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*

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6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase has been postulated to be a metabolic signaling enzyme, which acts as a switch between glycolysis and gluconeogenesis in mammalian liver by regulating the level of fructose 2,6-bisphosphate. The effect of overexpressing the bifunctional enzyme was studied in FAO cells transduced with recombinant adenoviral constructs of either the wild-type enzyme or a double mutant that has no bisphosphatase activity or protein kinase phosphorylation site. With both constructs, the mRNA and protein were overexpressed by 150- and 40-fold, respectively. Addition of cAMP to cells overexpressing the wild-type enzyme increased the S0.5 for fructose 6-phosphate of the kinase by 1.5-fold but had no effect on the overexpressed double mutant. When the wild-type enzyme was overexpressed, there was a decrease in fructose-2,6-bisphosphate levels, even though 6-phosphofructo-2-kinase maximal activity increased more than 22-fold and was in excess of fructose-2,6-bisphosphatase maximal activity. The kinase: bisphosphatase maximal activity ratio was decreased, indicating that the overexpressed enzyme was phosphorylated by cAMP-dependent protein kinase. Overexpression of the double mutant resulted in a 28-fold increase in kinase maximal activity and a 3-4-fold increase in fructose 2,6-bisphosphate levels. Overexpression of this form inhibited the rate of glucose production from dihydroxyacetone by 90% and stimulated the rate of lactate plus pyruvate production by 200%. In contrast, overexpression of the wild-type enzyme enhanced glucose production and inhibited lactate plus pyruvate production. These results provide direct support for fructose 2,6-bisphosphate as a regulator of gluconeogenic/glycolytic pathway flux and suggest that regulation of bifunctional enzyme activities by covalent modification is more important than the amount of the protein.

Gluconeogenic/glycolytic pathway flux is regulated by allosteric effectors and by covalent modification of key regulatory enzymes and/or by modulation of gene expression of these enzymes (1, 2). 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 (3). Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6-PF-2-K/Fru-2,6-P₂ase)1 is a regulatory enzyme in the gluconeogenic/glycolytic pathway, which catalyzes both the synthesis and degradation of the signal molecule, Fru-2,6-P₂, an allosteric activator of 6-phosphofructo-1-kinase and an inhibitor of fructose-1,6-bisphosphatase (4–6). The enzyme has been postulated to provide a switching mechanism between the glycolytic and gluconeogenic pathways in liver (7). The kinase and bisphosphatase activities are reciprocally regulated by cAMP-dependent protein kinase-catalyzed phosphorylation (1). Gene expression of the enzyme is subject to multihormonal regulation; insulin and glucocorticoids enhance and cAMP suppresses gene transcription (1). Therefore, the level of Fru-2,6-P₂ and, ipso facto, glycolytic and gluconeogenic flux depend in a complex way on hormonal milieu.

A number of questions with regard to the bifunctional enzyme and metabolic pathway flux remain unanswered: 1) what is the relative importance of covalent modification and the concentration of bifunctional enzyme protein in controlling Fru-2,6-P₂ levels; 2) what are the relative roles of the kinase and bisphosphatase activities in determining the level of Fru-2,6-P₂; and 3) what is the role of Fru-2,6-P₂ in determining gluconeogenic pathway flux? It was the object of this study to address these questions by using recombinant adenovirus to overexpress wild-type 6-PF-2-K/Fru-2,6-P₂ase and a double mutant (S32A/H258A) of the protein, which only possesses 6-PF-2-K activity and cannot be down-regulated by cAMP-dependent protein kinase-catalyzed phosphorylation. Mutation of Ser-32 to Ala prevents cAMP-dependent phosphorylation of the enzyme (8), while mutation of His-258 to alanine abolishes bisphosphatase activity, since this residue is known to mediate catalysis via a phosphohistidine intermediate (9, 10).

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1 6-PF-2-K/Fru-2,6-P₂ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase or bifunctional enzyme; Fru-2,6-P₂, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; FBS, fetal bovine serum; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; n.s., not significant; 8-CPT-cAMP, sodium cacodylate and 8-(4-chlorophylthio)adenosine 3'-5' cyclic AMP; kb, kilobase(s).

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they are capable of producing glucose from 3-carbon precursors (11–14), cultured rat hepatoma FAO cells were chosen to overexpress the enzyme forms. Thus, the switching mechanism between glycolysis and gluconeogenesis, hypothesized for the bifunctional enzyme and Fru-2,6-P₂ can be studied.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640, Dulbecco's modified Eagle's medium, and FBS were obtained from Life Technologies, Inc. Stat-60 reagent was from Tel-Test “B”, Inc. (Friendswood, TX). Gene Screen membrane was obtained from DuPont NEN, and Immobilon PVDF from Millipore Corp. The BlueScript SK⁺ plasmid was purchased from Stratagene (San Diego, CA). All enzymes and chemicals were obtained from Sigma. Secondary antibody and color reagents were purchased from Boehringer-Mannheim.

Cell Culture—Monolayer cultures of rat FAO hepatoma-derived cells (14) were grown in RPMI 1640 medium (11 mM glucose) supplemented with 10% FBS. All experiments were carried out before the cells became confluent. The Ad-E1A-transformed human embryonic kidney cell line 293 (15) was cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Preparation of Recombinant Adenovirus—Adenoviral vectors were prepared using cDNAs coding for wild-type and mutant forms of rat liver 6-PF-2-K/Fru-2,6-P₂-ase, including a S32A mutant (8) and a H258A mutant (10). Two HindII restriction sites were engineered at the 5' and 3' ends of the cDNA by polymerase chain reaction: one 172 base pairs upstream of the ATG site and another 1511 base pairs downstream of the 3'-untranslated region. Recombinant adenoviruses containing the cDNA encoding the wild-type or mutant 6-PF-2-K/Fru-2,6-P₂-ase mRNA and protein levels, activities, Fru-2,6-P₂, and production of glucose, lactate, and pyruvate were measured.

The experiments were performed 48 h after the viral incubation and repeated 5–10 times. The efficiency of adenovirus-mediated gene transfer was approximately 70% as measured by immunocytochemistry and with a recombinant adenovirus containing the bacterial β-galactosidase gene (data not shown). The survival of the FAO hepatoma cell line was unaffected by incubation of cells with the different adenovirus constructs since the dry mass of the attached cells in 55 cm² plates was the same after 48 h in treated or untreated cell plates (untreated, 6.5 ± 0.5 mg; AdWT, 6.7 ± 0.7 mg; Ad-PF2KWT, 6.8 ± 0.8 mg; Ad-PF2Kmut, 6.8 ± 0.3 mg; n = 5 for each group).

RNA Extraction and Northern Blot Analysis—Total RNA was prepared from plated FAO cells by extraction with Stat-60 following the protocol of the manufacturer. RNA was denatured, electrophoresed, and transferred to a nylon membrane (Gene Screen) as described by Hod et al. (20). A 1.4-kb EcoRI fragment of rat liver 6-PF-2-K/Fru-2,6-P₂-ase cDNA and a 2-kb PstI fragment of chicken brain β-actin cDNA (21) were labeled with [α-32P]dCTP by the random primer method (22). Northern blots were hybridized with the labeled probe and washed as described previously (23). Autoradiographs were scanned on a Bio-Rad imaging densitometer, model GS-670, and analyzed with the Bio-Rad Molecular Analyst software package. The β-actin mRNA was used to normalize the overexpressed 6-PF-2-K/Fru-2,6-P₂-ase mRNA.

Ribonuclease Protection Assay—An RNA probe was designed to distinguish the wild-type from the mutant form (S32A/H258A) of rat liver 6-PF-2-K/Fru-2,6-P₂-ase mRNA. The 0.5-kb EcoRI fragment previously described (16) was cloned into the EcoRI restriction site of the plasmid Bluescript SK⁺. The plasmid was linearized by digestion with BglII and was used to synthesize antisense RNA using its T3 RNA polymerase. The synthesized riboprobe was 297 bases long, 230 bases of which were complementary to the rat liver 6-PF-2-K/Fru-2,6-P₂-ase mRNA, including the DNA region mutated to code for an Ala instead of Ser at position 32. The wild-type 6-PF-2-K/Fru-2,6-P₂-ase mRNA would protect a 230-base fragment, and the mutant (S32A/H258A) would protect two fragments of 179 and 49 bases. Liver and skeletal muscle forms of mRNA were used as size standards. The ribonuclease protection assay was performed as described previously (24, 25).

Enzyme Extractions and Western Blot Analysis—6-PF-2-K/Fru-2,6-P₂-ase expression and/or on the activities of overexpressed enzymes. In each experiment, 6-PF-2-K/Fru-2,6-P₂-ase mRNA and protein levels, activities, Fru-2,6-P₂, and production of glucose, lactate, and pyruvate were measured.
P<sub>2</sub>ase protein was extracted in homogenizing buffer (20 mM TES, pH 7.8, 10 mM KC1, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride and 2.5 mg/liter leupeptin) and concentrated by precipitation with 65% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate was dissolved in 20 mM TES, pH 7.4, containing 20 mM dihydroxyacetone, 20 mM lactate plus pyruvate, and 5% CO<sub>2</sub> in 3 ml of Krebs bicarbonate buffer at pH 7.4 (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 24 mM NaHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>) containing 20 mM dihydroxyacetone, 20 mM lactate plus pyruvate as a gluconeogenic substrate, or 30 mM glucose. A volume of 1.8 ml of incubation medium was taken and acidified with HClO<sub>4</sub> (5%, w/v) and then neutralized as described previously by Argaud et al. (30, 31). At the end of the incubation, the cells were washed, scraped, and dried for 12 h at 180 °C for dry mass determination. Glucose, lactate, and pyruvate concentrations were measured spectrophotometrically using standard enzymatic methods (32) in neutralized protein-free extracts. Glucose, lactate, and pyruvate production are reported as nmol/plate in 0.1 μl NaOH and measured using the 6-phosphofructo-1-kinase activation assay (28).

Measurements of Gluconeogenesis and Glycolysis—Gluconeogenesis, lactate, and pyruvate production was measured in 55-cm<sup>2</sup> plates of FAO cells that were incubated from 30 min to 3 h at 37 °C with atmospheric air, 5% CO<sub>2</sub> in 3 ml of Krebs bicarbonate buffer at pH 7.4 (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 24 mM NaHCO<sub>3</sub>, 2.4 mM CaCl<sub>2</sub>) containing 20 mM dihydroxyacetone, 20 mM lactate plus pyruvate as a gluconeogenic substrate, or 30 mM glucose. A volume of 1.8 ml of incubation medium was taken and acidified with HClO<sub>4</sub> (5%, w/v) and then neutralized as described previously by Argaud et al. (30, 31). At the end of the incubation, the cells were washed, scraped, and dried for 12 h at 180 °C for dry mass determination. Glucose, lactate, and pyruvate concentrations were measured spectrophotometrically using standard enzymatic methods (32) in neutralized protein-free extracts. Glucose, lactate, and pyruvate production are reported as nmol/plate in 55-cm<sup>2</sup> plate (2 × 10<sup>7</sup> cells). All of the metabolic results are expressed as means ± S.E., and comparisons were made using Student’s t test for unpaired samples.

### RESULTS

**Overexpression of the Wild-type and Mutated Form of Rat Hepatic 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase—The levels of 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA and protein in FAO cells were quantified 48 h after treatment of FAO cells with Ad-PF2KW, Ad-PF2KMut, or wild-type adenovirus 5 (AdWT). As described previously (25), FAO cells express low levels of the liver form of 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA, and treatment with AdWT itself did not change the abundance of this mRNA (Fig. 2). When the FAO cells were treated with either Ad-PF2KW or Ad-PF2KMut, 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA increased about 150-fold (Fig. 2), whereas the β-actin mRNA increased less than 20% (data not shown).

**Overexpression of the wild-type and double mutant (S32A/H258A) 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNAs was verified by ribonuclease protection assay (Fig. 3). As expected, a 230-base complement fragment was protected with rat liver RNA (lane L) and with RNA from FAO cells treated with Ad-PF2KW (lane 2), while a 179-base fragment was observed with RNA from FAO cells transduced with Ad-PF2KMut (lane 3). The 297-base nucleotide riboprobe protects a fragment that includes part of exon I specific for the liver 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA and part of exon I which is shared by both the liver and skeletal muscle forms of 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA (25). Moreover, the 5′ end of exon I corresponds to the sequence encoding the S32A mutation site. Hence, the fragment protected by the rat skeletal muscle mRNA was the same size as the fragment protected by the mutant form (S32A/H258A) of 6-PF-2-K/Fru-2,6-P<sub>2</sub>ase mRNA, i.e. 179 bases (Fig. 3, lanes M and 3). These results provide definitive evidence that the wild-type and mutant bifunctional enzyme mRNAs were overexpressed.
Overexpression of Liver 6-PF-2-K/ Fru-2,6-P$_2$ase

Consequences of Overexpression of the Wild-type and Mutated Form of 6-PF-2-K/ Fru-2,6-P$_2$ases on Fru-2,6-P$_2$ase levels—Overexpression of the wild-type enzyme resulted in a 70% decrease in Fru-2,6-P$_2$ levels relative to the control level (Table I). In contrast, overexpression of the mutant form resulted in a 3–4-fold increase in the level of Fru-2,6-P$_2$. Since the wild-type enzyme is subject to CAMP-dependent phosphorylation with concomitant inhibition of the kinase and activation of the bisphosphatase, it is possible that phosphorylation of the enzyme in FAO cells accounts for decrease in Fru-2,6-P$_2$ levels.

Effect of 8-CPT-cAMP on FAO Cell 6-Phosphofructo-2-kinase Activity—In order to ascertain whether the overexpressed bifunctional enzyme in FAO cells was subject to regulation by CAMP-dependent protein kinase-catalyzed phosphorylation, 8-CPT-cAMP (100 μM) was added to cells 48 h after treatment with either Ad-PF2KWT or Ad-PF2KMut. As observed with endogenous 6-PF-2-K/Fru-2,6-P$_2$ase in H4IIE cells (33), a 20-min exposure to the cAMP analog increased by 1.5-fold the S$_{0.5}$ for Fru-6-P of the wild-type overexpressed 6-PF-2-K, from 320 μM to 466 μM. As expected, the S$_{0.5}$ was unchanged by incubation with the CAMP analog when the double mutant form was overexpressed (253 μM versus 203 μM with CAMP). The higher S$_{0.5}$ for Fru-6-P (320 μM versus 253 μM) of the wild-type enzyme compared with the double mutant in cells incubated without 8-CPT-cAMP is consistent with phosphorylation of the overexpressed wild-type enzyme.

Gluconeogenesis and Glycolysis in FAO Cells—FAO cells contain all of the gluconeogenic enzymes required for growth and survival in the absence of glucose (11, 12), and they can produce glucose from gluconeogenic precursors (13). However, quantitative studies on glucose metabolism in these cells have not been reported. As shown in Fig. 5, FAO cells produced glucose from lactate plus pyruvate (10:1) (2.5 nmol/min/plate) or from dihydroxyacetone (1.5 nmol/min/plate). In contrast to isolated hepatocytes (30, 31), the rate of glucose production was higher with lactate plus pyruvate than with dihydroxyacetone during the last 2 h, and the addition of 2 mM oleate did not stimulate glucose production from lactate plus pyruvate (data not shown). Since most cancer cell lines have high rates of glycolysis (34, 35), FAO cells were incubated with 30 mM glucose, and lactate plus pyruvate production was measured. In the first 30 min, the rate of lactate plus pyruvate production, after subtraction of endogenous lactate plus pyruvate production, was 8.3 nmol/min/plate, but thereafter, the rate declined to nearly 0. These results demonstrate that FAO cells synthesize glucose from 3-carbon precursors and that glycolysis from glucose is very low.

Effect of Overexpression of Wild-type and Mutated Bifunctional Enzyme on Dihydroxyacetone Metabolism—Since FAO cells did not produce a large amount of lactate plus pyruvate from glucose, dihydroxyacetone was used to study the effect of overexpression of the bifunctional enzyme forms and the associated changes in Fru-2,6-P$_2$ on glucose and lactate plus pyruvate production. Dihydroxyacetone is phosphorylated by glycero kinase or α-trikinase to dihydroxyacetone phosphate (36), which then can be converted either to glucose or to lactate plus pyruvate. Addition of 20 mM dihydroxyacetone increased Fru-2,6-P$_2$ levels by 5-fold in FAO cells during the 3-h incubation. However, as was the case with FAO cells incubated in RPMI medium containing 11 mM glucose, overexpression of the wild-type enzyme decreased and overexpression of the double mu-

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2 Endogenous glucose and lactate plus pyruvate production in the absence of added substrate were subtracted from rates in the presence of substrate (dihydroxyacetone, lactate plus pyruvate (10:1) and glucose).
Overexpression of Liver 6-PF-2-K/Fru-2,6-P$_2$ase

The effect of overexpression of bifunctional enzyme on kinase and bisphosphatase maximal activities, kinase:bisphosphatase maximal activity ratio (K/P) and Fru-2,6-P$_2$ level in FAO cells

Table I

| FAO treatment | Kinase (milliunits/mg of protein) | Bisphosphatase (milliunits/mg of protein) | K/P ratio | Fru-2,6-P$_2$ (nmol/plate) |
|---------------|----------------------------------|------------------------------------------|------------|--------------------------|
| Untreated     | 8 ± 0.3                          | 3 ± 0.6                                  | 3 ± 0.7    | 45 ± 4                   |
| AdWT          | 8 ± 0.2                          | 3.3 ± 0.4                                | 2.7 ± 0.4  | 48 ± 4                   |
| Ad-PF2KWT     | 173 ± 40                         | 97 ± 18                                  | 1.7 ± 0.2  | 15 ± 4                   |
| Ad-PF2KMut    | 225 ± 23                         | 20 ± 1                                   | 12 ± 1     | 146 ± 17                 |

Overexpression of Liver 6-PF-2-K/Fru-2,6-P$_2$ase activities were assayed under maximal velocity conditions at pH 6.9 as described under "Experimental Procedures." 100 μg of soluble protein extracted and treated with 65% (NH$_4$)$_2$SO$_4$ were used for each measurement. Like activities measurements, fructose-2,6-bisphosphate levels were measured 48 h after the treatment of FAO cells with different adenovirus constructs. Results are the mean ± S.E. of five independent experiments.

The rate of glucose production was higher during the first hour than in the last 2 h in both treated and untreated cells, but the rate of dihydroxyacetone metabolism to glucose or lactate plus pyruvate was constant for the first 2 h (Fig. 6C). The lack of linearity of glucose production reflects a change in the balance between glucose and lactate plus pyruvate production from dihydroxyacetone after the first hour. This balance was also affected by the overexpression of both forms of the enzyme. During the last 2 h, in AdWT-treated or untreated FAO cells, about 30% of the 3-carbon precursors were used for glucose synthesis and about 70% for lactate plus pyruvate formation. With overexpression of the double mutant and the associated increase in Fru-2,6-P$_2$ level, only about 20% of dihydroxyacetone was used for glucose formation and about 80% for lactate plus pyruvate formation, whereas with overexpression of the wild-type enzyme and the decrease in Fru-2,6-P$_2$ level, about 40% of dihydroxyacetone was converted to glucose and about 60% to lactate plus pyruvate.

Fig. 5. Glucose production in FAO cells. 95-cm$^2$ plates of FAO cells were incubated for 1, 2, or 3 h with 3 ml of Krebs-bicarbonate, pH 7.4, containing 2% bovine serum albumin and 20 mM lactate plus pyruvate (10:1) (△) or 20 mM dihydroxyacetone (●). Glucose was measured in the incubation medium of each plate as described under "Experimental Procedures." Results are the mean ± S.E. (n = 4) for dihydroxyacetone and n = 1 for lactate plus pyruvate.

A relationship between Fru-2,6-P$_2$ levels and hepatic gluconeogenesis and glycolysis has been established by studies on nutritional status and metabolic disease states, such as diabetes (1, 37). The role of Fru-2,6-P$_2$ in regulating metabolic fluxes has also been studied by correlating pathway flux with acute changes in Fru-2,6-P$_2$ level brought about by hormones like glucagon, insulin, and vasopressin (37). However, changes in nutritional status and/or hormones involves modulation of other regulatory enzymes as well (38-40), and this has made it difficult to determine the importance of any individual step. The ability to overexpress wild-type 6-PF-2-K/Fru-2,6-P$_2$ase or a double mutant that has no bisphosphatase activity allowed us to analyze the relative role of the kinase and bisphosphatase in regulating Fru-2,6-P$_2$ levels and metabolic flux through the gluconeogenic/glycolytic pathway. Overexpression of wild-type 6-PF-2-K/Fru-2,6-P$_2$ase decreased Fru-2,6-P$_2$ levels and increased glucose production from dihydroxyacetone while inhibiting lactate plus pyruvate production. In contrast, overexpression of the double mutant increased Fru-2,6-P$_2$ levels and inhibited glucose production from dihydroxyacetone, while stimulating lactate plus pyruvate production. These results support the importance of Fru-2,6-P$_2$ as a regulator of the gluconeogenic/glycolytic pathway in FAO cells.
There have been only a limited number of reports of overexpression of enzymes of mammalian glucose metabolism with concomitant analysis of the consequences on pathway flux (19, 41–44). In several instances there was not a perfect quantitative correlation between overexpression of an enzyme and the predicted metabolic consequences of that overexpression. For example, a 10–100-fold overexpression of hexokinase I in a pancreatic β-cell line (MIN6) (41) or in isolated islets of Langerhans (42) enhanced glucose utilization or insulin secretion by only 2-fold. Overexpression of glycogen phosphorylase by 46-fold in primary hepatocytes did not change glycogen content in the basal state, although preferential activation of glycogenolysis was evident upon treatment with pharmacologic agents (19). Overexpression of glucokinase in islets had no effect on glucose utilization or insulin secretion. These results suggest that other steps in a pathway may become rate-limiting when one enzymatic step is enhanced by overproduction of the protein and/or that other as yet unrecognized regulatory mechanisms may be revealed by overexpression. On the other hand, overexpression of phosphoenolpyruvate carboxykinase in H4IIE-C3 cells (43) or of glucokinase in the same cell line or in FTO-2B cells (44) was quantitatively correlated with enhanced pathway fluxes, gluconeogenesis, and glycolysis, respectively.

The changes in Fru-2,6-P\textsubscript{2} levels were better correlated with the change in the kinase:bisphosphatase maximal activity ratio than with the kinase maximal activity, which was enhanced 22–28-fold in the case of overexpression of the wild-type and double mutant enzymes. However, there were a number of unexpected findings: 1) mRNA abundances of the wild-type enzyme and its double mutant were increased to a greater extent than the protein level. This discrepancy may be due to a higher rate of expression of the mRNA relative to the FAO cells’ ability to translate and/or process the mRNA. The apparent discrepancy between massive overexpression of the enzyme and relatively modest effects on Fru-2,6-P\textsubscript{2} concentration may reflect the heterogeneity of the FAO cells since the efficiency of gene transfer was 70%. 2) Overexpression of the wild-type enzyme resulted in a decrease in Fru-2,6-P\textsubscript{2} level. 3) Overexpression of the double mutant resulted in a nearly 6-fold increase in bisphosphatase, even though this enzyme form is devoid of significant bisphosphatase activity (10).

The paradoxical drop in Fru-2,6-P\textsubscript{2} argues for covalent modification of the overexpressed wild-type enzyme in FAO cells. Cyclic AMP-dependent protein kinase-catalyzed phosphorylation increases the kinase \( S_{0.5} \) for Fru-6-P and has no effect on the kinase maximal velocity but enhances the bisphosphatase

| Table II | Balance between the effect on glucose and the effect on lactate plus pyruvate of overexpression of Ad-PF2KWT and Ad-PF2KMut |
|----------|-------------------------------------------------------------------------------------------------------------------|
| Ad-PF2KWT | +228                                                                                                               |
| Ad-PF2KMut | −192                                                                                                               |

FIG. 6. Effect of overexpression of wild-type or mutated 6-PF-2-K/Fru-2,6-P\textsubscript{2} on glucose and lactate plus pyruvate production in FAO cells. 55-cm\textsuperscript{2} plates of FAO cells treated with different adenovirus constructs; AdWT ( ), Ad-PF2KWT ( ● ), and Ad-PF2KMut ( ■ ) were incubated 1, 2, or 3 h with 3 ml of Krebs-bicarbonate, pH 7.4, containing 2% bovine serum albumin and 20 mM dihydroxyacetone. Glucose (A) and lactate plus pyruvate (B) productions were measured in the incubation medium of each plate as described under “Experimental Procedures.” \( 2 \text{Glc} + \text{Lac} + \text{Pyr} \) is the sum of glucose and lactate plus pyruvate production in 3-carbon equivalent (C). Results are the mean ± S.E., \( n = 4 \).
maximal velocity 2–3-fold (1). Phosphorylation of the overexpressed wild-type enzyme is supported by both the higher $S_{0.5}$ for Fru-6-P of the 6-PF-2-K, compared with the double mutant, and the decrease in the kinase:bisphosphatase maximal activity ratio compared with the ratio for the enzyme in untreated or vector-treated cells. The question remains as to why the overexpressed enzyme is phosphorylated to a greater extent than the endogenous enzyme. In untreated FAO cells, the enzyme would be predicted to have a low phosphate content, since the concentration of 6-PF-2-K/Fru-2,6-P$_2$ase was estimated to be less than 1 $\mu$M by Western blot analysis (data not shown), which is far below its $K_{m}$ (10 $\mu$M) for phosphorylation by cAMP-dependent protein kinase (45). An increase in bifunctional enzyme concentration to about 40 $\mu$M after overexpression would be predicted to enhance its extent of phosphorylation in FAO cells. However, the possibility that an unrecognized covalent modification of the enzyme inhibits maximal kinase activity cannot be ruled out. For example, it has recently been demonstrated that in vitro ADP-ribosylation of the liver enzyme inactivates the kinase, but has no effect on the bisphosphatase (46).

Overexpression of the double mutant increased the kinase:bisphosphatase ratio and increased Fru-6-P$_2$ levels, as expected. However, the 6-fold increase in bisphosphatase activity was surprising, since this mutant lacks the histidine residue needed to form the phosphoenzyme intermediate that mediates Fru-2,6-P$_2$ hydrolysis (9, 10). Despite the inability to form phosphoenzyme intermediate, the H258A mutant retains a very low residual activity, and overexpression of the protein by 40-fold may account for the increased bisphosphatase activity. Increased phosphorylation of the overexpressed enzyme is unlikely to have contributed to the increased bisphosphatase activity, since the phosphorylation site (Ser-32) also was mutated to Ala. It has been reported that Ser-33 can undergo phosphorylation in vitro but at a very low rate and with negligible effect on the bisphosphatase (8). It also cannot be ruled out that the endogenous enzyme underwent phosphorylation as a result of the large increase in total bifunctional enzyme protein, resulting in enhanced bisphosphatase activity. However, this phosphorylation would not fully explain the 6-fold increase in total bisphosphatase activity. This change probably involves both residual activity of the overexpressed protein and phosphorylation of the endogenous enzyme.

The results reported here also support previous work with H4IIE cells, which demonstrated that regulation of the activities of the bifunctional enzyme and Fru-2,6-P$_2$ levels by covalent modification is more important than changes in amount of this two-domain protein (33). For example, dexamethasone, which did not affect phosphorylation state, increased bifunctional enzyme mRNA (11-fold) and protein (3-fold) but had only a small effect on Fru-2,6-P$_2$ content. In contrast, insulin, which increased the kinase:bisphosphatase activity ratio by causing dephosphorylation of the enzyme, increased Fru-2,6-P$_2$ content by 15-fold (15). Although the phosphorylation state of the enzyme is probably the most important determinant of net synthesis or degradation of Fru-2,6-P$_2$, it is likely that the amount of the enzyme is important in some situations, i.e., lipogenic conditions, where elevated Fru-2,6-P$_2$ levels have been correlated with increased enzyme amount (16). In addition, insulin and dexamethasone had highly synergistic effects on Fru-2,6-P$_2$ in H4IIE cells by altering both the phosphorylation state and the amount of protein (33).

As shown previously, FAO cells are capable of synthesizing glucose from various 3-carbon precursors (13). Rates of glucose synthesis from lactate/pyruvate (10:1) or dihydroxyacetone are between 5 and 15% (0.3–0.7 mmol/min/dry mass) that of isolated hepatocytes (30, 31) and perfused liver (47), depending on the substrate and growth medium. The lower rate of glucose production may also be due to differences in incubation conditions and/ or to different rate-limiting steps in hepatocytes versus hepatoma cells, since the latter contain lower fructose-1,6-bisphosphatase activity and higher phosphoenolpyruvate carboxykinase activity (11, 12). However, the rate of glucose synthesis in FAO cells was high enough to study the role of Fru-2,6-P$_2$ in regulation of glycolytic and gluconeogenic flux, particularly with the use of dihydroxyacetone.

Tumor cells are usually thought to have high rates of glycolysis (34, 35). However, like other rat hepatoma cell lines such as FT0–2B and H4IIE (44), in contrast to isolated hepatocytes, FAO cells exhibit very low rates of glycolysis from glucose but a high lactate plus pyruvate production from dihydroxyacetone. The lack of expression of the glucokinase gene in FAO cells (data not shown) and in other rat hepatoma cell lines such as FT0–2B and H4IIE (44) compared with isolated hepatocytes (7) probably accounts for the low glycolytic rate from glucose and the substrate effect (dihydroxyacetone versus glucose) on this rate.

The results of this study point to the utility of FAO cells as a model system for glycolytic/gluconeogenic pathway engineering. Adenoviral and/or retroviral constructs containing the coding region for glucokinase can be used to enhance glucose utilization in these cells and test the role of this other enzymes in controlling glycolytic flux, substrate cycling, and Fru-2,6-P$_2$ levels. Such work is in progress.

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REFERENCES

1. Pilkis, S. J., Claus, T. H., Kurland, I. J., and Lange, A. J. (1995) Annu. Rev. Biochem., 64, 799–835.
2. Pilkis, S. J., and Granner, D. K. (1992) Annu. Rev. Physiol., 54, 885–909.
3. Becker, T. C., Nod, R. J., Coats, W. S., Gómez-Fox, A. M., Alam, T., Gerard, A. G., and Newgard, C. B. (1994) Methods Cell Biol., 43, 161–189.
4. Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., Claus, T. H., and Cumming, D. A. (1981) J. Biol. Chem., 256, 3171–3174.
5. Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., and Claus, T. (1983) J. Biol. Chem., 258, 3619–3626.
6. Van Schaftingen, E., and Hers, H. G. (1981) Proc. Natl. Acad. Sci. U. S. A., 78, 2861–2863.
7. Pilkis, S. J., El-Maghrabi, M. R., and Claus, T. H. (1988) Annu. Rev. Biochem., 57, 755–783.
8. Kurland, I. J., El-Maghrabi, M. R., Correia, J. J., and Pilkis, S. J. (1992) J. Biol. Chem., 267, 4416–4423.
9. Pilkis, S. J., Livély, M. O., and El-Maghrabi, M. R. (1987) J. Biol. Chem., 262, 12672–12675.
10. Tuder, A., Lin, K., and Pilkis, S. J. (1990) J. Biol. Chem., 265, 15617–15622.
11. Bertolotti, R. (1977) Somatic Cell Genet., 3, 365–380.
12. Deschatrette, J., Moore, E. E., Dubois, M., Caselio, D., and Weiss, M. C. (1979) Somatic Cell Genet., 5, 697–718.
13. Kahn, C. R., Lauren, F., Koch, S., Crettaz, M., and Granner, D. K. (1989) Mol. Endocrinol., 3, 840–845.
14. Deschatrette, J., and Weiss, M. C. (1974) Biochimie (Paris) 56, 1603–1611.
15. Graham, F. L., Simley, J., Russell, W. C., and Nairn, R. (1977) J. Gen. Virol. 36, 59–72.
16. Czecot, A. D., Marker, A. J., Lange, A. J., El-Maghrabi, M. R., Granner, D. K., Tauler, A., Pilkis, J., and Pilkis, S. J. (1988) J. Biol. Chem. 263, 18669–18677.
17. Gluzman, Y., Reichl, H., and Sønnick, D. (1982) Eucaryotic Viral Vectors (Gluzman, Y. ed.) pp 187–192, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988) J. Biol. Chem. 263, 59–72.
19. Colosia, A. D., Marker, A. J., Lange, A. J., El-Maghrabi, M. R., Granner, D. K., Tauler, A., Pilkis, J., and Pilkis, S. J. (1988) J. Biol. Chem. 263, 18669–18677.
20. Gluzman, Y., Reichl, H., and Sønnick, D. (1982) Eucaryotic Viral Vectors (Gluzman, Y. ed.) pp 187–192, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Cleveland, D. W., Lopala, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirshner, M. W. (1980) Cell 20, 85–105.
22. Feinberg, A. P., and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
23. El-Maghrabi, M. R., Corrêa, J. J., Heil, P. J., Pate, T., Cobb, C. E., and Pilkis, S. J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5005–5009.
24. Van Schaftingen, E., Lederer, B., Bartrons, R., and Hers, H. G. (1982) Eur. J. Biochem. 129, 191–195.
25. El-Maghrabi, M. R., Corrêa, J. J., Heil, P. J., Pate, T., Cobb, C. E., and Pilkis, S. J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5005–5009.
26. Van Schaftingen, E., Lederer, B., Bartrons, R., and Hers, H. G. (1982) Eur. J. Biochem. 129, 191–195.
27. El-Maghrabi, M. R., Corrêa, J. J., Heil, P. J., Pate, T., Cobb, C. E., and Pilkis, S. J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5005–5009.
28. Van Schaftingen, E., Lederer, B., Bartrons, R., and Hers, H. G. (1982) Eur. J. Biochem. 129, 191–195.