Nuclear localization of liver FBPase isoenzyme in kidney and liver

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Received 30 April 2003; revised 8 July 2003; accepted 9 July 2003

First published online 29 July 2003

Edited by Judit Ovádi

Abstract Nuclear localization has been observed for glycolytic enzymes but not for key gluconeogenic enzymes. We report our findings on the intracellular localization of liver FBPase in rat liver and kidney, the main organs in the endogenous glucose production. Immunofluorescence and confocal analysis revealed that FBPase was present in the cytosol and, unexpectedly, inside the nucleus of hepatocytes and proximal cells of the nephron. Additionally, FBPase was found in the plasma membrane area of adjacent hepatocytes where glycogen is synthesized and in the apical region of proximal kidney cells. This subcellular distribution in multiple compartments suggests the presence of different localization signals on FBPase for diverse metabolic functions.

Key words: Gluconeogenesis; Glucose metabolism; Fructose-1,6-bisphosphatase; Localization; Nucleus; Nuclear translocation

1. Introduction

Synthesis of endogenous glucose occurs mainly in the liver and kidney by gluconeogenesis from precursors such as glycerol, amino acids, and lactate [1,2]. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), a rate-limiting enzyme, catalyzes the irreversible conversion of fructose-1,6-bisphosphate (Fru-1,6-P_2) to fructose-6-phosphate (F-6-P) and inorganic phosphate [3–5]. The existence of three FBPase isoenzymes: brain, muscle and liver, has been proposed considering immunological and kinetic data [3,6,7]. Although the rat muscle isoform displays 70% identity with the hepatic form, the specific physiological role of the muscle and brain isoenzymes remains unclear [8,9]. The liver FBPase isoenzyme is recognized to be one of the major regulatory enzymes of gluconeogenesis [1,2]. This isoenzyme, which has been isolated from different organisms, is a tetramer composed of identical subunits (MW 36 000–41 000) and is mainly regulated by two synergistic inhibitors: AMP and fructose-2,6-bisphosphate (F-2,6-P_2) [3–5].

We recently demonstrated, by using immunohistochemical analysis in human tissues, that FBPase is expressed not only in kidney and liver, but also in a variety of organs such as small intestine, stomach, adrenal gland, testis and prostate, which might also contribute to gluconeogenesis [10]. In human and rat kidney, the specialized distribution of liver FBPase and cytosolic phosphoenolpyruvate carboxykinase (PEPCK) only in the proximal convoluted tubules of the nephron supports the idea that renal endogenous glucose production occurs mainly in this region, whereas the distal tubules specifically contribute to the glycolytic activity [10,11]. Our results, showing aldolase B exclusively localized in the proximal tubules in the cortex of rat kidney and preferentially in hepatocytes of the periportal region of rat liver, are consistent with this idea [12]. In rat and human liver FBPase is expressed throughout the parenchyma in the hepatocytes. Similarly to kidney, FBPase expression is compartmentalized in rat liver with the periportal hepatocytes showing a higher expression level with a gradient of concentration from this region to the perivenous hepatocytes [10,11]. Additionally, subcellular localization studies demonstrated that FBPase is located in the perinuclear region of the positive staining cells [10,11]. The presence of glycolytic enzymes inside the nuclei has been reported [13–15]. However, due to the immunocytochemistry technique used in the analysis we were unable to distinguish between the nuclear and perinuclear location of liver FBPase. In this study, we have taken advantage of the immunofluorescence and confocal analysis to show that the liver FBPase is localized in a specialized plasma membrane compartment and inside the nuclei in rat kidney and liver cells, suggesting that the physical separation of metabolic pathway within the cell is an interesting regulatory mechanism.

2. Materials and methods

2.1. Antibody production. FBPase antibodies

FBPase was purified from pig kidney as described by Reyes et al. [16]. Rabbit policlonal antiserum against pure FBPase was prepared in white rabbits. Ten days after the booster injection the rabbits were bled, and the presence of antibodies was checked by immunoblotting. Rabbit serum immunoglobulins were prepared by ammonium sulfate precipitation followed by DEAE–cellulose chromatography. This antiserum has a high specificity for the liver isoform and does not cross-react with the muscle isoform [11].

2.2. Western blot

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a minigel apparatus (Bio-Rad, Hercules, CA, USA). The final acrylamide monomer concentration in the slab gels was 10% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Extracted proteins from rat liver were electrophoretically transferred from polyacrylamide gels to nitrocellulose membranes using a semi-dry Trans-Blot SD (Bio-Rad). The nitrocellulose-bound protein was probed with the anti-FBPase sera, and the antibody-antigen interaction was detected using secondary antibodies coupled to peroxidase detection (Dako, Carpenteria, CA, USA).

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Abbreviations: FBPase, fructose-1,6-bisphosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GS, glycogen synthase; GK, glucokinase; PAGE, polyacrylamide gel electrophoresis; NLS, nuclear localization sequence

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doi:10.1016/S0014-5793(03)00809-3

2.3. Immunohistochemistry

Liver and renal tissues obtained from fasted adult Holtzman rats (body weight 300–350 g) were fixed in Bouin's fluid, 4% (v/v) for 24 h at room temperature. Fixed samples were dehydrated in ethanol and embedded in paraplast plus (Monoject Scientific, St. Louis, MO, USA) or Hiscosec (Merck, Darmstadt, Germany). Immunostaining was performed using the 'Universal ICQ LSAB plus' kit. The sections were incubated with anti-FBPase antibodies (1:1000) washed and then incubated with anti-rabbit IgG-biotin and the streptavidin/PAP complex coupled to peroxidase detection. Control sections were incubated with antibodies preabsorbed with FBPase or without the first antibody. The immunofluorescence was performed using the same procedure but replacing the secondary antibody with Alexa fluor 594 conjugated to goat anti-rabbit IgG (diluted 1/300) purchased from Molecular Probes (Eugene, OR, USA). Controls were performed by substituting the second antibodies by buffers. The cross-reactivity of the secondary antibodies was tested by substituting the target primary antibody with normal serum or buffer. Stained sections were examined with a Zeiss (Thomwood, NY, USA) laser scanning confocal microscope at the Centro de Estudios Científico, Valdivia (CECS). The light source was argon/krypton laser and optical sections of 1 μm were obtained.

3. Results

The immunoblot analysis demonstrated the presence of similar immunoreactivity by liver FBPase in hepatic and renal extracts. These results indicate that FBPase is present in a similar amount in both tissues. Moreover, the analysis revealed that the antibody detected two single bands with a MW of 36 000 and 40 000, in kidney and liver extracts, respectively (Fig. 1). The immunoreactive bands migrated at the predicted subunit molecular weight, demonstrating the high specificity of the prepared antibody. Minimal degradation of these proteins was observed on both total tissue extracts. The antibody does not react with other proteins and does not recognize the muscle isoform (Fig. 1, lane 4).

Immunohistochemical detection of FBPase in kidney, using the peroxidase reaction (DAB) and light microscopy, yielded a strong signal at the proximal straight and convoluted tubules located at the cortex but not in cells of the kidney medulla (Fig. 2). The very low immunoreaction detected in the distal convoluted tubules indicated low or non-existent expression of FBPase (Fig. 2), with the distal cells providing an internal control for the primary and secondary antibodies. At the subcellular level, FBPase showed a strong reaction in the nuclei of the proximal convoluted cells. The cytosol was also stained and showed an apical localization of the enzyme. This unusual

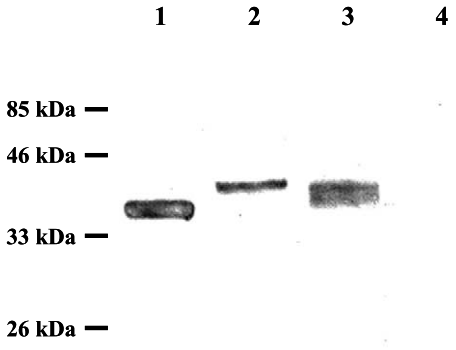

Fig. 1. Immunoblot with anti-liver FBPase. Lane 1, 2 μg purified pig kidney FBPase; lane 2, 20 μg of rat liver homogenate; lane 3, 20 μg of rat kidney homogenate; lane 4, 20 μg of rat muscle homogenate.

organization of FBPase correlates with a subcellular compartmentalization of metabolic enzymes. FBPase exhibited a similar immunoreaction level in the liver. This technique is unable to quantify the amount of expressed enzymes; however, qualitative values show that FBPase is similarly expressed in proximal tubule cells and periportal hepatocytes. FBPase immunostaining was observed in the cytoplasm of the hepatocytes; however, the periportal vein and endothelial cells were negative (Fig. 2). Particularly intense staining was seen in the plasma membranes of adjacent cells (Figs. 2 and 4), suggesting a special subcellular distribution of the FBPase, which could be related to the glycogen synthesis. In addition, a prominent perinuclear and nuclear staining was also observed in hepatocytes from periportal regions. Immunostaining controls replacing the primary antibody by non-immune rabbit serum showed no immune reaction (data not shown).

The immunostaining pattern seen with conventional light microcopy showed the association of FBPase to the nuclei of rat liver and kidney cells. To identify the presence of FBPase inside the nuclei, we performed immunofluorescence and confocal analysis taking optical sections of 1 μm. The results revealed a clear nuclear localization of FBPase in kidney proximal tubule cells. The white arrow (Fig. 3) shows several stained nuclei through the 1 μm optical series, confirming this unexpected observation. Moreover, this analysis demonstrated that FBPase is concentrated in the apical mem-

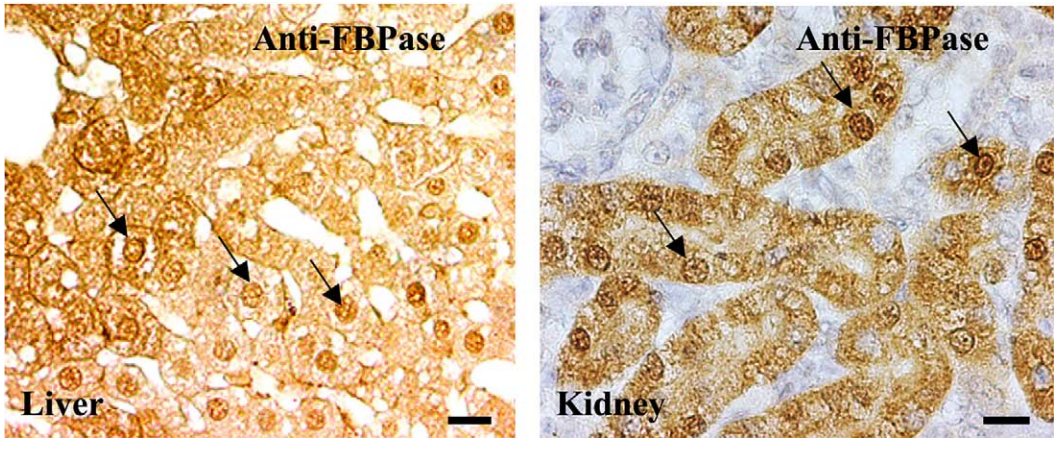

Fig. 2. Localization of liver FBPase in kidney and liver by conventional light microscopy. Nuclear reaction (arrows). Scale bars, 10 μM.

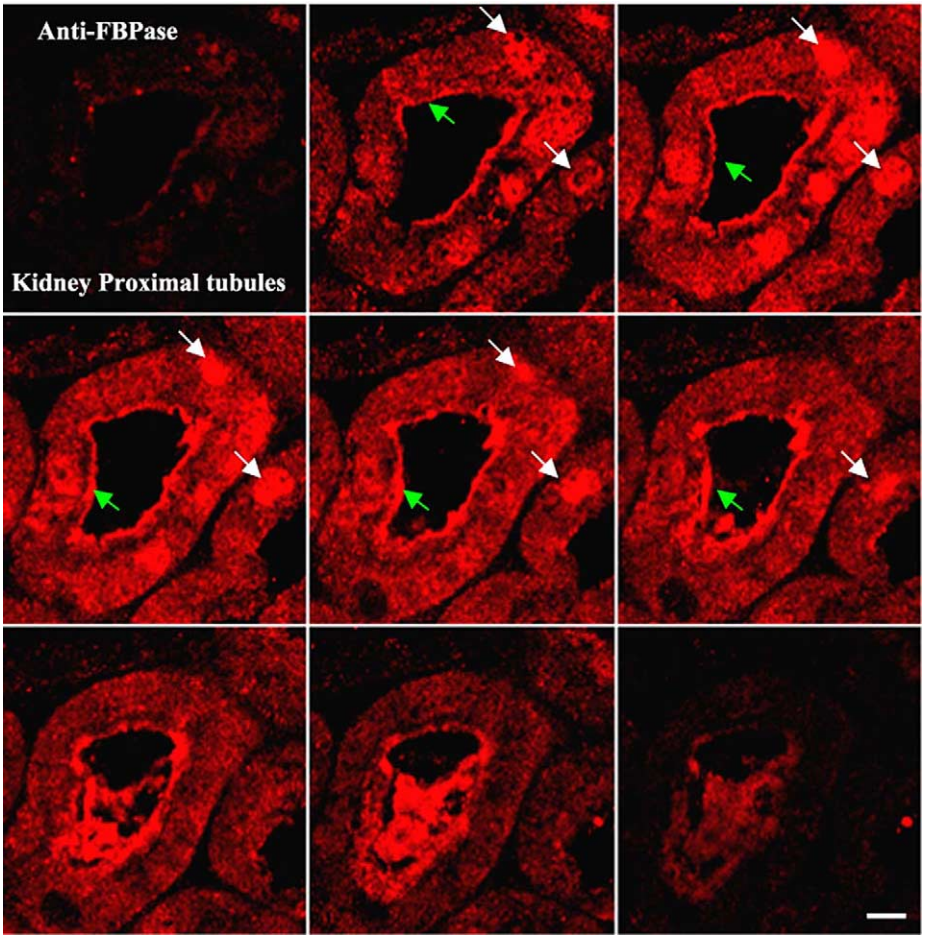

Fig. 3. Immunofluorescence of FBPase (red label) in kidney tissue. Proximal convoluted tubule optical sections of 1 μm were obtained. Nuclei (white arrows). Apical membrane (green arrows). Scale bars, 5 μm.

brane region. The nuclear localization of FBPase was detected in several kidney samples; however, not all the cells of this epithelium exhibited this staining. These results indicate that not all the proximal tubule cells are in the same metabolic state that allows the translocation of FBPase from the cytosol to the nucleus. Similar results were observed in liver, confirming the relative abundance of this enzyme in the cytoplasm of hepatocytes (Fig. 4A). The nuclear localization of FBPase in these cells was also clearly observed (Fig. 4A). Additionally, the nuclear staining was not uniform and the nucleolus was negative for FBPase staining. Furthermore, the rotation of a three-dimensional reconstruction of optical sections from hepatocytes clearly shows that FBPase is concentrated at the hepatocyte cell periphery forming uneven clumps, close to the plasma membrane (Fig. 4B). No specific staining was detected in any of the negative controls without primary or secondary antibodies against rabbit IgG (data not shown).

4. Discussion

Glucose metabolic pathways are well defined but the subcellular organization of these pathways is poorly understood in liver and kidney. Several factors affect the net flux of gluconeogenesis and allow the cells to respond to physiological changes [1]. Ovadi and Srere have suggested that a new regulatory mechanism for metabolic pathways is the subcellular compartmentalization and the physical separation of them in a single cell [17,18]. These authors also underline the need for a thorough characterization of the subcellular organization of metabolic enzymes [17,18]. In this context, in muscle there is convincing evidence about the interaction between some glycolytic enzymes and the subcellular cytoskeletal network, association that regulates the location of glycolytic enzymes and the kinetic rates of individual enzymes in the contraction process [19–21]. In this report, we present the first data available on the subcellular localization of liver FBPase in rat kidney and liver cells that clearly describe the localization of FBPase in the nucleus and in a compartment near the apical membrane in kidney cells and in an area adjacent to the plasma membrane in the hepatocytes.

Which are the physiological reasons for the localization of FBPase in the periphery of these cells? Kidney contributes with a significant fraction of the systemic gluconeogenesis [22,23]. Recently, we have established that liver FBPase and C-PEPCK are expressed mainly in the human proximal tubule cells localized in the kidney cortex, indicating that the glucose synthesis is compartmentalized to the proximal tubules [10,11]. A similar distribution of glycolytic and gluconeogenic enzymes has been described in kidney and liver [11,12,24,25], suggesting that the separate localization of both pathways within the nephron, coupled with the elevation of renal precursors in the proximal tubule, may represent an additional physiological mechanism that stimulates the gluconeogenic pathway and avoids the consumption of ATP by the forma-

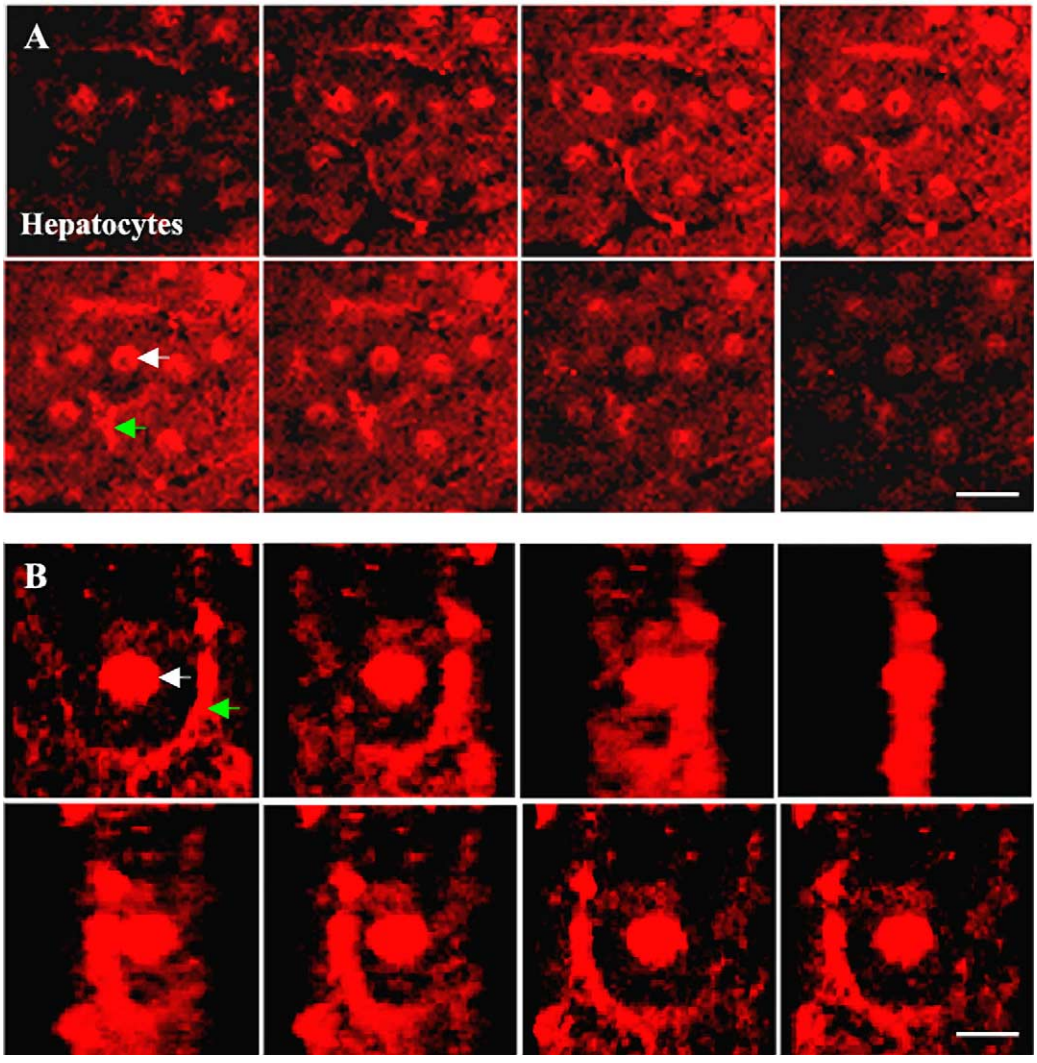

Fig. 4. Immunofluorescence of FBPase (red label) in liver tissue. A: Hepatocyte optical sections of 1 μm were obtained. Nuclei (white arrow). Plasma membrane (green arrows). Scale bars, 10 μm. B: Rotation of a three-dimensional reconstruction of hepatocyte optical sections (1 μm). Scale bars, 5 μm.

tion of futile cycles in a single cell [10,11]. Moreover, this analysis suggests that the presence of these catalytically active key enzymes is not sufficient to explain the mechanism responsible for the variation in gluconeogenic rates from lactate in the proximal tubules. Our findings [10], showing the expression of the monocarboxylated transporter 1 in the S1 segment, may explain these different rates in the proximal tubule segments. The FBPase shows a specialized subcellular compartmentalization along rat kidney proximal tubules, being clearly localized in the apical area of these cells. The treatment of a kidney crude extract with Triton X-100 led to a significant increase of 250% of the total activity of FBPase. From these data we propose that FBPase may interact with transport elements in the cellular membrane and play an important function in the metabolic heterogeneity observed among the proximal tubules. This functional duality of gluconeogenic enzymes has been demonstrated for aldolase [26,27]. Indeed, this enzyme plays a structural role in the polymerization of actin and in the dynamic association of GLUT 4 vesicles with actin, regulating glucose transport [26,27]. Also, we can postulate that glucose synthesis in these cells occurs in a subcellular compartment adjacent to the apical membrane.

A similar metabolic zonation has been observed in rat liver, demonstrating that hepatocytes from the periportal region of rat liver mainly expressed the gluconeogenic enzymes, whereas the hepatocytes located in the pericentral region mainly expressed glycolytic enzymes [11,24,28,29]. These data and other interesting results strengthen the notion of the importance of different metabolic functions for particular cells in bi-functional organs [10,11,24,30]. The negative immunostaining in non-hepatocyte cells is a good control for our antiserum and supports the specificity of our subcellular localization. Interestingly, FBPase was located at the periphery of the plasma membrane of adjacent hepatocytes. Studies by Guinovart's laboratory have demonstrated that liver glycogen synthase (GS) has a cytosolic distribution in the absence of glucose and concentrates at the periphery of the hepatocyte when the concentration of the hexose increases [31]. Additionally, these authors showed that GS colocalizes with the glycogen deposit stores in the membrane compartment, suggesting that this enzyme remains attached to its product [32]. Glucose depletion causes glycogen degradation and the redistribution of the GS to the cytosol [33]. Our results show that the intracellular distribution of FBPase closely resembles the GS and

glycogen localization and lead us to propose that FBPase in this compartment is participating in the production of glucose 6-phosphate as a precursor for glycogen synthesis. Therefore, the existence of this organized subcellular distribution and the possible separation of glycogenogenesis and gluconeogenesis pathways within a single cell might be the regulatory mechanism that may explain how the kidney and hepatic cells can segregate intermediates of competing metabolic pathways. This idea is sustained by the capacity of the liver GS to differentiate between glucose 6-phosphate produced by glucokinase (GK) or hexokinase I and by the capacity of GS to use the glucose 6-phosphate produced by gluconeogenesis from dihydroxyacetone [34]. Together these observations support the proposal that the physical separation of FBPase in multiple compartments and its interaction with enzymes that participate in glycogen synthesis and gluconeogenesis are required for the regulation of these pathways.

Interestingly, the subcellular localization of FBPase in hepatocytes and proximal tubule cells reveals that FBPase is also able to translocate to the nucleus in these cell types. The nuclear localization of muscle-FBPase was recently reported [35]. This data corroborate our results on the ability of liver FBPase to localize to the nuclei of liver and kidney cells. Nevertheless, the authors discussed that rat liver FBPase does not contain a nuclear localization sequence (NLS) compared to the muscle isoform [35]. We agree that liver FBPase does not contain this specific canonical NLS, but the nature of these signals is broad and liver FBPase may contain other NLSs or a bipartite signal. Moreover, using neural networks that predict subcellular location we found that liver FBPase contains other classical NLSs (PXKRXKX) [36], that may allow the translocation of FBPase into the nuclei.

Which are the physiological reasons for the localization of FBPase inside the nuclei of kidney and liver cells? Broad evidence exists about the nuclear translocation and cytosolic redistribution of several enzymes in response to metabolic conditions [13–15,37]. In the absence of glucose, GK is localized in the nucleus of the hepatocytes, bound to its regulatory protein, but moves into the cytoplasm when the levels of sugar increase [15,38]. Muscular GS (M-GS) is also concentrated in the nucleus at low glucose and at high glucose concentrations translocates to the cytosol, where it adopts a particulate pattern [37]. The nuclear role of GK in response to high glucose concentration was proposed as a mechanism to minimize the futile cycle between glucose and glucose 6-phosphate, an event that represents an important mechanism for the regulation of glucose metabolism [38]. We can also suggest a similar mechanism for the nuclear localization of FBPase in the cell avoiding the formation of a futile cycle between F-6-P and Fru-1,6-P_2, as well as the degradation of FBPase. Moreover, it is possible to postulate that other FBPase putative functions in the nuclei might be the participation of the enzyme as transcription factor, involvement in the phosphorylation/dephosphorylation process that regulates the activation of transcription factors, or participation in the DNA metabolism. These unusual ideas are supported by the demonstration that the glycolytic enzymes lactate dehydrogenase and 3-phosphoglycerate kinase can reside in the nuclei of mammalian cells and exert functions in DNA replication, transcription and DNA repair, in addition to their role as catalysts in the cytoplasm [13,14,39]. The transfer of these glycolytic enzymes to cell nuclei requires phosphorylation [39] and notably rat liver FBPase at the COOH terminus contains two AMP-dependent phosphorylation sites [40]. The catalytic cytosolic function is not altered by this modification; however, it can be responsible for the FBPase nuclear localization and also for the regulation of a new phosphatase function exerted by this enzyme in the nuclei.

On the other hand, it is also plausible to think that the nuclear FBPase function is similar to the function of the cytosolic form; participating in the glucose 6-phosphate production. However, the amount of FBPase present in purified nuclei from liver and kidney tissues is lower than 2% of the total activity present in the organs (data not shown), but the staining pattern indicates that FBPase concentration is high. These two observations can be reconciled if we considerer the possibility that nuclear accumulation is a way to regulate the pool of active/inactive enzyme. Finally, the binding ability of FBPase to recognize several important metabolites can be a regulatory linkage between the energy state of the cell and nuclear functions.

Acknowledgements: This work was supported in part by Research Grants from the Fondo Nacional de Ciencia y Tecnología, Chile, FONDECYT, 1010720 and from the Dirección de Investigación, Universidad Austral de Chile, DID-UACH 200302. We are grateful to Dr. Joan Guinovart (Universitat Barcelona, Spain) for his continuous interest and support in our work. We thank Dr. Alejandro Claude R. and Dr. Juan G. Cárcamo (Universidad Austral de Chile) for their critical reading of the manuscript and Mr. Cristían Droppelmann (Universidad Austral de Chile) for the FBPase NLS analysis. We also thank Dr. Felipe Barros and Mr. Joel Castro (Centro de Estudios Científicos, Valdivia) for their assistance in the confocal microscopy analysis and Fundación Andes for a grant to CECS.

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