Effects of overexpression of the liver subunit of 6-phosphofructo-1-kinase on the metabolism of a cultured mammalian cell line

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Overexpression of the liver subunit of 6-phosphofructo-1-kinase in Chinese hamster ovary K1 cells was shown to increase the steady-state level of the enzyme's product, fructose 1,6-bisphosphate, and to produce a small but significant decrease in the concentration of fructose 2,6-bisphosphate, which is an allosteric activator of the enzyme. However, overexpression of the enzyme had no effect on glycolytic flux under a variety of

different substrate conditions. This latter observation is consistent with similar studies in fungi and in potato tubers which indicate that 6-phosphofructo-1-kinase has very little control over flux in glycolysis.

Key words: Down's syndrome, flux, fructose-2,6-bisphosphate, glycolysis, tumours.

INTRODUCTION

6-Phosphofructo-1-kinase (PFK-1; EC 2.7.1.11) is frequently considered to catalyse the 'rate-limiting' step of the glycolytic pathway [1]. This view seems to have arisen for a number of reasons. These include the fact that the reaction is effectively irreversible, and that it is the first unique step in the pathway. The enzyme also displays complex allosteric regulation by effectors and metabolites, the levels of which are controlled by the hormonal and nutritional status of the tissue. For example, the role of fructose 2,6-bisphosphate, which is the most potent allosteric activator of the enzyme in regulating glycolytic and gluconeogenic fluxes in the liver, is well established [2]. There is also, in tumours, a correlation between the high glycolytic flux maintained by these tissues and the activity of the liver isoform of PFK-1 (PFK-L) [3]. Furthermore, the level of fructose 2,6-bisphosphate in tumour cells is often increased, and transformation of cells with retroviruses containing either the *v-src* or *v-fps* oncogene has been shown to increase both the fructose 2,6-bisphosphate concentration and glycolytic flux [4,5]. However, although it is clear that the enzyme is tightly regulated and that there is a correlation between its activity and glycolytic flux, the evidence that it is rate-limiting for glycolytic flux is very poor. For example, in the liver other steps in the glycolytic pathway are also regulated [2], and in tumours there is up-regulation of many of the other glycolytic enzymes in addition to PFK-1 [3,6]. Furthermore, selective increases in the expression of the enzyme in a variety of organisms, using molecular genetic techniques, has failed to produce significant increases in glycolytic flux. These include studies in the yeast *Saccharomyces cerevisiae* [7], in the filamentous fungus *Aspergillus niger* [8] and in the potato (*Solanum tuberosum*) tuber [9]. These latter studies are in accordance with many other studies which have shown that increasing the activity of a single enzyme in a metabolic pathway will only very rarely have a significant effect on flux [10]. These findings are also in agreement with the predictions of Metabolic Control Analysis, that control of flux will be distributed among the enzymes of a pathway, with no single enzyme having a large effect on pathway flux. However, there are, in the case of

PFK-1, several studies in mammalian systems which appear to contradict these specific and more general findings.

In Down's syndrome (trisomy 21), the gene for the liver-type subunit of the enzyme, which is located on the long arm of chromosome 21, is present in three copies [11]. This increase in gene dosage results in a 50 % increase in the specific activity of PFK-1 in erythrocytes [12] and fibroblasts [13] obtained from Down's syndrome patients. The erythrocytes showed an increase in the ratio of the intracellular concentration of fructose 1,6-bisphosphate to that of fructose 6-phosphate, consistent with enhanced PFK-1 activity in the cell [14]. These cells also showed a 20–40 % increase in glycolytic flux, compared with cells from controls, when flux in the pathway was stimulated by treatment of the cells with Methylene Blue [15]. In a series of experiments [16–20], Groner and co-workers have overexpressed, in cells and transgenic mice, some of the genes located on the long arm of chromosome 21, including the gene for PFK-L. Transfection of PC12 cells with a vector expressing PFK-L resulted in clones with ~ 50 % increases in PFK-L activity and ~ 40 % increases in glycolytic flux [16]. Similarly, in transgenic mice overexpressing PFK-L, a 60 % increase in the specific activity of brain PFK-1 was associated with a 58 % increase in the rate of glucose metabolism [19]. These studies led to the remarkable conclusion that PFK-1 has a flux control coefficient for glycolysis that is near to unity in these tissues. Given the disparity between these results and the general expectations of control analysis and the more specific studies on the effects of overexpressing PFK-1 in other organisms, we set out to repeat these experiments in another mammalian cell line.

EXPERIMENTAL

Materials

All chemicals used were of analytical grade and were purchased from BDH (Poole, Dorset, U.K.), Fisher Scientific (Loughborough, Leics., U.K.), Fluka Chemicals (Gillingham, Dorset, U.K.) or Sigma-Aldrich (Poole, Dorset, U.K.). Bio-

Abbreviations used: PFK-1, 6-phosphofructo-1-kinase; PFK-L, liver isoform of PFK-1; PP_i-PFK, PP_i-dependent PFK-1; CHO, Chinese hamster ovary; MSX, methionine sulfoximine; MTT, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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chemicals were obtained from Boehringer Mannheim (Lewes, E. Sussex, U.K.), Sigma, Life Technologies (Paisley, Scotland, U.K.), Fisher or BDH. Enzymes and other materials used in the manipulation of DNA were obtained from Sigma, Boehringer, Amersham Pharmacia Biotech (St. Albans, Herts., U.K.), Promega (Southampton, U.K.), QIAGEN Ltd (Crawley, W. Sussex, U.K.) and Fisher. Tissue culture materials and media were obtained from Becton Dickinson (Oxford, U.K.), Greiner Labortechnik Ltd (Stonehouse, Gloucs., U.K.), Life Technologies and Sera-Lab Ltd (Crawley Down, W. Sussex, U.K.). Media components for bacterial growth were supplied by Difco Laboratories (East Molesley, Surrey, U.K.). D-[3-³H]Glucose (specific radioactivity either 16.8 Ci/mmol or 17.7 Ci/mmol; 1 mCi/ml) was obtained from Nycomed Amersham plc (Amersham, Bucks., U.K.).

Construction of the PFK-1 expression vector (pBF143)

All DNA manipulations were carried out essentially as described in [21]. Human liver-type PFK-1 cDNA (cPFKL3.0 [22]) was excised from plasmid pGcPFKL3.0 by digestion with *Eco*RI and *Xba*I. This cDNA fragment contains all of the coding sequence (2.337 kb), 0.055 kb of the 5'-untranslated region and 0.515 kb of the 3'-untranslated region. After removing the 5'-cohesive termini generated by digestion with *Eco*RI and *Xba*I, the fragment was subcloned into the *Sma*I site of pEE14 (obtained from Dr C. R. Bebbington, Celltech, U.K. [23]) to give plasmid pBF143. This expression vector uses the human CMV (cytomegalovirus) promoter and a glutamine synthetase mini-gene as the selectable marker.

Mammalian cell lines

The anchorage-dependent Chinese hamster ovary (CHO) cell line CHO-K1 (E.C.A.C.C. no. 85051005) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts., U.K.). All other cell lines were derived from this cell line by stable transfection.

Mammalian cell culture

Cultures were grown as monolayers and maintained in exponential-phase growth. Wild-type CHO-K1 cells were maintained in Dulbecco's modified Eagle medium containing 10% (v/v) heat-inactivated foetal calf serum, 4 mM L-glutamine and 25 mM glucose. For the generation of new cell lines, cultures were maintained in transfection medium (GMEM-S). This medium contained 25 mM glucose and 10% (w/v) dialysed foetal calf serum, and was supplemented with glutamate, asparagine and nucleosides [24]. During the selection procedure, cells were grown in transfection medium containing 30 μ M methionine sulphoximine (MSX) (Sigma). For subsequent experiments, cell cultures were typically maintained in a medium referred to as standard growth medium. This glutamine-free medium contained 1 mM sodium pyruvate, 25 mM glucose (unless stated otherwise) and 10% (v/v) heat-inactivated foetal calf serum. All media contained 50 units/ml penicillin and 50 μ g/ml streptomycin. Cells were typically plated at $(1.5\text{--}2) \times 10^4$ cells/cm² and grown for 40–48 h before extraction. All experiments were carried out before the cultures became confluent, and at the time of the experiments all cultures were at identical cell densities (confirmed by protein content and/or cell numbers). Cell counts were performed on cell suspensions, using a haemocytometer. Cell viability was assessed by Trypan Blue dye exclusion.

Generation of cell lines overexpressing PFK-1

Cells were transfected with plasmid pBF143, according to the protocol described by Bebbington [24]. At 1 day after transfection, cells were switched to transfection medium containing 30 μ M MSX. MSX-resistant colonies were screened for increased PFK-1 expression. Clone C27, transfected with pEE14 alone, was used as a control in all experiments.

Determination of protein content

All protein determinations were performed using the Bradford method [25], using a dye reagent concentrate from Bio-Rad and BSA standards.

Determination of PFK-1 activity

The assay conditions were similar to those described in [26]. Cell pellets (approx. 1×10^6 cells) were resuspended in 300 μ l of fresh homogenization buffer containing 50 mM Tris/HCl, pH 8.2, 2 mM dithiothreitol, 2 mM EDTA, 1% (v/v) Triton X-100 and 1 mM fructose 6-phosphate. Activity was determined at 25 °C, by measuring the rate of formation of ADP. The assay mixture contained, in a final volume of 1 ml, 50 mM Tris/HCl (pH 8.0 at 25 °C), 100 mM KCl, 2 mM dithiothreitol, 0.1 mM EDTA, 20 mM (NH₄)₂SO₄, 1 mM fructose 6-phosphate, 1 mM ATP, 3 mM MgCl₂, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 4 units of pyruvate kinase, 10 units of lactate dehydrogenase and 50 μ l of cell homogenate, containing between 20 and 30 μ g of protein. The assay was initiated by the addition of ATP.

Determination of growth rates

Cells were seeded at 0.5×10^4 cells/cm² in 6-well plates and grown for approx. 36 h in 2 ml of standard growth medium. The number of viable cells in the culture was then determined at different times, using the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [27] (Sigma).

Determination of flux through PFK-1 *in vivo*

Flux was determined by measuring the detritiation of [3-³H]-glucose [28]. Cells were seeded in 140 mm-diameter culture dishes and grown for approx. 24 h in 30 ml of growth medium supplemented with 10% (v/v) foetal calf serum. The growth medium was then removed and, after washing the monolayers with PBS, replaced with 30 ml of the same medium (unless stated otherwise). All cultures were grown for a further ~20 h, before determination of flux through PFK-1. The experiment was initiated by quickly removing the medium and adding 18 ml of fresh medium supplemented with tracer [3-³H]glucose (giving a final specific radioactivity for glucose in the medium of approx. 16 nCi/ μ mol). Samples of incubation medium were removed every 20 min for a total of 100 min and treated with HClO₄, and precipitated protein and cell debris were removed by centrifugation. The supernatants were neutralized (pH 7–8) with a 2 M KOH solution containing 0.5 M triethanolamine, and precipitated KClO₄ was removed by centrifugation. Tritiated water was separated from the labelled glucose by column chromatography [29]. Samples were applied to a semi-micro chromatography column (0.8 cm $\times$ 4 cm; 2 ml bed volume; Bio-Rad) filled with Amberlite IRA-420 (chloride form; wet mesh: 100–200; Sigma), converted into the borate form by batch treatment. After elution, columns were washed with 2 ml of water, and 0.2 ml portions of the eluate were mixed with 2 ml of scintillation fluid (OptiPhase 'HiSafe' 2; Fisher) and the radioactivity was counted using a Beckman LS 3801 scintillation

counter (Beckman Instruments, Fullerton, CA, U.S.A.). To determine protein content, the cell monolayers were washed twice with PBS and then overlaid with 3 ml of a 1% (v/v) Triton X-100 solution and left at 4 °C for at least 2 h. The cellular material was scraped into the Triton X-100 solution and, after homogenization, cell suspensions were centrifuged to remove any insoluble material. Protein levels were determined in the supernatants.

Determination of concentrations of glycolytic intermediates

Concentrations were determined spectrophotometrically in neutralized, protein-free HClO_4 extracts using standard enzymic methods. During the preparation of cell extracts, culture dishes were kept on ice and all steps were carried out at 4 °C.

Cell extraction

Cell monolayers, which had been washed twice with ice-cold PBS, were extracted with an ice-cold 0.5 M HClO_4 solution. After centrifugation to remove precipitated protein and insoluble cell debris, the pH of the clear supernatants was adjusted to 4–6 with a 5 M potassium carbonate solution. After a 5 min incubation on ice, samples were centrifuged to remove the precipitated KClO_4 . The supernatants were frozen in liquid nitrogen and stored at –80 °C until assayed. To determine protein levels, protein precipitates were dissolved in 0.5 ml of a 1 M NaOH solution overnight at 4 °C.

Triose phosphates and fructose 1,6-bisphosphate

The above compounds were determined as described in [30]. The assay mixture contained, in a final volume of 1 ml, 0.2 M triethanolamine (pH 7.6), 20 mM EDTA, 1.7 mM NADH and a deproteinized sample corresponding to 3.5–4.5 mg of total cell protein. The concentrations of triose phosphates and fructose 1,6-bisphosphate were determined by measuring the change in A_{340} following sequential additions of glycerol-3-phosphate dehydrogenase (0.3 unit/ml) plus triose phosphate isomerase (4 units/ml) or aldolase (0.4 unit/ml) respectively. The triose phosphate concentrations quoted are the sums of the dihydroxyacetone phosphate and glyceraldehyde 3-phosphate concentrations.

Glucose 6-phosphate

Glucose 6-phosphate was determined as described in [31]. The assay mixture contained, in a final volume of 1 ml, 0.2 M triethanolamine (pH 7.6), 5 mM MgCl_2 , 0.2 mM NADP^+ and a deproteinized sample corresponding to 2.5–3.0 mg of total cell protein. The level of glucose 6-phosphate was determined by measuring the change in A_{340} following the addition of 0.9 unit of glucose 6-phosphate dehydrogenase.

Fructose 2,6-bisphosphate assay

The assay is based on the ability of fructose 2,6-bisphosphate to stimulate the activity of PP_i -dependent PFK-1 (PP_i -PFK) from potato tubers [32]. Cell monolayers were extracted using an ice-cold 50 mM NaOH solution containing 1% (v/v) Triton X-100. The cellular material was scraped from the dishes, and the resulting alkali extracts were heated for 20 min at 80 °C. PP_i -PFK activity was determined at 25 °C, in the presence of 50 mM Tris/acetate, pH 7.8, 2 mM magnesium acetate, 0.12 mM NADH, 1.8 mM dithiothreitol, 1 mM fructose 6-phosphate, 0.5 mM pyrophosphate, 1.8 units of glycerol-3-phosphate de-

hydrogenase, approx. 18 units of triose phosphate isomerase, 1.8 units of aldolase and 0.04 unit of PP_i -PFK, in a final volume of 1 ml. The fructose 6-phosphate was treated to remove any contaminating fructose 2,6-bisphosphate, as described in [33]. Samples of the cell extracts were added to the assay; after a 10 min preincubation, the assay was started by the addition of PP_i , and the rate of oxidation of NADH was followed for 2–3 min. A saturating amount of fructose 2,6-bisphosphate (2 μM) was then added, and the maximum PP_i -PFK activity under the experimental conditions employed was determined. Results, expressed as a percentage of the maximum rate, were converted into pmol of fructose 2,6-bisphosphate using a calibration curve.

Presentation of results

Enzyme activities, metabolite and protein concentrations, growth rates and fluxes through PFK-1 were determined in experiments in which the control cell line and the transformants were cultured and assayed in parallel. One unit of PFK-1 activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of fructose 1,6-bisphosphate in 1 min, under the assay conditions described. The statistical significance of the differences from the control was assessed using Student's *t* test.

RESULTS

Cell lines expressing the human liver-type subunit of PFK-1 were generated by transfection with plasmid pBF143 (see the Experimental section). After 2 weeks in selective medium, 42 clones that were resistant to 30 μM MSX were isolated. When screened for increased PFK-1 expression, 14 clones were found to overexpress PFK-1, with PFK-1 activities ranging between 1.2 and 3.8 times greater than the activity determined in a control cell line, C27, which had been transfected with the empty vector. The control cell line and three PFK-1-overexpressing cell lines were selected for examination and, unless otherwise specified, experiments were performed within 11 passages after transfection. The activity of PFK-1 in the control cell line was comparable with that reported in a PC12 cell line, where an increase in PFK-1 activity was reported previously to result in an increase in glycolytic flux [16].

PFK-1 activities in cell lines C27, PF25, PF36 and PF40

PFK-1 activities in cell-free extracts prepared from cell lines PF25, PF36 and PF40 were 3.8-, 3.4- and 2.3-fold higher respectively than in the control cell line, C27 (Table 1). The activities measured after 15 passages were similar to those

Table 1 PFK-1 activities in control (C27) and PFK-1 overexpressing cell lines

PFK-1 activities were determined in crude cell extracts as described in the Experimental section. Each extract was assayed twice, and the two values, typically differing by less than 10%, were averaged. Values are from one representative experiment (out of five independent experiments for cell line C27, four for cell line PF25, two for cell line PF36 and one for cell line PF40) and represent means $\pm$ S.D. for three different cell cultures. Significance of differences from control cell line: * $P < 0.02$, ** $P < 0.005$.

Cell line	PFK-1 activity (units/mg of protein)
C27 (control)	0.067 $\pm$ 0.002
PF25	0.25 $\pm$ 0.02*
PF36	0.23 $\pm$ 0.03*
PF40	0.152 $\pm$ 0.009**

Table 2 Doubling times for the control cell line (C27) and three cell lines overexpressing PFK-1

The number of cells in culture was assessed at different times, over a period of 36 h, by the MTT colorimetric method, as described in the Experimental section. Doubling times were calculated by plotting the logarithm of MTT formazan absorbance against time. Values were calculated using linear regression. The correlation coefficients (*r*) for the doubling times varied between 0.973 and 0.997, showing that during the experiment the cells were in exponential growth. For each time point, three different cell cultures were assayed in parallel and the results averaged. Data are from one independent experiment (for cell lines PF36 and PF40) or two independent experiments (for cell lines C27 and PF25). Values in parentheses are from the second experiment.

Cell line	Doubling time (h)
C27 (control)	11.8 (10.5)
PF25	11.1 (10.7)
PF36	11.0
PF40	11.2

measured in cells within 11 passages after transfection (results not shown), thus confirming that integration of plasmid pBF143 was stable under the experimental conditions employed.

Effects of PFK-1 overexpression on cell growth rates

Overexpression of PFK-1 had no significant effect on the growth rate of CHO-K1 cells. The doubling times for the control cell line and the three PFK-1-overexpressing cell lines were essentially the same (Table 2), and are in good agreement with the values reported in the literature for wild-type CHO-K1 cells [34].

Effects of PFK-1 overexpression on glycolytic flux

Glucose consumption was estimated by measuring the production of $^3\text{H}_2\text{O}$ from $[3\text{-}^3\text{H}]\text{glucose}$. Loss of ^3H to water occurs in the reactions catalysed by aldolase and triose phosphate isomerase, and therefore the rate of detritiation is not strictly equivalent to glycolytic flux. However, the rate of detritiation of $[3\text{-}^3\text{H}]\text{glucose}$ in hepatocytes, at glucose concentrations of less than 20 mM, has been shown to be similar to the rate of lactate production in the perfused liver [28]. Therefore we have assumed that the rate of detritiation of $[3\text{-}^3\text{H}]\text{glucose}$ is a reasonable measure of glycolytic flux and is, at the very least, a measure of flux through the hexose part of the pathway. This flux can be

expected to be more sensitive to changes in PFK-1 activity, since it depends on fewer steps in the glycolytic pathway.

Initial experiments were performed using standard growth medium [25 mM glucose and 10% (v/v) foetal calf serum]. No significant differences could be detected in the rates of $[3\text{-}^3\text{H}]\text{glucose}$ detritiation by the control cell line (C27) and by cell line PF25 (Table 3). These rates are high compared with those measured in isolated hepatocytes [28]. However, this was expected, since cultured cells tend to be very glycolytic, oxidizing very little glucose carbon via the citric acid cycle [35,36]. For instance, in wild-type Chinese hamster fibroblasts, more than 97% of the glucose consumed is converted into lactate [37,38].

The flux control coefficient displayed by an enzyme is a systemic property and will depend on a number of factors, including the activities of the other enzymes in the pathway and the pathway flux [10]. Therefore we considered the possibility that, under these conditions, the flux control coefficient of PFK-1 might be very low. To investigate this we examined the effects of overexpressing PFK-1 in cells grown under conditions where a change in glycolytic flux could be expected. CHO-K1 cells cultured in the absence of foetal calf serum exhibited $[3\text{-}^3\text{H}]\text{glucose}$ detritiation rates that were approx. 30% lower than those of CHO-K1 cells grown in the presence of 10% (v/v) foetal calf serum. When cells were cultured both in the absence of serum and in the presence of much lower concentrations of glucose, no further decrease in the $[3\text{-}^3\text{H}]\text{glucose}$ detritiation rate was observed. Glucose and glutamine may be used as both energy and carbon sources by cells in culture, and it has been reported that glucose utilization can decrease as the concentration of glutamine in the growth medium is increased [39,40]. Supplementation of the medium with glutamine, in the presence or absence of serum, also lowered the rate of $[3\text{-}^3\text{H}]\text{glucose}$ detritiation. However, under all of these different substrate conditions, there was no difference in the rate of $[3\text{-}^3\text{H}]\text{glucose}$ detritiation between control cells (C27) and cells overexpressing PFK-1 (PF25) (Table 3).

Effects of PFK-1 overexpression on concentrations of glycolytic intermediates

The concentration of fructose 1,6-bisphosphate was significantly higher (approx. 30%) in a cell line overexpressing PFK-1 (PF25) compared with the control cell line (C27) ($P < 0.05$). This cell

Table 3 Rates of $[3\text{-}^3\text{H}]\text{glucose}$ detritiation under different growth conditions for the control cell line (C27) and for a cell line overexpressing PFK-1 (PF25)

The formation of $^3\text{H}_2\text{O}$ was determined in subconfluent cultures, as described in the Experimental section. Rates were calculated using linear regression. The correlation coefficients (*r*) for glucose consumption varied between 0.975 and 0.999, showing that there was a constant rate of glucose detritiation over the 100 min incubation period. For each growth condition, values are from one representative experiment (out of three independent experiments for cells grown under standard growth conditions and two independent experiments for serum-starved cells in the presence of 25 mM glucose). Values represent means $\pm$ S.D. for three different cell cultures, established and assayed in parallel. Significance of differences from cells cultured in standard growth medium (experiment 1): * $P < 0.05$, ** $P < 0.02$. For each of the five growth conditions described, no significant differences were observed between the control cell line (C27) and the cell line overexpressing PFK-1 (PF25).

Growth conditions			Rate of $[3\text{-}^3\text{H}]\text{glucose}$ detritiation (nmol/min per mg of protein)	
Experiment	Glucose (mM)	Foetal calf serum (% v/v)	C27	PF25
In the absence of glutamine†				
1	25	10	16 $\pm$ 1	15.9 $\pm$ 0.5
2	25	0	11.7 $\pm$ 0.2**	11 $\pm$ 2*
3	5.6	0	13.5 $\pm$ 0.9	14 $\pm$ 1
In the presence of 4 mM glutamine				
4	25	10	13.5 $\pm$ 0.8	12.2 $\pm$ 0.6
5	25	0	11 $\pm$ 2	11 $\pm$ 1*

† Excluding any traces of glutamine present in the dialysed foetal calf serum.

Table 4 Concentrations of glycolytic intermediates in the control cell line (C27) and in a cell line overexpressing PFK-1 (PF25)

Metabolite concentrations were determined in HClO₄ extracts as described in the Experimental section. Values represent means $\pm$ S.D. for four different cell cultures. Significance of difference from control cell line: * $P < 0.05$. All cell cultures were established, extracted and assayed in parallel. The triose phosphate concentration is the sum of the concentrations of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

Metabolite	Metabolite concentration (nmol/mg of protein)	
	C27	PF25
Fructose 1,6-bisphosphate	1.0 $\pm$ 0.1	1.3 $\pm$ 0.1*
Triose phosphates	0.7 $\pm$ 0.1	0.9 $\pm$ 0.1
Glucose 6-phosphate	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1

Table 5 Concentrations of fructose 2,6-bisphosphate in the control cell line (C27) and in three cell lines overexpressing PFK-1 (PF25, PF36 and PF40)

Levels of fructose 2,6-bisphosphate were determined in cell extracts as described in the Experimental section. For experiments 1 and 2 the cells were grown in standard growth medium and each extract was assayed twice and the two values (typically differing by less than 10%) were averaged. In experiment 3 the cells were cultured in the absence of serum. The values shown are the means $\pm$ S.D. for the specified number of independent cell cultures. Significance of differences with respect to the corresponding control cell line: ** $P < 0.02$, *** $P < 0.005$. All cell cultures were established, extracted and assayed in parallel. n.d., not determined.

Experiment	Fructose 2,6-bisphosphate levels (pmol/mg of protein)			
	C27	PF25	PF36	PF40
1 ($n = 5$)	66 $\pm$ 4	43 $\pm$ 2***	57 $\pm$ 4**	48 $\pm$ 3***
2 ($n = 4$)†	60 $\pm$ 1	52.5 $\pm$ 0.9***	n.d.	54 $\pm$ 2**
3 ($n = 10$)	33 $\pm$ 5	25 $\pm$ 3***	n.d.	n.d.

† Except for C27 where $n = 3$.

line also exhibited higher levels of triose phosphates (approx. 30 % higher) and lower levels of glucose 6-phosphate, although these latter differences were not statistically significant (Table 4).

Qualitatively similar results to these were obtained following overexpression of *Escherichia coli* PFK-1 in potato tubers [9]. However, in potato tubers the much larger increase in PFK-1 activity had more significant effects on the concentrations of glycolytic intermediates, with the levels of most metabolites downstream of PFK-1 showing much larger increases in concentration, and upstream metabolites showing significant decreases in concentration.

Effect of PFK-1 overexpression on fructose 2,6-bisphosphate concentration

Levels of fructose 2,6-bisphosphate were determined in the control cell line and in the three cell lines overexpressing PFK-1 (Table 5). Although the experiments were carried out under similar conditions, there were significant variations in the levels of fructose 2,6-bisphosphate between experiments. However, the levels of fructose 2,6-bisphosphate were always lower in all cell lines overexpressing PFK-1 than in the control cell line. Even where the decreases in the levels of fructose 2,6-bisphosphate were very small (less than 15 %), the differences were nevertheless statistically significant. Levels of fructose 2,6-bisphosphate were also determined in cell lines C27 and PF36 that had been serum-starved for 17 h. Again there was a small but statistically significant difference between the control cell line and the cell line overexpressing PFK-1, with cell line PF36 exhibiting levels of

fructose 2,6-bisphosphate approx. 25 % lower than those of C27 (Table 5).

DISCUSSION

Increasing PFK-1 activity in CHO-K1 cells by up to a factor of 3.8 over that found in control cells had no effect on cell growth rate or glucose utilization (Tables 2 and 3). The flux control coefficient of PFK-1 for glycolytic flux, and therefore the extent to which PFK-1 overexpression could affect glucose utilization, might depend on the absolute value of this flux. Therefore we tried to decrease glycolytic flux by removing serum from the medium, by lowering the glucose concentration and by supplementing the medium with glutamine. Both serum removal and glutamine supplementation produced a significant decrease in glucose utilization. However, under all of these substrate conditions, overexpression of PFK-1 had no effect on glucose utilization (Table 3).

The overexpressed PFK-1 was demonstrated to be functional in the cells by the increased steady-state levels of fructose 1,6-bisphosphate observed compared with control cells. There was also a slight increase in the levels of the triose phosphates, but this was not statistically significant (Table 4). Similar changes in metabolite concentrations were observed in potato tubers overexpressing *E. coli* PFK-1 [9], although the increases in PFK-1 activity and the increases in the levels of glycolytic intermediates downstream of PFK-1 were considerably larger in the potato. The study on potato tubers also showed that the extent of the increase in the steady-state concentrations of glycolytic intermediates downstream of PFK-1 decreased down the pathway. A simulation of the pathway showed that 'near-equilibrium' enzymes downstream of PFK-1, i.e. those enzymes from aldolase to enolase, would respond passively to activation of an upstream enzyme by increasing their rates of activity and simultaneously transmitting an increased level of metabolites to downstream enzymes [41]. However, the simulation showed that there would be a significant attenuation of this effect down the pathway, in agreement with what was observed experimentally. Thus in the CHO-K1 cells studied here, the modest increases in steady-state fructose 1,6-bisphosphate and triose phosphate concentrations are unlikely to be communicated to downstream metabolites in the pathway, and are therefore unlikely to lead to significantly increased flux in the lower half of the pathway.

We showed previously in the yeast *S. cerevisiae* that a 5-fold increase in PFK-1 activity had no effect on glycolytic flux in anaerobic cells or, in contrast with what was observed in the present study, the steady-state level of the enzyme's product, fructose 1,6-bisphosphate. In yeast there was, instead, a decrease in the steady-state level of the allosteric activator of PFK-1, fructose 2,6-bisphosphate [7]. We suggested that this would decrease the specific activity of the enzyme, thus compensating, at least semi-quantitatively, for the increased concentration of the enzyme, and that this accounted for the absence of a change in flux through the enzyme and in the fructose 1,6-bisphosphate concentration. A similar decrease in the fructose 2,6-bisphosphate concentration was observed following overexpression of PFK-1 in *A. niger* [8]. Subsequent work in *S. cerevisiae* indicated that the decrease in the fructose 2,6-bisphosphate concentration was due to a decrease in the concentration of 6-phosphofructo-2-kinase [42]. We have observed a similar phenomenon in the CHO-K1 cells studied here, where overexpression of PFK-1 led to a small, but consistent, decrease in the steady-state level of fructose 2,6-bisphosphate (Table 5). The calculated intracellular concentration of this metabolite is between 5 and 12 μ M, assuming that there is 0.64 ml of intracellular water/120 mg of

cell protein [35]. These concentrations fall within the range where they would have a significant effect on PFK-1 activity [43]. However, in marked contrast with what we found in yeast, this was not sufficient to compensate totally for the increase in PFK-1 concentration, as we still observed an increase in the steady-state fructose 1,6-bisphosphate concentration. Whether the decrease in the fructose 2,6-bisphosphate concentration was due to a decrease in 6-phosphofructo-2-kinase activity or to a decrease in the level of its substrate, fructose 6-phosphate, was not determined. However, we were unable to detect a significant change in the glucose 6-phosphate concentration in cells overexpressing PFK-1, and therefore presumably there was little change in the fructose 6-phosphate concentration.

These studies on PFK-1 overexpression in CHO-K1 cells are in accordance, therefore, with the previous studies in *S. cerevisiae*, *A. niger* and *S. tuberosum* [7–9], which showed that overexpression of PFK-1 had little or no effect on glycolytic flux, and with mathematical modelling studies of erythrocyte glycolysis which indicated that PFK-1 has a relatively low flux control coefficient [14]. These data are also in agreement with the more general finding that overexpression of an individual enzyme in any metabolic pathway is unlikely to have a significant effect on pathway flux [10,44]. This observation is predicted by Metabolic Control Analysis, which shows that only relatively small increases in flux can be achieved even by manifold overexpression of enzymes with relatively high flux-control coefficients. In order to effect large increases in pathway flux, the activities of several enzymes need to be increased simultaneously. This was demonstrated experimentally in *S. cerevisiae*, where a large increase in tryptophan synthesis was achieved only by simultaneous overexpression of five enzymes, when increased expression of between one and four enzymes produced relatively poor results [45]. This requirement for increasing the activity of several enzymes in order to increase pathway flux is echoed in the long-term response of the glycolytic pathway to tissue hypoxia. Hypoxia, which increases glycolytic flux in normal and tumour tissues, increases not only the concentrations of hexokinase, PFK-1 and pyruvate kinase, which are traditionally thought to control flux in the pathway, but also the concentrations of the other enzymes [6,46]. This latter group are generally thought to catalyse reactions which are near to equilibrium in the cell, and to play no part in controlling flux. This co-ordinate increase in the concentrations of the glycolytic enzymes in hypoxic tissues is mediated by the hypoxia-inducible transcription factor, HIF-1 [46].

Even if PFK-1 were catalysing a truly rate-limiting step in the glycolytic pathway, there is no reason to expect, *a priori*, that increasing its activity will increase flux in the glycolytic pathway unless there is a corresponding increase in demand for glycolytically derived ATP or for glycolytic intermediates for biosynthesis. In yeast we did observe a small but significant increase in glycolytic flux when we overexpressed PFK-1 in aerobic cells. However, this occurred at the expense of a decrease in oxygen consumption, such that the overall calculated rate of ATP turnover in the cell remained unchanged [7].

What, then, is the explanation for the metabolic changes observed in the tissues of Down's syndrome patients [12,13,15] and in the previous studies that have characterized the effects of overexpressing PFK-1 in cultured mammalian cells [16] and in transgenic mice [18–20]? In Down's syndrome, many genes will be overexpressed, and therefore it is impossible to assign any of the metabolic effects observed specifically to the overexpression of PFK-1. The increased glycolytic flux in PC12 cells overexpressing transfected PFK-1 suggests that there was an increased demand for ATP in these cells [16]. Subsequent studies on these cells showed elevated levels of protein kinase C [17]. The

activation of such a promiscuous protein kinase could have pleiotropic effects on cell activities, and it could be these that are responsible for an increased demand for ATP and the consequent increase in glycolytic flux. Overexpression of PFK-1 in the brains of transgenic mice was shown to result in enhanced glucose utilization [19]. However, it was not possible in that study to show whether this was due to an increased rate of glycolysis. Even if there were an increase in glycolytic flux, it would still not be possible to assign this unequivocally to the effect of increased PFK-1 activity in the brain. These animals also showed an impairment of glucose-induced insulin secretion as a result of PFK-1 overexpression in the pancreatic islets [20]. This effect on insulin secretion, and possibly other systemic effects resulting from PFK-1 overexpression in other tissues, could be responsible for the observed changes in glucose handling by the brain.

In conclusion, we have shown that overexpression of PFK-1 in CHO-K1 cells increases the steady-state level of its product, fructose 1,6-bisphosphate, but has no effect on glycolytic flux. This finding accords with similar studies on the overexpression of PFK-1 in other systems [7–9], and the finding that, in order to obtain a significant increase in flux in a metabolic pathway, the activity of several enzymes must be increased [10,45]. This is also consistent with the expectation that flux will not increase unless there is an increased demand for glycolytically derived ATP or for glycolytic intermediates. In those cases where there is evidence for an effect of PFK-1 overexpression on glycolytic flux, this is probably a secondary consequence of an increased demand for ATP, which has been shown to exert significant control over glycolytic flux [47,48].

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