

Overexpression of fructose 2,6-bisphosphatase decreases glycolysis and delays cell cycle progression

J. XAVIER PEREZ,¹ TERESA ROIG,² ANNA MANZANO,¹ MIREIA DALMAU,³ JORDI BOADA,² FRANCESC VENTURA,¹ JOSE L. ROSA,¹ JORDI BERMUDEZ,² AND RAMON BARTRONS¹
¹Unitat de Bioquímica, ²Unitat de Biofísica, ³Laboratori de Citometria, Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, Barcelona, Spain

Received 4 October 1999; accepted in final form 1 May 2000

Perez, J. Xavier, Teresa Roig, Anna Manzano, Mireia Dalmau, Jordi Boada, Francesc Ventura, Jose L. Rosa, Jordi Bermudez, and Ramon Bartrons. Overexpression of fructose 2,6-bisphosphatase decreases glycolysis and delays cell cycle progression. *Am J Physiol Cell Physiol* 279: C1359–C1365, 2000.—The ability to overexpress 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2)/FBPase-2 or a truncated form of the enzyme with only the bisphosphatase domain allowed us to analyze the relative role of the kinase and the bisphosphatase activities in regulating fructose 2,6-bisphosphate (Fru-2,6-P₂) concentration and to elucidate their differential metabolic impact in epithelial Mv1Lu cells. The effect of overexpressing PFK-2/FBPase-2 resulted in a small increase in the kinase activity and in the activity ratio of the bifunctional enzyme, increasing Fru-2,6-P₂ levels, but these changes had no major effects on cell metabolism. In contrast, expression of the bisphosphatase domain increased the bisphosphatase activity, producing a significant decrease in Fru-2,6-P₂ concentration. The fall in the bisphosphorylated metabolite correlated with a decrease in lactate production and ATP concentration, as well as a delay in cell cycle. These results provide support for Fru-2,6-P₂ as a regulator of glycolytic flux and point out the role of glycolysis in cell cycle progression.

metabolism; 6-phosphofructo-2-kinase

FRUCTOSE 2,6-BISPHOSPHATE (Fru-2,6-P₂) is a potent positive effector of 6-phosphofructo-1-kinase (PFK-1) and inhibitor of fructose 1,6-bisphosphatase (FBPase-1), enzymes of the fructose-6-phosphate/fructose 1,6-bisphosphate (Fru-1,6-P₂) cycle (22, 34, 43). Its synthesis and breakdown are catalyzed by the enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) (22, 34, 43). There are different tissue-specific mammalian isoforms, all of which have kinase and bisphosphatase activities. They have been termed L (liver), M (muscle), H (heart), and T (testis) (22, 30, 34, 43) and, recently, an ubiquitously expressed isoform has also been described (20, 32, 36). PFK-2/FBPase-2 isoenzymes differ in their kinetic properties, molecular masses, and responses to phosphorylation by protein kinases. In liver, the bifunctional enzyme, via regula-

tion of gene expression and phosphorylation state, controls the cellular level of Fru-2,6-P₂ and is responsible for directing hepatic metabolic flux toward either glycolysis or gluconeogenesis, whereas in extrahepatic tissues that do not produce glucose, it contributes to the control of glycolysis (22, 34, 43).

The kinase and bisphosphatase reactions are catalyzed at separate sites on each subunit of a homodimeric protein. The PFK-2 reaction is catalyzed in the NH₂-terminal half domain of the subunit and involves ternary complex formation between the enzyme and the two substrates, fructose-6-phosphate and MgATP. The bisphosphatase domain, comprising the COOH-terminal region of the enzyme, catalyzes the hydrolysis of Fru-2,6-P₂ to fructose-6-phosphate and P_i through a covalent phosphoenzyme intermediate (22, 34, 43).

Until recently, it has not been possible to alter properties of an enzyme in a metabolic pathway in a systematic manner and then test the effect on the function of the entire pathway in an intact cell. The development of host/vector systems containing powerful promoters has allowed transfer and expression of normal and mutant cDNAs of proteins in mammalian cells and evaluation of regulatory enzymes in controlling pathway flux. There are different reports of overexpressing enzymes of mammalian glucose metabolism with concomitant analysis of the consequences on pathway flux. Argaud et al. (1) studied the effect of the overexpression of the wild-type liver L-PFK-2/FBPase-2 and a double mutant of the protein, which only possessed PFK-2 activity in FAO cells (rat hepatoma cells). When the wild-type enzyme was overexpressed, there was a decrease in Fru-2,6-P₂ levels, even though PFK-2 maximal activity increased >22-fold, was in excess of FBPase-2 maximal activity, and enhanced glucose and inhibited lactate production. Overexpression of the double mutant resulted in a 28-fold increase in kinase activity and a 3-fold increase in Fru-2,6-P₂ levels, inhibiting the rate of glucose synthesis and stimulating the rate of lactate production (1).

Address for reprint requests and other correspondence: R. Bartrons, Unitat de Bioquímica, Campus de Bellvitge, Universitat de Barcelona, Feixa Llargà s/n, E-08907 Hospitalet, Barcelona, Spain (E-mail: bartrons@bellvitge.bvg.ub.es).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In this work, rat liver PFK-2/FBPase-2 and a truncated form with only the bisphosphatase domain of the enzyme have been expressed in epithelial Mv1Lu cells to study the role of this domain in regulating Fru-2,6-P₂ concentration, the cellular metabolic activity, and cell cycle progression.

MATERIALS AND METHODS

Chemicals. [α -³²P]dCTP (3,000 Ci/mmol), [γ -³²P]ATP (3,000 Ci/mmol), [1-¹⁴C]deoxy-D-glucose, [U-¹⁴C]glucose, Rediprime DNA labeling system, and Hybond-N membranes were from Amersham International (Amersham, Bucks, United Kingdom). CLONfectin was from Clontech (Palo Alto, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Biological Industries (Kibbutz Reit, Haewek, Israel). Other materials and chemicals were of the highest quality available.

Cell culture and transfections. Cells were derived in earlier studies by chemical mutagenesis of the mink lung epithelial cell line Mv1Lu (CCL-64, American Type Culture Collection). A highly transfectable subclone of R-1B cells was generated by stable transfection of the parental R-1B cell line with a plasmid containing a histidinol marker (2). Cells were maintained in DMEM, supplemented with 10% (vol/vol) FCS, 100 units of penicillin/ml, and 100 μ g of streptomycin/ml. In stable transfected cultures, 0.4 mg/ml of G418 was added. Stable liposome-mediated transfection of R-1B-Mv1Lu cells was performed at 70–80% confluence, using 4 μ g of CLONfectin and 4 μ g of plasmid DNA per 35-mm dish, following the manufacturer's instructions. After transfection (4 h), cells were washed in phosphate-buffered saline (PBS), and R-1B-Mv1Lu cells were incubated in complete medium. After 24 h, the cells were trypsinized and replated at 1:4 dilution. G418 (0.4 mg/ml) was added 24 h later. Colonies from the transfections were isolated and analyzed for experimentation.

Plasmid constructions. Standard molecular cloning techniques were used throughout (37). The full-length cDNA from rat liver PFK-2/FBPase-2 was used to prepare the plasmid constructions (41). For the full-length construction (pPFK-2/FBPase-2), a 417-bp fragment of PFK-2/FBPase-2 cDNA was amplified by PCR using the sense primer AGTAGGATCCAAGATGTCTCGAGAGATGGGAGAACT, which contains the *Bam*H I site upstream at the start codon (under-scored) and seven complete codons complementary to amino acids 1–7 of PFK-2/FBPase-2, and the antisense primer CCTTCCTCGCGCTGAGA, which is complementary to nucleotides 530–547 (12). The PCR product (417 bp) was digested with *Bam*H I/*Eco*R I, and the resulting 317-bp product was subcloned in the *Bam*H I/*Eco*R I sites of the previously digested pPFK-2/FBPase-2 plasmid. The new pPFK-2/FBPase-2 plasmid was digested with *Bam*H I/*Sal* I and then subcloned in the *Bam*H I/*Xho* I sites of the pcDNA3 plasmids (Invitrogen). For the truncated construction of the PFK-2/FBPase-2 with only the bisphosphatase domain (pFBPase-2), a 256-bp fragment was amplified by PCR using the sense primers GATGTTCTGATTATGCTTCCATCTTCGACGTGGGCACACG and AGTAGGATCCAAGATGTATCCTTATGATGTTCTGATTATGCTTCC, which contain the *Bam*H I site (under-scored), a new start codon (bold), the epitope of the Hemagglutinin, and six complete codons complementary to amino acids Ile₂₁₉-Tyr₂₂₄ of PFK-2/FBPase-2, and the antisense primer GTTGGCTAGTGCATAGGC that is complementary to nucleotides 1,023–1,040 (12). The PCR product (256 bp) was digested with *Bam*H I/*Nco* I. The resulting 163-bp product was subcloned in the *Bam*H I/*Nco* I sites

of the previously digested pFBPase-2 plasmid. The new pFBPase-2 plasmid was digested and subcloned in the pcDNA3 plasmid, as was done with the full-length construction.

RNA extraction and Northern blot analysis. Total RNA was extracted from plated R-1B-Mv1Lu cells by the LiCl/urea method (3). Northern blot analyses were carried out by standard procedures (37). To detect the full-length L-PFK-2/FBPase-2 and the truncated construction mRNAs, a 1.4-kb *Eco*R I fragment from the rat liver PFK-2/FBPase-2 cDNA, labeled with [α -³²P]dCTP by the random primer method, was used as a probe (10). The integrity of the RNA was verified by observing the rRNA bands in the ethidium bromide gel under ultra-violet irradiation. The level of mRNA was evaluated by densitometric scanning of the autoradiograms and corrected by the amount of the 18S rRNA with the use of a ribosomal cDNA probe.

Metabolite determination. To measure Fru-2,6-P₂ levels, R-1B-Mv1Lu cells were homogenized in 0.1 M NaOH, heated to 80°C for 15 min, and centrifuged at 12,000 *g* for 5 min. Fru-2,6-P₂ was determined in supernatants by its ability to activate pyrophosphate-dependent PFK-1 from potato tubers as described by Van Schaftingen et al. (44). ATP and glycolytic metabolites were measured spectrophotometrically in neutralized perchloric extracts, using standard enzymatic methods (19).

Measurement of enzyme activities. R-1B-Mv1Lu cells were homogenized in medium composed of 100 mM KCl, 20 mM TES, 5 mM potassium phosphate, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2.5 mg/l leupeptin, pH 7.8 (*buffer A*). The enzyme was partially purified by 6–21% polyethylene glycol 6000 precipitation. PFK-2 activity was assayed by measuring the rate of Fru-2,6-P₂ production from 5 mM fructose-6-phosphate and 5 mM MgATP, pH 7.8 (4). FBPase-2 was assayed by ³²P_i released from 5 μ M [2-³²P]Fru-2,6-P₂ in the presence of 5 mM P_i at 30°C. The ³²P_i was separated from [2-³²P]Fru-2,6-P₂ by anion exchange chromatography (DEAE-Sephadex A25) and elution with triethylamine/HCO₃⁻ (41).

Western blot analysis. Immunoblot analysis was performed by a modification of the method described by Burnette (6) with a 1:2,000 dilution of polyclonal antibody raised against rat liver protein (16). Protein was extracted in homogenizing *buffer A*. For Western blot analysis, 50 μ g of total protein was electrophoresed on SDS and transferred overnight to a membrane that was incubated for 90 min at 37°C with a 1:2,000 dilution of polyclonal antibody. Before Western blot analysis, the concentration of protein was determined (31). Bound antibody was detected by the enhanced chemiluminescence method.

Heat and lactate production and oxygen consumption associated to glucose metabolism. To measure heat production, oxygen consumption, and lactate production, cell suspensions attached to microcarriers were used. For a 100-ml culture, ~3·10⁷ cells were added to 0.3 g (dry wt) of preswollen Cytodex-1 microcarriers (Pharmacia Biotech) and suspended up to 100 ml with supplemented DMEM. To achieve a maximum yield of cells attached to microcarriers, the cultures were stirred for 5 min every 30 min. After 2 h, the medium was changed and the cultures were stirred continuously at 60 rpm. Microcarrier cultures were incubated for up to 48 h. Before each determination, cells attached to microcarriers were rinsed and suspended in Krebs bicarbonate buffer containing 2.5 mM CaCl₂, 2% BSA, and 10 mM glucose. To measure heat production, 2.7 ml of the microcarrier suspension (10⁵ cell/ml) was introduced into each measurement vessel of a thermal activity monitor (LKB-Thermometrics,

Järfälla, Sweden) thermostatted at 37°C, as described (31). The gas phase remaining in the vessels (0.8 ml) and stirring at 120 rpm provided aerobic conditions during the measurements. The resulting value indicated the heat evolved from the metabolic activity of cell suspensions. Oxygen consumption was measured using Clark-type oxygen electrodes (Rank Brothers, Cambridge, England) (31). Samples were taken to measure lactate production in the same conditions. To measure glucose uptake and the phosphorylated intermediate production associated with glucose consumption, the same medium containing glucose as the only glycolytic substrate was used. The transport of 2-[1-¹⁴C]deoxy-D-glucose was determined as described by Simons (40), and the rate of glucose uptake into the glycolytic flux was determined as the conversion of [U-¹⁴C]glucose in phosphorylated or charged intermediates (29).

Cell proliferation and cell cycle analysis. For synchronization, R-1B-Mv1Lu cells were cultured in DMEM supplemented with 10% FCS for 4 days up to confluence. After this period, cells were harvested, counted, and plated (240,000 cells) into 6-mm dishes to start the experiment. At the indicated times (0, 12, 13, 15, 16, 22, and 24 h) and after trypsination, cells were washed twice in 1% BSA/PBS, fixed with ice-cold 70% methanol/PBS, and incubated at -20°C for 30 min. After being washed in 1% BSA/PBS, the DNA was stained by incubating at 37°C for 30 min with 40 µg/ml propidium iodide and 50 µg/ml RNase A. Stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) with Cell Quest software. Cell cycle analysis of DNA histograms was performed with the ModFit program (Verity Software House).

Data analysis. Differences were tested by ANOVA and the appropriate a priori contrasts.

RESULTS

Overexpression of PFK-2/FBPase-2 and FBPase-2 domain. The full length and the bisphosphatase domain of the rat liver bifunctional enzyme (Fig. 1) was expressed in Mv1Lu cells using the pcDNA3 vector. G418-resistant colonies of each construction (pcDNA3, pPFK-2/FBPase-2, and pFBPase-2) were tested for the ability to express the corresponding mRNA by Northern blot analysis. As shown in Fig. 2A, control cells (pcDNA3) did not express PFK-2/FBPase-2 liver isoform. When the cells were transfected with pPFK-2/

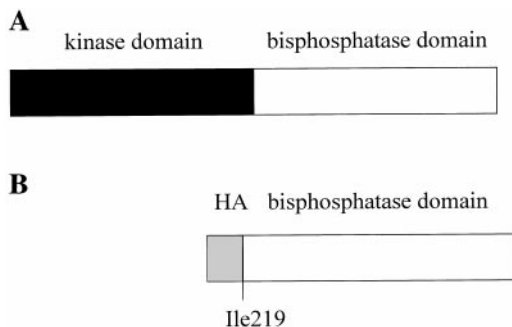


Fig. 1. Schematic representation of the construction of plasmid 6-phosphofructo-2-kinase (pPFK-2)/fructose 2,6-bisphosphatase (FBPase-2) and plasmid FBPase-2. The pPFK-2/FBPase-2 plasmid contains the rat liver full-length cDNA. The pFBPase-2 plasmid results from a 256-bp fragment of PFK-2/FBPase-2 that corresponds to the bisphosphatase domain and the pcDNA3 plasmid. Hemagglutinin epitope, HA.

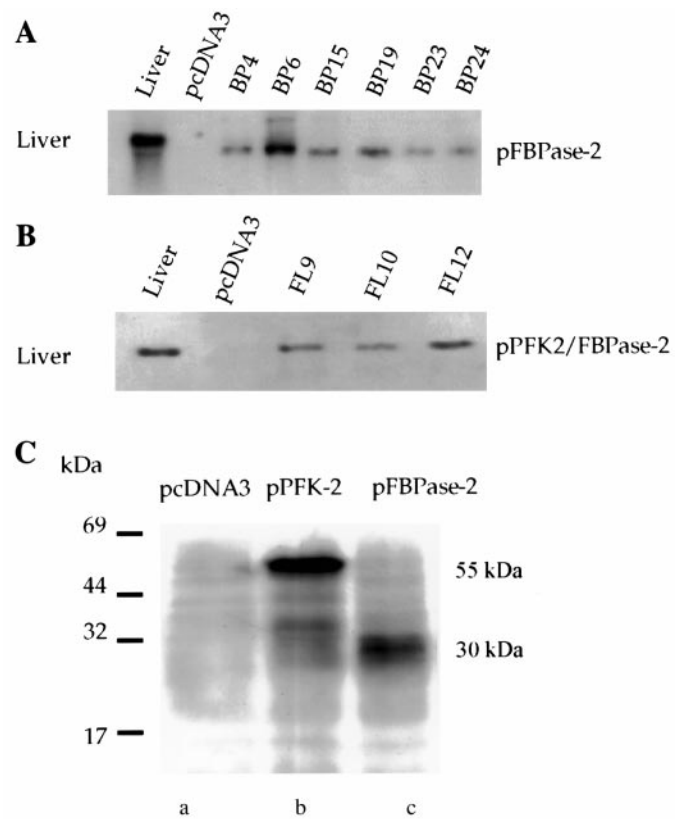


Fig. 2. Northern and Western blot analysis of expressed full-length (FL) PFK-2/FBPase-2 or the bisphosphatase domain (BP). Total RNA (20 µg/lane) extracted from R-1B-Mv1Lu cells of the different constructs (pcDNA3 plasmid, FL pPFK-2/FBPase-2, or BP pFBPase-2 clones) were transferred to nylon membranes after electrophoresis in 1.5% agarose and hybridized with a 1.4-kb *EcoR* I fragment of rat liver PFK-2/FBPase-2 cDNA as a probe, as described in MATERIALS AND METHODS. Representative Northern blots are shown in A and B. Cell extracts transfected with the full-length (b) or the bisphosphatase domain (c) of the liver PFK-2/FBPase-2 cDNA in pcDNA3 or vector alone (a) were analyzed by Western blotting using antibody against liver PFK-2/FBPase-2, as described in MATERIALS AND METHODS. Total protein (20 µg/lane) obtained from cells were transferred to nitrocellulose membranes after electrophoresis in SDS-PAGE (10%) and incubated with specific antibody. C: representative of 3 different Western blots.

FBPase-2 or pFBPase-2 constructs (Fig. 2, A and B), colonies expressed complete or truncated liver isoforms, respectively. The mRNA sizes were different for the two constructs: the full-length PFK-2/FBPase-2 mRNA had a similar electrophoretic migration to the control liver isozyme (2.2 kb), whereas the mRNA size of the truncated form was lower (1.4 kb). Protein expression was verified by Western blot analysis, using a polyclonal antibody against rat liver PFK-2/FBPase-2, which has been shown to recognize distinct PFK-2/FBPase-2 isoforms (16). Figure 2C shows the Western blot analysis of both constructions (pPFK-2/FBPase-2 and pFBPase-2) leading to the expression of two proteins of 55 kDa (lane b) and 30 kDa (lane c), respectively, recognized by the anti-bifunctional enzyme antibody, whereas the protein was at low levels in control cells. These results provided evidence that the full-

Table 1. Effect of overexpression of PFK-2/FBPase-2 and bisphosphatase domain on kinase and bisphosphatase activities and Fru-2,6-P₂ concentration in Mv1Lu cells

Clone	Fru-2,6-P ₂ , pmol × mg protein ⁻¹	PFK-2/FBPase-2		
		Kinase, μU × mg protein ⁻¹	Bisphosphatase, μU × mg protein ⁻¹	Kinase-to-Bisphosphatase Activity Ratio
pcDNA3	25 ± 4	36 ± 3	1.0 ± 0.2	36
pFBPase-2	5 ± 2*	40 ± 3	12 ± 1*	3.3*
pPFK-2/FBPase-2	43 ± 8*	73 ± 9*	1.3 ± 0.2	56

Results are means ± SE for 3–10 independent experiments. Experiments were performed as described in MATERIALS AND METHODS. *Indicate $P < 0.001$ statistically significant differences vs. pcDNA3 clones. 6-Phosphofructo-2-kinase, PFK-2; fructose 2,6-bisphosphatase, FBPase-2; fructose 2,6-bisphosphate, Fru-2,6-P₂.

length form and the truncated form of the bifunctional enzyme were correctly expressed in Mv1Lu cells.

Effect of the PFK-2/FBPase-2 or FBPase-2 overexpression on Fru-2,6-P₂ levels and kinase and bisphosphatase activities. Because maximal velocity values for the kinase and bisphosphatase reflect the amount of enzyme (10), kinase and bisphosphatase activities were assayed in FL12 and FL9 clones to determine whether activity correlated with the amount of protein. Expression of the L-type PFK-2/FBPase-2 increased the kinase activity twofold, whereas the bisphosphatase activity remained unchanged, increasing the kinase-to-bisphosphatase activity ratio. When the truncated form with the only bisphosphatase domain was overexpressed in BP6 and BP19 clones, kinase activity did not change significantly, whereas the bisphosphatase activity increased 12-fold, decreasing the kinase-to-bisphosphatase activity ratio 10-fold, with respect to the control cells (Table 1). The consequences of the expression of the L-type PFK-2/FBPase-2 isozyme resulted in a 70% increase in Fru-2,6-P₂ concentration relative to the control levels. In contrast, expression of the truncated form resulted in an 80% decrease in the bisphosphorylated metabolite.

Effects on the metabolic activity. To ascertain whether the changes in Fru-2,6-P₂ concentration had an immediate effect on the metabolic activity of the Mv1Lu cells, we measured the heat production rate, O₂ consumption, and several metabolic parameters in the different cell constructions. Results shown in Table 2 indicate that cells overexpressing FBPase-2, the truncated form of PFK-2/FBPase-2, reduced their metabolic activity by 40 μW/mg protein (–25%) with respect to control cells. This reduction in heat production rate coincided with a slight decrease in oxygen uptake and was consistent with measurements of glucose uptake, since reduced 2-[1-¹⁴C]deoxy-D-glucose transport in those cells was observed. This reduction was also followed by a decrease in [U-¹⁴C]glucose incorporation in phosphorylated or charged intermediates, Fru-1,6-P₂ and phosphoenolpyruvate concentrations, in lactate production, and by a concomitant increase in hexoses-6-phosphate concentration. These metabolic changes reduced by 17% the ATP pool in the cells that expressed the truncated bisphosphatase form. In contrast, no significant differences were observed in any of these parameters between control and L-type PFK-2/FBPase-2-expressing cells (Tables 1 and 2).

Effects on cell cycle. Most cancer cells (9, 23, 33) and treated cells with growth-promoting agents (8, 11, 14, 23, 25) have high glycolytic rates. Moreover, recent studies suggest that glycolysis is necessary for proliferation, because it ensures supply of energy and phosphometabolites (18). Because Mv1Lu cells expressing the FBPase-2 domain have decreased glycolytic capacity, we studied its involvement in their cell cycle. As shown in Fig. 3, the percentage of cells in the different cell cycle phases, as determined by flow cytometry DNA measurements, changed in the different clones studied. The percentage of cells in G₀/G₁ decreased as the percentage of cells in S phase increased. After 14 h of incubation, the percentage of cells in S phase increased from 14% to 36% or 31% of the population of control cells and those overexpressing PFK-2/FBPase-2, respectively, the differences not being significant. In contrast, cells expressing the FBPase-2 truncated form

Table 2. Metabolic changes in Mv1Lu cells

	Clone		
	pcDNA3	pFBPase-2	pPFK-2/FBPase-2
O ₂ consumption, μmol · mg protein ⁻¹ · h ⁻¹ · 10 ⁻²	62.5 ± 7.5	57.5 ± 5.0	60.6 ± 5.0
Heat production, μW × mg protein ⁻¹	155 ± 7	115 ± 20*	152 ± 17
2-Deoxy-D-glucose uptake, pmol · mg protein ⁻¹ · h ⁻¹	19.3 ± 0.1	12.2 ± 0.1†	19.1 ± 0.1
Phosphorylated glucose metabolites, nmol × mg protein ⁻¹	69.0 ± 2.8	41.1 ± 2.4†	62.1 ± 4.1
Hexoses-6-phosphate, nmol × mg protein ⁻¹	1.4 ± 0.2	2.8 ± 0.1†	1.5 ± 0.1
Fru-1,6-P ₂ , nmol × mg protein ⁻¹	0.6 ± 0.1	0.3 ± 0.1*	0.5 ± 0.1
Phosphoenolpyruvate, nmol × mg protein ⁻¹	0.6 ± 0.1	0.4 ± 0.1*	0.6 ± 0.1
Lactate production, μmol · mg protein ⁻¹ · h ⁻¹	3.88 ± 0.1	2.97 ± 0.12	3.72 ± 0.15
ATP, nmol × mg protein ⁻¹	157 ± 5	130 ± 6	155 ± 4

Results are means ± SE for 4–10 different experiments. Experiments were performed as described in MATERIALS AND METHODS. The 2-deoxy-D-glucose uptake and the phosphorylated glucose metabolites were determined after 60-min incubation with 2-[1-¹⁴C]deoxy-glucose and [U-¹⁴C]-D-glucose, respectively. * and †, respectively, indicate $P < 0.05$ and $P < 0.001$ statistically significant differences vs. pcDNA3 clones.

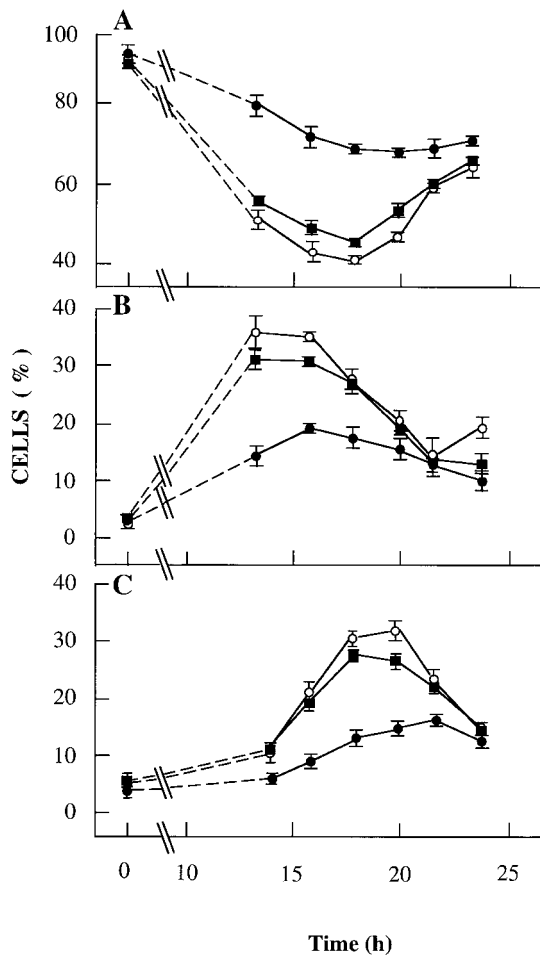


Fig. 3. Effect of overexpression of the full-length bifunctional enzyme and the bisphosphatase domain on cell cycle progression. A: G_0/G_1 ; B: S; C: G_2/M phase on cell cycle progression. Cells were incubated with DMEM supplemented with 10% FCS (vol/vol). At the indicated times, cells were processed as described in MATERIALS AND METHODS. Percentage of control pcDNA3 (○); pFBPase-2 (●); or pPFK2/FBPase-2 cells (■). Each determination was made in triplicate, and the values represented correspond to the means \pm SE of 1 representative of 3 independent experiments.

showed a reduction in the number of cells cycling (30% of truncated form expressing cells vs. 60% full-length clones had left G_0/G_1 phase after 18-h incubation), therefore, decreasing the number of cells entering the S and G_2/M phases. These results indicate that the lower metabolic activity of the cells expressing the bisphosphatase domain has an immediate influence on cell cycle progression.

DISCUSSION

Fru-2,6- P_2 is an important determinant of the glycolytic flux, and its physiological significance has been well documented in different cell types (5, 8, 11, 22, 23, 25, 33, 34, 43). The fact that it is now possible to express L-type PFK-2/FBPase-2 or a truncated form of the enzyme with only the bisphosphatase domain allowed us to analyze the relative role of the kinase and the bisphosphatase activities in regulating Fru-2,6- P_2

concentration and allowed us to elucidate the differential metabolic impact of PFK-2/FBPase-2 or the FBPase-2 domain overexpression on cellular metabolic activity in a highly transfectable clone (R-1B) of epithelial Mv1Lu mink lung cells (2).

Previous studies of expression of the bisphosphatase domain of rat (27, 41) or chicken (28) liver PFK-2/FBPase-2 in bacteria showed that the turnover and kinetic properties of the separate domain of the rat liver enzyme were similar to those of the bifunctional enzyme, except that its maximal velocity was 5- to 10-fold higher. Furthermore, studies in FAO cells (1) have shown that the overexpression of liver L-PFK-2/FBPase-2 decreases Fru-2,6- P_2 levels, even though PFK-2 activity increased >22-fold and it was in excess of FBPase-2 maximal activity. These changes produced an enhanced glucose synthesis and inhibited lactate production. The paradoxical drop in Fru-2,6- P_2 was argued for covalent modification of the overexpressed enzyme in these cells. Overexpression of a double mutant of the protein, which only possessed PFK-2 activity, resulted in a 28-fold increase in the kinase activity and a 3-fold increase in Fru-2,6- P_2 levels, inhibiting the rate of glucose production and stimulating the rate of lactate production (1).

Other studies have reported the overexpression of glycolytic or gluconeogenic regulatory enzymes. Glucokinase overexpression in FTO-2B cells correlated quantitatively with enhanced glycolytic flux (42), whereas phosphoenolpyruvate carboxykinase enhanced gluconeogenesis in H4IIE-C3 cells (35). In several instances, there was no quantitative correlation between overexpression of an enzyme and the predicted metabolic consequences. For example, a 10- to 100-fold overexpression of hexokinase I in a pancreatic β -cell line (MIN 6) enhanced glucose utilization only 2-fold (24). Overexpression of glycogen phosphorylase 46-fold in primary hepatocytes did not change glycogen content in the basal state, although preferential activation of glycogenolysis was evident upon treatment with pharmacological agents (17). These results suggest that other steps in a pathway may become rate limiting when one enzymatic step is enhanced by overproduction of the protein. The impact of overexpression of several glycolytic enzymes on overall flux has also been studied in yeast without obtaining a marked effect on the rate of ethanol production (38). The poor response is probably the consequence of the effect of overexpression of a given enzyme on the expression of other enzymes, indicating that the transfected cells adapt to the new conditions. As reported by Kacser and Burns (26), the control of fluxes or metabolite concentrations in a metabolic pathway is found to be generally shared among all the enzymes, although a smaller number may share the majority of this control.

In this paper, we have produced stable Mv1Lu cell lines expressing L-type PFK-2/FBPase-2 or the truncated form of FBPase-2. The overexpression of PFK-2/FBPase-2 resulted in a small increase in the kinase activity and in the activity ratio of the bifunctional enzyme, increasing Fru-2,6- P_2 concentration. How-

ever, these changes did not modify any of the parameters studied, suggesting that the basal Fru-2,6-P₂ levels present in these cells are high enough to activate PFK-1 and glycolysis. In contrast, when the truncated form of the enzyme coding the bisphosphatase domain was expressed, the change in the PFK-2/FBPase-2 activity ratio caused by the increased bisphosphatase activity produced a significant decrease in Fru-2,6-P₂ concentration. The fall in this bisphosphorylated metabolite could lead to a low PFK-1 activity and the restriction of glycolytic flux at the level of the fructose-6-phosphate/Fru-1,6-P₂ cycle. This is corroborated by the increase in hexoses-6-phosphate concentration and a decrease in 2-[1-¹⁴C]deoxy-D-glucose transport, [¹⁴C]glucose incorporation in phosphorylated intermediates, Fru-1,6-P₂, phosphoenolpyruvate concentrations, and lactate production. All these differences in enzyme activities and in metabolite concentrations render a reduction of the metabolic activity of the overexpressing FBPase-2 cells, as also indicated by the measurements of heat production. Consequently, the activity of the ATP-consuming processes must be reduced in cells overexpressing FBPase-2 to balance the ATP supply and demand and maintain cell homeostasis (21).

When quiescent cells are stimulated to proliferate, the expression of several genes is induced at the G₁/S transition. These genes code for proteins involved in progression through the cell cycle, for enzymes of DNA synthesis and, as it has been shown previously, for PFK-2/FBPase-2 (13, 25). Several reports have pointed out the role of Fru-2,6-P₂ in the activation of glycolytic flux in growing cells (5, 8, 11, 23, 25). The concentration of this metabolite increases in different proliferative states, and this change has been correlated with an increase in PFK-2/FBPase-2 activity and in its mRNA levels (5, 8, 11, 23, 25). Transformation of chick embryo fibroblasts by retrovirus carrying either the v-src or v-fps oncogenes has been observed to induce Fru-2,6-P₂ synthesis and to cause increased glycolytic flux and cell proliferation (23), and it has been shown that the modulation of the expression of PFK-2/FBPase-2 by growth factors is concomitant with their mitogenic response (7, 25). In a recent work, Durante et al. (15) observed, in Rat-1 fibroblasts that stably express transgenes coding for the kinase domain of PFK-2/FBPase-2, high Fru-2,6-P₂ concentrations that resulted in a parallel increase in lactate production but maintained similar proliferation rates than control cells. Whereas serum deprivation in culture reduced the growth rate of control cells, it caused apoptosis in cells overproducing Fru-2,6-P₂. These results could discount the hypothesis that activation and/or overexpression of PFK-2-catalyzed Fru-2,6-P₂ synthesis may contribute to the mitogenic potential of cells. Other authors (39) have observed that glucose deprivation or treatment with the glucose antimetabolite 2-deoxyglucose caused nontransformed cells to arrest in the G₀/G₁ phase of the cell cycle. In contrast, overexpression of lactate dehydrogenase alone was sufficient to induce apoptosis with glucose deprivation (39). These results

suggest that regulatory points exist in the cell cycle and in the apoptotic pathway that are sensitive to glucose metabolism.

In conclusion, the data reported here show that the cells overexpressing the phosphatase domain of the bifunctional enzyme have decreased Fru-2,6-P₂ content, reduced lactate production, and delayed cell cycle progression, providing support for Fru-2,6-P₂ as a regulator of glycolytic flux and pointing out the role of glycolysis in cell cycle progression.

We are grateful to Drs. S. Ambrosio, G. Pons, and J. Gil for help and valuable advice during the course of this work. We thank R. Rycroft for language assistance. The skillful technical assistance of E. Adanero and M. Vallés is also acknowledged.

This work has been supported by the Ministerio de Educación y Cultura [MEC; (PM 97/0114)] and Fondo de Investigaciones Sanitarias (FIS 97/0763).

A. Manzano and J. Boada are recipients of research fellowships from MEC and Universitat de Barcelona, respectively.

REFERENCES

1. Argaud D, Lange AJ, Becker TC, Okar DA, El-Maghrabi R, Newgard CB, and Pilkis SJ. Adenovirus-mediated overexpression of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in gluconeogenic rat hepatoma cells. Paradoxical effect on Fru-2,6-P₂ levels. *J Biol Chem* 270: 24229–24236, 1995.
2. Attisano L, Cárcamo J, Ventura F, Weis FM, Massagué J, and Wrana JL. Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* 75: 671–680, 1993.
3. Auffray C and Rougeon F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor. *Eur J Biochem* 107: 303–314, 1980.
4. Bartrons R, Hue L, Van Schaftingen E, and Hers HG. Hormonal control of fructose 2,6-bisphosphate concentration in isolated rat hepatocytes. *Biochem J* 214: 829–837, 1982.
5. Bosca L, Rousseau GG, and Hue L. Phorbol 12-myristate 13-acetate and insulin increase the concentration of fructose 2,6-bisphosphate and stimulate glycolysis in chicken embryo fibroblasts. *Proc Natl Acad Sci USA* 82: 6440–6444, 1985.
6. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112: 195–203, 1981.
7. Chesney J, Mitchell R, Benigni F, Bacher M, Spiegel L, Al-Abed Y, Hee J, Metz C, and Bucala R. An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect. *Proc Natl Acad Sci USA* 96: 3047–3052, 1999.
8. Colomer D, Vives JL, and Bartrons R. Effect of TPA on fructose 2,6-bisphosphate levels and protein kinase C activity in B-chronic lymphocytic leukemia (B-CLL). *Biochim Biophys Acta* 1097: 270–274, 1991.
9. Colomer D, Vives JL, Pujades A, and Bartrons R. Control of phosphofructokinase by fructose-2,6-bisphosphate in B-lymphocytes and B-chronic lymphocytic leukemia cells. *Cancer Res* 47: 1859–1862, 1987.
10. Colosia AD, Marker AJ, Lange AJ, El-Maghrabi MR, Graner DK, Tauler A, Pilkis J, and Pilkis SJ. Induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA by refeeding and insulin. *J Biol Chem* 263: 18669–18677, 1988.
11. Dalmau M, Bartrons R, and Gil J. Control of fructose 2,6-bisphosphate metabolism by different mitogenic signals in Swiss 3T3 fibroblasts. *Exp Cell Res* 212: 93–96, 1994.
12. Darville MI, Crepin KM, Vandekerckhove J, Van-Damme J, Octave JN, Rider MH, Marchand MJ, Hue L, and Rousseau GG. Complete nucleotide sequence coding for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone. *FEBS Lett* 224: 317–321, 1987.

13. **Darville MI and Rousseau GG.** E2F-dependent mitogenic stimulation of the splicing of transcripts from an S phase-regulated gene. *Nucleic Acids Res* 25: 2759–2765, 1997.
14. **Diamond I, Legg A, Schneider JA, and Rozengurt E.** Glycolysis in quiescent cultures of 3T3 cells. Stimulation by serum, epidermal growth factor, and insulin in intact cells and persistence of the stimulation after cell homogenization. *J Biol Chem* 253: 866–871, 1978.
15. **Durante P, Gueuning M, Darville MI, Hue L, and Rousseau GG.** Apoptosis induced by growth factor withdrawal in fibroblasts overproducing fructose 2,6-bisphosphate. *FEBS Lett* 448: 239–243, 1999.
16. **El-Maghrabi MR, Correia JJ, Heil PJ, Pate TM, Cobb C, and Pilkis SJ.** Tissue distribution, immunoreactivity, and physical properties of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Proc Natl Acad Sci USA* 83: 5005–5009, 1986.
17. **Gómez-Foix AM, Coats WS, Baqué S, Alam T, Gerard RD, and Newgard CB.** Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. *J Biol Chem* 267: 25129–25134, 1992.
18. **Greiner EF, Guppy M, and Brand K.** Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J Biol Chem* 269: 31484–31490, 1994.
19. **Gutman I and Wahlefeld AW.** In: *Methods of Enzymatic Analysis*, edited by Bergmeyer HU. New York: Verlag, 1974, p. 1464–1468.
20. **Hamilton JA, Callaghan MJ, Sutherland RL, and Watts CKW.** Identification of PRG1, a novel progesterin-responsive gene with sequence homology to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Mol Endocrinol* 11: 490–502, 1997.
21. **Hochachka PW, Buck LT, Doll CJ, and Land SC.** Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA* 93: 9493–9498, 1996.
22. **Hue L and Rider MH.** Role of fructose-2,6-bisphosphate in the control of glycolysis in mammalian tissues. *Biochem J* 245: 313–324, 1987.
23. **Hue L and Rousseau GG.** Fructose-2,6-bisphosphate and the control of glycolysis by growth factors, tumor promoters and oncogenes. *Adv Enzyme Regul* 33: 97–110, 1993.
24. **Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki J, and Oka Y.** Overexpression of hexokinase I but not GLUT1 glucose transporter alters concentration dependence of glucose-stimulated insulin secretion in pancreatic beta-cell line MIN6. *J Biol Chem* 269: 3081–3087, 1994.
25. **Joaquin M, Salvadó C, Bellosillo B, Lange AJ, Gil J, and Tauler A.** Effect of growth factors on the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in Rat-1 fibroblasts. *J Biol Chem* 272: 2846–2851, 1997.
26. **Kacser H and Burns JA.** The control of flux. *Symp Soc Exp Biol* 27: 65–104, 1973.
27. **Lee YH, Okar D, Lin K, and Pilkis SJ.** Mechanism of modulation of rat liver fructose-2,6-bisphosphatase by nucleoside triphosphates. *J Biol Chem* 269: 11002–11010, 1994.
28. **Li L, Ling S, Wu C, Yao W, and Xu G.** Separate bisphosphatase domain of chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: the role of the C-terminal tail in modulating enzyme activity. *Biochem J* 328: 751–756, 1997.
29. **Lucas M.** Stimulation by calcium of glucose uptake and lactate production in pigeon erythrocytes. *Biomed Biochim Acta* 46: 253–257, 1987.
30. **Manzano A, Pérez JX, Nadal M, Estivill X, Lange AJ, and Bartrons R.** Cloning, expression and chromosomal localization of a human testis 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase gene. *Gene* 229: 83–89, 1999.
31. **Manzano A, Roig T, Bermudez J, and Bartrons R.** Effects of taxol on isolated rat hepatocyte metabolism. *Am J Physiol Cell Physiol* 271: C1957–C1962, 1996.
32. **Manzano A, Rosa JL, Ventura F, Pérez JX, Nadal M, Estivill X, Ambrosio S, Gil J, and Bartrons R.** Molecular cloning, expression and chromosomal localization of a ubiquitously expressed human 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase gene (PFKFB3). *Cytogenet Cell Genet* 83: 214–217, 1998.
33. **Miralpeix M, Azcon J, Bartrons R, and Argiles JM.** The impairment of respiration by glycolysis in the Lewis lung carcinoma. *Cancer Lett* 50: 173–178, 1990.
34. **Pilkis SJ, Claus TH, Kurland IJ, and Lange AJ.** 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. *Annu Rev Biochem* 64: 799–835, 1995.
35. **Rosella G, Zajac JD, Kaczmarczyk SJ, Andrikopoulos S, and Proietto J.** Impaired suppression of gluconeogenesis induced by overexpression of a noninsulin-responsive phosphoenolpyruvate carboxykinase gene. *Mol Endocrinol* 7: 1456–1462, 1993.
36. **Sakai A, Kato M, Fukasawa M, Ishiguro M, Furuya E, and Sakakibara R.** Cloning of cDNA encoding for a novel isozyme of fructose-6-phosphate, 2-kinase/fructose 2,6-bisphosphatase from human placenta. *J Biochem (Tokyo)* 119: 506–511, 1996.
37. **Sambrook J, Fritsch EF, and Maniatis T.** *Molecular Cloning: A Laboratory Manual*, edited by Nolan C. Cold Spring Harbor, NY: Cold Spring Harbor, 1989, p. 7.3–7.5.
38. **Schaaff I, Heinisch J, and Zimmermann FK.** Overproduction of glycolytic enzymes in yeast. *Yeast* 5: 285–290, 1989.
39. **Shim H, Chun YS, Lewis BC, and Dang CV.** A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc Natl Acad Sci USA* 95: 1511–1516, 1998.
40. **Simons TJB.** Characterization of sugar transport in the pigeon red blood cells. *J Physiol (Lond)* 338: 477–499, 1983.
41. **Tauler A, Rosenberg AH, Colosia A, Studier FW, and Pilkis S.** Expression of the bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in *Escherichia coli*. *Proc Natl Acad Sci USA* 85: 6642–6646, 1988.
42. **Valera A and Bosch F.** Glucokinase expression in rat hepatoma cells induces glucose uptake and is rate limiting in glucose utilization. *Eur J Biochem* 222: 533–539, 1994.
43. **Van Schaftingen E.** Fructose 2,6-bisphosphate. *Adv Enzymol Relat Areas Mol Biol* 59: 315–395, 1987.
44. **Van Schaftingen E, Lederer B, Bartrons R, and Hers HG.** A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2,6-bisphosphate. *Eur J Biochem* 129: 191–195, 1982.