Subcellular localization of liver FBPase is modulated by metabolic conditions

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Abstract In primary cultured hepatocytes, fructose-1,6-bisphosphatase (FBPase) localization is modulated by glucose, dihydroxyacetone (DHA) and insulin. In the absence of these substrates, FBPase was present in the cytoplasm, but the addition of glucose or DHA induced its translocation to the nucleus. As expected, we observed the opposite effect of glucose on glucokinase localization. The addition of insulin in the absence of glucose largely increased the amount of nuclear FBPase. Moreover, at high concentrations of glucose or DHA, FBPase shifted from the cytosol to the cell periphery and co-localized with GS. Interestingly, the synthesis of Glu-6-P and glycogen induced by DHA was not inhibited by insulin. These results indicate that FBPase is involved in glycogen synthesis from gluconeogenic precursors. Overall, these findings show that translocation may be a new integrative mechanism for gluconeogenesis and glyconeogenesis.
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1. Introduction

The synthesis of glucose from non-glucidic precursors, such as lactate, amino acids and glycerol [1,2], occurs mainly in liver and kidney and is essential for glucose homeostasis during fasting. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) is a key enzyme in the gluconeogenic pathway as it catalyzes the irreversible hydrolysis of fructose-1,6-bisphosphate (Fru-1,6-P_2) to fructose-6-phosphate and P_i [3]. On the basis of immunological and kinetic data, at least three distinct forms of FBPase isoenzymes have been proposed: brain, muscle and liver [3–5]. The liver isoform is the main regulatory enzyme of gluconeogenesis in liver and kidney [1,2]; however, the physiological roles of the brain and muscle isoenzymes are not fully understood [6,7].

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Liver FBPase has been isolated from several species and presents a homotetrameric structure (36–41 kDa per subunit) and is regulated mainly at the posttranslational level by two synergistic negative effectors, AMP and fructose-2,6-bisphosphate (Fru-2,6-P_2) [8,9]. These metabolites play a crucial function by modulating the rate of gluconeogenic/glycolytic fluxes. Although the liver isoform is found in various human and rat tissues [10], it is expressed mainly in liver and kidney, bifunctional organs that perform glycolysis and gluconeogenesis. In addition, FBPase exhibits a cellular compartmentation in these two tissues, with almost exclusive expression in kidney proximal tubules and higher expression levels in periportal hepatocytes than the perivenous region [11]. In these tissues, FBPase also shows a particular subcellular distribution. In kidney, it is distributed throughout the cytoplasm and in an apical peripheral compartment of proximal epithelial cells. Similar results are observed in liver, where the enzyme is mainly localized in the cytoplasm of periportal hepatocytes, and in a specific compartment close to the plasma membrane of adjacent hepatocytes [11]. Interestingly, FBPase is also observed in the nuclei of hepatocytes and proximal epithelial cells.

The subcellular redistribution of some metabolic enzymes is dynamic and responsive to metabolic conditions. In the absence of glucose, Glucokinase (GK) is bound to its regulatory protein in the nucleus of the hepatocyte and translocates to the cytoplasm when sugar levels increase [12]. Muscle glycogen synthase (GS) is also concentrated in the nucleus at low glucose and translocates to the cytosol, where it adopts a particulate pattern at high glucose concentrations [13,14]. In contrast, liver GS presents a cytosolic distribution in the absence of glucose and concentrates at the periphery of the hepatocytes when the hexose concentration increases [15].

Here, we examined the subcellular distribution of FBPase in comparison with GK and GS localization in primary cultured hepatocytes. Our results indicate that the subcellular location of FBPase is modulated by the action of glucose, dihydroxyacetone (DHA) and insulin.

2. Materials and methods

2.1. Hepatocyte isolation and culture
Fasted male rats weighing 300 g were used. Hepatocytes were isolated by liver perfusion with collagenase (Sigma) as described [16]. Cells were suspended in 50 ml of Dulbecco's modified Eagle's medium

(DMEM; Invitrogen) supplemented with 5 mM glucose, 10% (v/v) fetal bovine serum (FBS; Sigma), 100 nM insulin (Sigma), 100 nM dexamethasone (Sigma) and 1% (v/v) penicillin/streptomycin (Invitrogen), and seeded onto gelatin-coated coverslips placed in 24-well plates. After 5 h incubation at 37 °C in humidified air/CO$_2$ (19:1) chamber, cells were washed with sterile PBS and the culture medium was replaced with glucose-free DMEM including 10% FBS. After overnight incubation, the hepatocytes were incubated for 6 h in one of the following DMEM conditions: (1) glucose-free, (2) 25 mM glucose, (3) glucose-free supplemented with 10 mM DHA (Sigma), (4) glucose-free + 10 nM insulin, and (5) 25 mM glucose + 10 nM insulin (Sigma). The hepatocytes were then fixed for immunohistochemistry and cell monolayers were scraped to prepare total cell extracts.

2.2. Antibodies

Rabbit antiserum against purified pig FBPase was used [11]. FBPase was purified from pig kidney as described by Reyes et al. [17]. Chicken egg yolk antibodies against FBPase were prepared by immunizing two Leghorn hens through two subcutaneous injections of an emulsified mixture of purified proteins (2 mg) with Freund's complete adjuvant (first injection) or incomplete adjuvant (second injection) at 2-week intervals. Two weeks after the second injection, a booster of 4 mg of pure enzymes was given. The eggs were collected daily throughout the study period and stored at 4 °C until analysis. The isolation of polyclonal IgY from egg yolk granules was performed following the method described by Akita and Nakai [18]. GS antibody was raised in rabbit against a peptide that contains the fourteen C-terminal amino acids of rat liver GS. This antiserum has a high specificity for liver GS and does not cross-react with the muscle isoform [15]. The anti-rat liver GK antibody was raised in rabbit [12].

2.3. Immunofluorescence analysis

The analysis was performed in three ($n = 3$) independent experiments. Hepatocytes seeded onto gelatin-coated coverslips were washed with PBS and fixed with 4% (w/v) paraformaldehyde (Polysciences, Inc.) in PBS for 20 min. The cells were then treated with 1 mg/ml NaBH$_4$ (Sigma) in PBS permeabilized with 0.3% (v/v) Triton X-100 (Sigma) in PBS and blocked with 3% (w/v) BSA (Calbiochem) in PBS. The samples were dually incubated for 2 h at room temperature with primary antibodies against FBPase and GS or FBPase and GK, respectively, diluted in 1% BSA in PBS. Cells were then washed with PBS and incubated for 1 h at room temperature with Alexa Fluor 594 and Alexa Fluor 488-conjugated secondary antibodies against rabbit IgG and chicken IgY (Molecular Probes, Eugene, OR) respectively. Finally, the hepatocytes were washed again with PBS and mounted onto microscope glass slides with Fluorescent Mounting Medium (DAKO Corporation, Carpinteria, CA). Optical sections of 1 μm were obtained using a Zeiss (Jena, Germany) LSM5 Pascal Laser scanning confocal microscope at the Centro de Estudios Científicos-Valdivia (CECS). Alexa Fluor 594 and Alexa Fluor 488 fluorescence were excited at 568 and 488 nm, respectively.

2.4. Metabolite determinations

To measure glycogen content, cell monolayers were scraped into 30% KOH, the extract was then boiled for 15 min and centrifuged at $5000 \times g$ for 15 min. Glycogen was measured in the cleared supernatants as described [19]. The intracellular concentration of glucose-6-phosphate (Glu-6-P) was measured using a spectrophotometric assay. Glucose concentration in the incubation medium was measured as described [19]. The metabolites were measured in three independent experiments ($n = 3$).

3. Results

We studied the effect of glucose and DHA (substrates) and insulin on the intracellular distribution of liver FBPase by immunofluorescence and confocal analysis in cultured rat hepatocytes. We concomitantly analyzed the subcellular distribution patterns of GK and GS and compared them with that of FBPase. In addition, we evaluated the effect of glucose, DHA and insulin on the intracellular Glu-6-P concentration and glycogen content. Hepatocytes were maintained for 16 h in

a medium devoid of substrates and then incubated for 6 h with 25 mM glucose or 10 mM DHA in the absence or presence of 10 nM insulin. In the hepatocytes incubated in the absence of substrates, the level of glycogen and Glu-6-P was very low (Table 1). In this condition, FBPase was observed in the cytoplasm of the hepatocytes, showing a strong immunostaining in the cell periphery around the group of the cells. Low immunoreaction was detected in the nuclei (Fig. 1B and H). When the cells were incubated with 25 mM glucose, the enzyme changed its subcellular localization and concentrated within the nucleus (Fig. 2E and K). As previously reported, GK was present in the nucleus in the absence of glucose and translocated to the cytosol in the presence of the sugar, and showed a visible juxtanuclear localization (Fig. 1A and D). Detailed co-localization analysis of FBPase with GK showed that these two enzymes translocated in opposite directions in response to glucose (Fig. 1A and D, respectively). Additionally, in response to high glucose concentration, FBPase was recruited to the cell periphery (Fig. 1D–F and J–L). As previously described, GS changed its cellular distribution from a uniform cytoplasmatic distribution to an accumulation at the cell periphery (Fig. 1G and J), where it showed a high degree of co-localization with FBPase (Fig. 1J–L). In the hepatocytes incubated with 25 mM glucose, there was an 8- and 12-fold increase in the intracellular concentration of glycogen and Glu-6-P, respectively, when compared with those incubated in the absence of substrates (Table 1).

DHA is an effective gluconeogenic substrate, thus we analyzed its effect on the intracellular distribution of FBPase. DHA (10 mM) induced the same pattern of FBPase and GS localization as glucose. DHA stimulus produced the recruitment of the two enzymes in peripheral compartments of the hepatocytes. Moreover, DHA induced FBPase translocation into the nucleus, but to a lesser extent than that produced by glucose. By contrast, this gluconeogenic precursor was unable to elicit any change in GK subcellular distribution and the enzyme remained in the nucleus (data not shown). Incubation with this substrate led to a marked increase in Glu-6-P levels, which were about 22-fold higher than in control cells (Table 1). Under these conditions, the hepatocytes produced the same amount of glycogen as those incubated with 25 mM glucose (Table 1).

We next analyzed the effect of insulin in the absence and in the presence of glucose. In hepatocytes incubated with 10 nM insulin in the absence of glucose, FBPase translocated largely into the nucleus (Fig. 2D–F), although it presented a less significant peripheral distribution than that observed when cells were incubated with glucose alone. In this condition, there was

Table 1
Hepatocytes were maintained for 16 h in a medium devoid of glucose and DHA and then incubated for 6 h with glucose or DHA in the absence or presence of 10 nM insulin ($n = 3$)

	Glycogen (μg/mg protein)	Glu-6-P (nmol/mg protein)
No substrates	3.80 ± 0.2	0.25 ± 0.03
25 mM glucose	31.8 ± 1.8	3.1 ± 0.14
10 mM DHA	30.0 ± 2.0	5.5 ± 0.2
No substrates + insulin	7.91 ± 0.5	0.15 ± 0.03
25 mM glucose + insulin	39.2 ± 1.9	4.2 ± 0.15
10 mM DHA + insulin	33.5 ± 1.5	5.2 ± 0.2

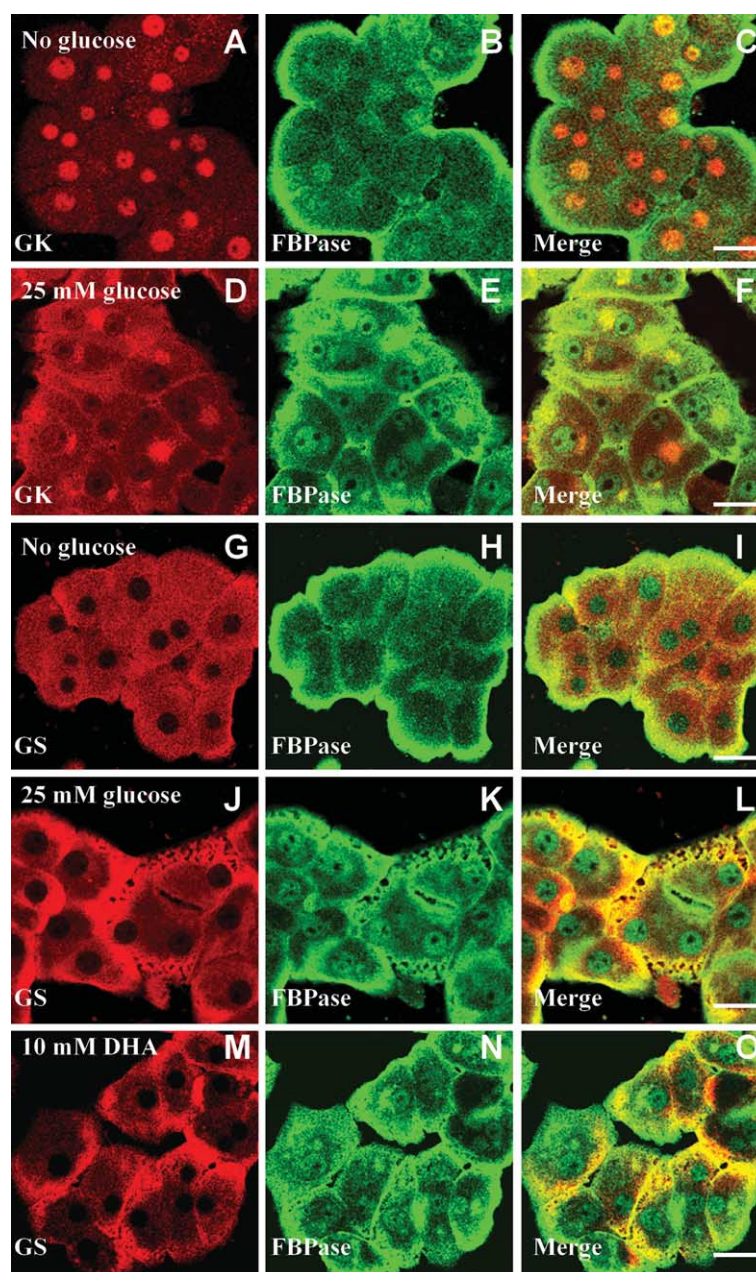The total intracellular content of glycogen and Glu-6-P was measured.

Fig. 1. Subcellular distribution of FBPase, GS and GK in hepatocytes cultured with 25 mM glucose or 10 mM dihydroxyacetone. Cultured hepatocytes treated differentially were fixed and co-incubated with rabbit anti-GK and chicken anti-FBPase (A–F) and with rabbit anti-GS and chicken anti-FBPase (G–O). The enzymes were detected using Alexa Fluor 594-labeled anti-rabbit IgG (red label) and Alexa Fluor 488-labeled anti-chicken IgY (green label) as secondary antibodies. The images C, F, I, L and O correspond to the merging images, respectively. Scale bar 20 μm.

a slight increase in glycogen content (Table 1). When incubated with 10 nM insulin plus 25 mM glucose, FBPase showed a similar accumulation at the cell periphery but was clearly more concentrated in the nucleus of the hepatocytes than when incubated with glucose alone (Fig. 2G–L). The same distribution pattern was observed in hepatocytes co-incubated with 10 mM DHA and 10 nM insulin (data not shown). Insulin alone produced a limited effect on GS distribution; nevertheless, this hormone potentiated the glucose-induced recruitment of GS towards the cell periphery (Fig. 2G and J). Interestingly, hepatocytes co-incubated with 10 mM DHA and 10 nM insulin used this substrate in the gluconeogenic production of Glu-6-P and glycogen.

4. Discussion

The subcellular organization and metabolite modulation of gluconeogenic enzymes are poorly understood. Here, we show for the first time that the subcellular localization of the liver isoenzyme of rat FBPase is modulated by the metabolic conditions of the cultured hepatocytes. These findings suggest that the modulation of the subcellular location of this enzyme may be a new regulatory mechanism of the gluconeogenic pathway.

In response to an increase in glucose or DHA concentration, FBPase showed nuclear import and recruitment in a peripheral cell compartment. Interestingly, FBPase and GS were co-recruited to the periphery of the cells. This is precisely the site
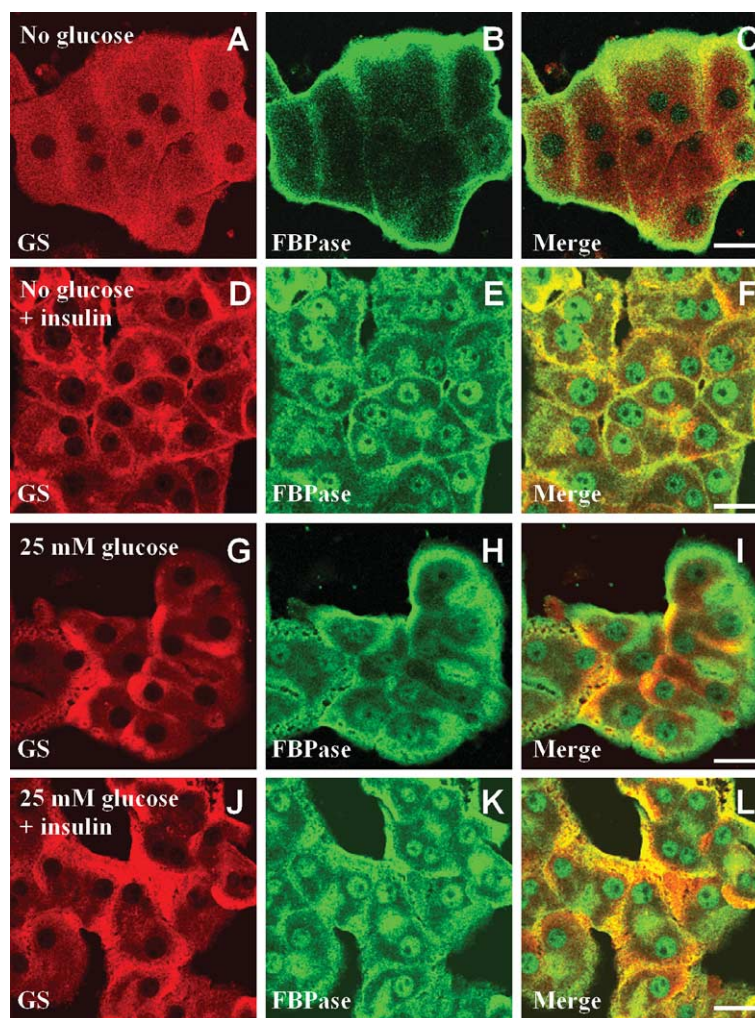
Fig. 2. Subcellular distribution of FBPase and GS in hepatocytes cultured with 10 nM insulin in the absence or in the presence of 25 mM glucose. Cultured hepatocytes treated differentially were fixed and co-incubated with rabbit anti-GS and chicken anti-FBPase. The enzymes were detected using Alexa Fluor 594-labeled anti-rabbit IgG (red label) and Alexa Fluor 488-labeled anti-chicken IgY (green label), as secondary antibodies. The images C, F, I and L correspond to the merging images. Scale bar 20 μm.

where glycogen is initially synthesized and the deposits of the polysaccharide grow from the periphery of the hepatocytes towards the interior [15]. This finding supports the notion of the formation of a complex between FBPase and the glycogen synthesis enzymes. This complex may allow the channeling of substrates and the enhancement of glycogen synthesis. In this context, the importance of the compartmentation of enzymes and the concomitant channeling of substrates has been discussed by Ovadi and Srere [20,21]. This micro-compartmentation, together with other factors involved in channeling, provides many potential biological advantages, such as the isolation of intermediates from competing reactions and a new means of metabolic regulation by modulating enzyme associations, which ensure the proper function and integration of metabolic pathways [20,21]. We observed similar capacities of glucose and DHA to stimulate the redistribution of FBPase and GS to the cell periphery. This result indicates that the direct pathway of glycogen synthesis from glucose and the indirect pathway from gluconeogenic precursors are located in the same subcellular functional compartment. By contrast, DHA has no effect on the subcellular localization of GK (data

not shown). We measured Glu-6-P as an intermediate precursor of glycogen synthesis, and as previously reported [19], we found that part of the Glu-6-P produced by gluconeogenesis from DHA was channeled to glycogen deposition.

Moreover, the colocalization of FBPase with GS was not inhibited by the action of insulin. This hormone stimulates glycogen synthesis, inhibits glycogen breakdown, and suppresses gluconeogenesis [22]. However, our data reveal that the synthesis of Glu-6-P and glycogen induced by DHA was not inhibited by insulin. These results show that the major effect of insulin is on neither Glu-6-P synthesis nor on the synthesis of glycogen, indicating that under these conditions the indirect pathway of glyconeogenesis is active in hepatocytes. These results are consistent with current data that demonstrate that liver glycogenolysis is acutely sensitive to small changes in plasma insulin, whereas gluconeogenic flux is not [23]. On the basis of the observation of the recruitment of FBPase and GS in the cell periphery induced by high glucose, we hypothesize that, even in this condition, FBPase participates in glycogen synthesis. This notion is also supported by studies which show that the postprandial synthesis of glycogen is dramatically

decreased when the gluconeogenic pathway is blocked by specific inhibitors [24,25]. Glucose utilization raises Glu-6-P and glycogen in hepatocytes, but also induces the increase of gluconeogenic/glycolytic intermediates. Thus, the recruitment and binding of FBPase to the GS compartment may prevent the inhibition of FBPase by the allosteric effector (AMP), thereby allowing the recycling and channeling of glycolytic intermediates to glycogen. Studies by Dzugaj's laboratory support this hypothesis and demonstrate that FBPase in a complex with aldolase is insensitive to AMP inhibition [26].

Furthermore, we found that insulin strongly induced a reversible FBPase translocation into the nucleus. This result confirms our previous data that showed the capacity of liver FBPase to translocate into the nuclei of hepatocytes and reveals, for the first time, a hormonal regulation mechanism for the nuclear localization of a gluconeogenic enzyme [11,27]. The entry of proteins of a size greater than the exclusion limit of the nuclear pore complex depends on the presence of a nuclear localization signal (NLS) sequence within the protein. In the absence of this sequence, large proteins enter the nucleus via interactions with other proteins by a piggy-back mechanism. Rat liver FBPase is an homotetrameric enzyme of approximately 155 KDa, whereas the exclusion limit of the nuclear pore permits only the passive diffusion of proteins of less than 45 KDa [28]. Similarly, the export of proteins from the nucleus is an active process that depends on a distinct motif called a nuclear export signal (NES) sequence. Using neural networks that predict subcellular localization, we found that liver FBPase contains a classical NLS [11,29,30]. Nevertheless, we cannot rule out a piggy-back mechanism to explain FBPase nuclear import.

Glucose-induced nuclear translocation of several enzymes, including GK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), muscle GS, aldolase, lactate dehydrogenase (LDH) and phosphoglycerate kinase (PGK) is a well-known phenomenon. Additional functions have been proposed for these enzymes in the nuclei. Of particular interest are their putative roles in gene transcription, nuclear stabilization, DNA repair and DNA synthesis [13,31–34]. Recently, we proposed distinct roles for FBPase in the nuclei of liver and kidney cells [11]; however, we cannot rule out that the function of nuclear FBPase is to produce Glu-6-P. This nuclear pool of Glu-6-P may generate the NADPH required for the generation of reduced glutathione, which exerts essential antioxidant and detoxifying functions for the maintenance of key protein thiol groups [32,35].

In conclusion, this is the first report that shows the capacity of glucose and insulin to induce the nuclear translocation of a gluconeogenic enzyme. Considering the broad metabolic function of hepatocytes, the present study reveals a further sophistication of the regulatory mechanism that controls the glucose pathways, and the integration of gluconeogenesis and glyconeogenesis in hepatocytes.

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