β proteins, have identical kinetic characteristics. All are acid labile, readily inhibited by vanadate, with [vanadate]₀.₅ of 1.3 to 1.4 mM, and all share optimal assay conditions of pH 6.5 and 37 ºC differing from those of G6Pase-α at pH 6.5 and 30 ºC. These along with the RNA and protein expression profiles, support the conclusion that the acid-labile G6P hydrolytic activity in brain is G6Pase-β. Of particular significance, the brain G6Pase is capable of coupling to the ubiquitous G6PT to create a functional G6Pase complex capable of converting G6P to free glucose. In refining the site of this activity, we have shown that astrocytes, the main site of glycogen storage in the brain, contain a functional G6Pase-β/G6PT complex in their microsomes, suggesting they are capable of hydrolyzing G6P to glucose, and might be a source of endogenous brain glucose.
While the G6P hydrolase activity in the brain (or astrocytes) represents only ~5% to 7% of the activity in the liver, the brain (or astrocytes) G6Pase-β/G6PT complex has ~30% to 32% of G6P transport activity of the liver G6Pase-α/G6PT complex. This is consistent with the previous reports that G6P translocation by G6PT is the rate-limiting step in G6Pase catalysis (19, 36). Our results indicate that despite the lower hydrolase activity in the brain, the overall activity of the G6Pase/G6PT complex in the brain is still sufficient to convert glycogen to glucose at a significant rate.

The potential role of brain glycogen in glucose homeostasis has been of significant interest. One concern that is expressed about the role of non-hepatic contributions to glucose homeostasis is based on kinetics. G6P is the intracellular form of extracellular glucose, trapped within the cell by the charged phosphate that prevents ready diffusion across the lipid cell membrane. The ability of the liver to maintain blood glucose homeostasis between meals is believed to stem, in part, from the balance between the G6P phosphohydrolase and glucose kinase activities present in the liver. The G6Pase-α activity in the liver is high, which allows for the rapid conversion of G6P derived from gluconeogenesis or glycogenolysis, to glucose. Of the two glucose kinase activities, glucokinase and hexokinase, glucokinase predominates in the liver. Since glucokinase has the higher $K_m$ (~20 mM) (37, 38) compared to hexokinase ($K_m$ ~50 µM) (24, 38), G6Pase-mediated glucose release to the blood can compete with rephosphorylation by glucokinase. On the other hand, most other tissues, including the kidney, express the low $K_m$ hexokinase. This might imply that in non-hepatic tissues, G6Pase-derived glucose is more likely to be rephosphorylated than released into the circulation. There is one clear inconsistency with this explanation, namely the observation that the kidney does play a proven role in blood glucose homeostasis, especially during prolonged starving (reviewed in Ref. 39, 40). One possible explanation is that the kinetic reasoning does not consider the subcellular compartmentalization of the different activities. The fate of cytoplasmic glucose, exposed to the glucokinase and hexokinase activities, might be very different from that of glucose produced within the ER lumen by the G6Pase complex. Within the lumen, glucose is protected from the glucose kinase
activities and may be released directly via the ER/Golgi pathway (2) where it is promptly taken up and re-phosphorylated to G6P by adjacent cells. Indeed, using GLUT2-deficient cells, it was demonstrated that hepatic (41) or intestinal (42) glucose release does not require the presence of the plasma membrane glucose facilitative diffusion mechanism, implying the existence of an alternative pathway for glucose release via a membrane traffic mechanism. This might explain why the cytoplasmic pathways of gluconeogenesis and glycogenolysis evolved a final step dependent upon a compartmentalized G6Pase enzyme complex.

The finding of a functional G6Pase complex in the brain astrocytes is consistent with a number of earlier observations and the known roles of astrocytes within the brain. It has been shown that the astrocytes are the primary source of glycogen storage in the brain, containing sufficient glycogen reserves to buffer the majority of the glucose supply deficit for more than 100 min of hypoglycemia (24). It is also known that sensory stimulation causes a breakdown of astrocyte glycogen, while anesthesia and hibernation increase glycogen stores in the brain (35). These findings suggest a role of astrocyte glycogen metabolism and neuronal activity. The known roles of astrocytes, not only in structural support and regulating the external chemical environment of the neurons, but also in their roles directly supporting neuronal maturation and neurogenesis would be consistent with an ability to regulate glucose release.

Although glucose export from the brain has been demonstrated in children undergoing elective cardiopulmonary bypass surgery for congenital heart disease (23) and we now show that astrocytes express an active G6Pase-β/G6PT complex, there is very limited evidence that confers a glucose-export capacity on astrocytes per se. Under most conditions in vitro, lactate export predominates. Primary cultures of astrocytes convert their glycogen into extracellular lactate upon glucose deprivation (38) and glycogen-derived lactate supports intact rodent optic nerve function in vitro during aglycemia and increased metabolic demand (43). Moreover, neuronal activation leads to an increase in lactate which can be used as a metabolic substrate by neurons, suggesting that energy is transferred from astrocytes to axons in the form of lactate (reviewed in Ref. 44, 45).
While the proposal that astrocytes have the ability to convert glycogen to glucose can explain the source of glucose reported to be released from the brain under certain conditions of cardiopulmonary bypass (23), we do not know under what conditions the astrocyte glycogen is cycled into glucose \textit{in vivo}. There are many fates of G6P in the cell, depending on the physiological status of the cell and its environment. These include: lactate and/or energy production via glycolysis; entry into the pentose phosphate pathway; and glucose production via the G6Pase-\(\beta\) complex in the ER. Since the level of glycogen storage in astrocytes is low, compared to the liver and muscle, we do not consider glucose production a primary pathway in astrocytes, but rather a pathway that may be used during periods of stress or metabolic restriction, to buffer the brain function. The relative importance of the production and use of lactate and glucose in the brain remains a question for careful experimental scrutiny.

In conclusion, we have demonstrated that there is a functional G6Pase activity in the mouse brain, which is present in astrocytes. The activity is independent of G6Pase-\(\alpha\) and has the expected characteristics of G6Pase-\(\beta\). The activity can couple functionally with G6PT to produce an active G6Pase complex and the complex can metabolize G6P to glucose and phosphate. These results can explain previous reports of the release of glucose from the brain, and suggest that there is reason to think beyond simple lactate energy in the brain. The biological significance of this finding awaits more detailed studies.
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FIGURE LEGENDS

Fig. 1. mG6Pase-β is a G6P phosphohydrolase. A, expression and activity of Ad-mG6Pase-β-3FLAG and Ad-hG6Pase-α-3FLAG in infected COS-1 lysates. COS-1 cells were infected with Ad-mG6Pase-β-3FLAG or Ad-hG6Pase-α-3FLAG at various multiplicity of infection. Expression of Ad-mG6Pase-β-3FLAG and Ad-hG6Pase-α-3FLAG was detected by Western-blot analysis using an anti-FLAG monoclonal antibody. The G6P phosphohydrolase activity was assayed at pH 6.5 and 37 ºC for mG6Pase-β and pH 6.5 and 30 ºC for hG6Pase-α. The background activity of mock-infected cultures (5.4 ± 0.2 nmol/mg/min for mG6Pase-β) or (8.2 ± 0.5 nmol/mg/min for hG6Pase-α) was subtracted from the data presented. The Ad-mG6Pase-β has an equivalent activity. B, comparison of the G6P phosphohydrolase activity of mG6Pase-β, hG6Pase-β and hG6Pase-α. Microsomes isolated from COS-1 cells infected with the appropriate recombinant adenoviral construct at 50 pfu/cell were assayed at pH 6.5 and 37 ºC for G6Pase-β, and pH 6.5 and 30 ºC for G6Pase-α. Acid stability was determined by assaying G6P phosphohydrolase activity in deoxycholate (0.2%) disrupted microsomes before and after pre-incubation at pH 5.0 for 10 min at 37 ºC. Latency was measured as the rate of hydrolysis of mannose-6-phosphate in intact (I) versus detergent-disrupted (D) microsomes, and defined as (1-I/D) x 100 (19). The pH and temperature optima were determined by assaying G6P phosphohydrolase activity in deoxycholate-disrupted microsomes. C, mG6Pase-β couples with G6PT to mediate microsomal G6P uptake. Microsomal G6P uptake activity in COS-1 cells infected with Ad-mG6Pase-β (25 pfu/cell) (▽), Ad-hG6PT (50 pfu/cell) (▼), co-infected with Ad-mG6Pase-β (25 pfu/cell) and Ad-hG6PT (50 pfu/cell) (○) or co-infected with Ad-hG6Pase-β (25 pfu/cell) and Ad-hG6PT (50 pfu/cell) (●) was measured over time. The radioactivity accumulated in the lumen of the ER consists of both [U-14C]G6P and [U-14C]glucose.

Fig. 2. Expression of G6Pase-β and G6PT in the brain. A, Northern-blot analysis. Total RNA was isolated from the brain and kidney of wild-type mice, separated on a formaldehyde-agarose gel, and duplicate blots were hybridized with a riboprobe for G6Pase-β, G6PT, or β-actin. B,
Western-blot analysis. G6Pase-β in microsomes isolated from the brains of G6Pase-α<sup>+/−</sup>, G6PT<sup>+/−</sup> and wild-type mice were detected using an anti-hG6Pase-β antibody (22) as described under Experimental Procedures. The lane marked mG6Pase-β represents protein from Ad-mG6Pase-β-infected COS-1 lysates.

Fig. 3. The G6Pase-β complex in the brain is functional. G6P uptake assays were performed using microsomes isolated from the brain or liver of wild-type, G6Pase-α<sup>+/−</sup> and G6PT<sup>+/−</sup> mice as described under “Experimental Procedures.” A, time course of G6P uptake activity in intact microsomes isolated from the brain of wild-type (○), G6Pase-α<sup>+/−</sup> (●), or G6PT<sup>+/−</sup> (▼) mice. B, microsomal G6P uptake activity. The radioactivity accumulated in the lumen of the ER consists of both [U-<sup>14</sup>C]G6P and [U-<sup>14</sup>C]glucose. The results are given as mean ± SEM.

Fig. 4. Mouse astrocytes in primary culture express an active G6Pase-β/G6PT complex. A, cell-specific immunostaining. Mouse astrocytes were isolated from 2- to 3-day-old wild-type, G6Pase-α<sup>+/−</sup> or G6PT<sup>+/−</sup> pups and plated at a density of 9 x 10<sup>3</sup> cells/6-mm dish. After 21 days in culture, the cells were stained for GFAP (a marker for astrocytes), fibronectin (a marker for fibroblasts), or myelin basic protein (a marker for oligodendrocytes) as described under Experimental Procedures. The data shown are for astrocytes obtained from wild-type mice at magnifications of x1 (upper panels) and x50 (lower panels). Similar results were obtained with primary astrocytes from G6Pase-α<sup>+/−</sup> and G6PT<sup>+/−</sup> pups. B, Western-blot analysis. G6Pase-β in microsomes isolated from primary astrocytes of wild-type, G6Pase-α<sup>+/−</sup> or G6PT<sup>+/−</sup> mice were detected using anti-hG6Pase-β (22). The lane marked mG6Pase-β represents protein from Ad-mG6Pase-β-infected COS-1 lysates. GFAP in cell lysates was detected using a rabbit anti-GFAP antibody. C, Astrocyte phosphohydrolase and G6P uptake activities. G6P hydrolytic and uptake assays were performed using microsomes isolated from primary astrocytes of wild-type, G6Pase-α<sup>+/−</sup> and G6PT<sup>+/−</sup> mice as described under “Experimental Procedures.” The results are given as mean ± SEM.
Table 1. Brain acid-sensitive G6P phosphohydrolase activity and inhibition by vanadate

| Mice     | Phosphohydrolase Activity (Vanadate)_{0.5} |
|----------|--------------------------------------------|
|          | nmol/mg/min | mM  |
| Wild-type| 10.07 ± 0.84 | 1.32 |
| G6Pase-α⁻/⁻| 9.76 ± 0.69 | 1.34 |
| G6PT⁻/⁻  | 9.94 ± 0.81 | 1.30 |

G6P phosphohydrolase activity was assayed using three independent brain microsomal preparations and was performed in two measurements; one on disrupted microsomes, to measure total phosphatase activity; the other on disrupted microsomes pre-incubated at pH 5.0, 37 °C for 10 min to measure the residual acid-resistant non-specific phosphatases. The difference between these measurements reflects the acid-sensitive G6Pase specific activity. Data are present as the mean ± SEM. The concentration of vanadate for 50% inhibition of G6P phosphohydrolase activity [(Vanadate)_{0.5}] was determined in deoxycholate-disrupted microsomes as previously described (14).
Fig. 1

### Table A

| MOI | mG6Pase-β Phosphohydrolase Activity (nmol/mg/min) | hG6Pase-α Phosphohydrolase Activity (nmol/mg/min) |
|-----|-----------------------------------------------|-----------------------------------------------|
| 5   | 11.8 ± 0.3                                    | 0.0                                          |
| 0   | 0.0                                          | 208.2 ± 7.9                                  |
| 10  | 17.2 ± 1.2                                    | 347.6 ± 9.9                                  |
| 25  | 32.0 ± 0.8                                    |                                               |
| 50  | 62.6 ± 2.7                                    |                                               |
| 100 | 82.4 ± 5.1                                    |                                               |

### Table B

|                      | mG6Pase-β | hG6Pase-β | hG6Pase-α |
|----------------------|-----------|-----------|-----------|
| Acid Stability       | <2%       | <2%       | <2%       |
| Latency              | 83.1%     | 73.2%     | 62.5%     |
| pH Optimum           | 6.5       | 6.5       | 6.5       |
| Temperature Optimum  | 37 °C     | 37 °C     | 30 °C     |

### Graph C

G6P Uptake (nmol/mg) vs Time (sec)
Fig. 2
Fig. 3

|       | Brain     | Liver     |
|-------|-----------|-----------|
| Wild-type       | 0.165 ± 0.010 | 0.543 ± 0.015 |
| G6Pase-α/−     | 0.155 ± 0.013 | 0.027 ± 0.002 |
| G6PT−/−         | 0.029 ± 0.005 | 0.019 ± 0.001 |

G6P Uptake
nmol/mg/3 min
Fig. 4
Brain contains a functional glucose-6-phosphatase complex capable of endogenous glucose production
Abhijit Ghosh, Yuk Yin Cheung, Brian C. Mansfiled and Janice Yang Chou

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