Activation of phosphatidylinositol 3-kinase is required for transcriptional activity of F-type 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: assessment of the role of protein kinase B and p70 S6 kinase

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Previous studies have demonstrated that the F isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase) is transcriptionally regulated by growth factors. The aim of this study was to investigate the importance of the phosphatidylinositol 3-kinase (PI 3-kinase) pathway in the regulation of 6PF2K/Fru-2,6-BPase gene expression. We have completed studies using chemical inhibitors and expression vectors for the proteins involved in this signalling cascade. Treatment of cells with LY 294002, an inhibitor of PI 3-kinase, blocked the epidermal growth factor (EGF)-dependent stimulation of 6PF2K/Fru-2,6-BPase gene transcription. Transient transfection of a constitutively active PI 3-kinase was sufficient to activate transcription from the F-type 6PF2K/Fru-2,6-BPase promoter. In contrast, co-transfection with a dominant-negative form of PI 3-kinase completely abrogated the stimulation by EGF, and down-regulated the basal promoter activity. In an

attempt to determine downstream proteins that lie between PI 3-kinase and 6PF2K/Fru-2,6-BPase gene expression, the over-expression of a constitutively active form of protein kinase B (PKB) was sufficient to activate 6PF2K/Fru-2,6-BPase gene expression, even in the presence of either a dominant-negative form of PI 3-kinase or LY 294002. The over-expression of p70/p85 ribosomal S6 kinase or the treatment with its inhibitor rapamycin did not affect 6PF2K/Fru-2,6-BPase transcription. We conclude that PI 3-kinase is necessary for the transcriptional activity of F-type 6PF2K/Fru-2,6-BPase, and that PKB is a downstream effector of PI 3-kinase directly involved in the regulation of 6PF2K/Fru-2,6-BPase gene expression.

Key words: fructose 2,6-bisphosphate, gene expression, glycolysis, LY 294002, signal transduction.

INTRODUCTION

Activation of the glycolytic pathway is common to such distinct processes as glucose metabolism in liver and cell proliferation. In all these processes, fructose 2,6-bisphosphate, a potent allosteric stimulator of 6-phosphofructo-1-kinase, determines the strength of glycolytic flux. The content of this metabolite is regulated by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase; EC 2.7.1.105/3.1.3.46), which catalyses both its synthesis and its degradation. Various 6PF2K/Fru-2,6-BPase isoenzymes have been described in different tissues. In rat, liver, muscle and F-type isoforms are encoded by the same gene arising from distinct promoters. These isoenzymes differ in their N-terminal sequences, regulation by phosphorylation and kinetic properties (for review see [1,2]).

The expression of 6PF2K/Fru-2,6-BPase isoenzymes depends on cellular processes. The F-type isoform is more specific to proliferative tissues and is transcriptionally activated by growth factors [3–6]. In accordance with this, several binding sites for different transcription factors have been implicated in the regulation of this isoform during cell proliferation, such as binding sites for Sp1 and for members of the *ets* oncogene family located on the F promoter [4], and a binding site for the E2F transcription factor located in the first exon of F-type mRNA [5]. The signalling pathways that mediate growth-factor regulation of 6PF2K/Fru-2,6-BPase gene expression are not fully understood. Previously we have demonstrated that activation of mitogen-

activated protein kinase (MAPK) is required for the stimulation of the transcription of 6PF2K/Fru-2,6-BPase by epidermal growth factor (EGF) [6]. However, this does not rule out the possibility that other pathways could also be involved in this regulation.

A large body of evidence supports the hypothesis that the activation of the phosphatidylinositol 3-kinase (PI 3-kinase) pathway by growth factors or insulin mediates the expression of several metabolic genes [7–15]. An increase in PI 3-kinase activity occurs following ligand-induced autophosphorylation of the receptor in response to a wide range of extracellular stimuli. This results in the generation of membrane-restricted second messengers, phosphatidylinositides containing a 3'-phosphate. Recent studies have identified a number of downstream targets of these phospholipid products, including the serine/threonine kinases 3-phosphoinositide-dependent kinase 1 (PDK1), protein kinase B (PKB) and, indirectly, p70/p85 ribosomal protein S6 kinase (p70^{S6K}; for review see [16]).

The aim of the present study was to investigate the role of PI 3-kinase on the regulation of 6PF2K/Fru-2,6-BPase gene expression. We now demonstrate that activation of PI-3 kinase is not only essential for the stimulation of the transcription of 6PF2K/Fru-2,6-BPase by EGF, but is also required for basal transcriptional activity. We also show that PI 3-kinase action occurs through activation of PKB, which is activated upon generation of 3-phosphorylated inositol lipids, the products of PI 3-kinase.

Abbreviations used: 6PF2K/Fru-2,6-BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PDK1, 3-phosphoinositide-dependent kinase 1; PKB, protein kinase B; p70^{S6K}, p70/p85 ribosomal S6 kinase; CAT, chloramphenicol acetyltransferase; FCS, foetal calf serum; AP-1, activator protein 1; mTOR, mammalian target of rapamycin.

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EXPERIMENTAL

Materials

Foetal calf serum (FCS), Dulbecco's modified Eagle's medium, and penicillin/streptomycin were from Bio-Whittaker. [α - 32 P]-dCTP (3000 Ci/mmol), D-threo-[dichloroacetyl-L- 14 C]chloramphenicol (56 mCi/mmol) and Hybond-N membranes were from Amersham Pharmacia Biotech. The random-primer DNA-labelling kit was from Boehringer Mannheim. EGF was from Sigma. LY 294002 and rapamycin were from Calbiochem. Tfx-10, Transfast Reagents and luciferase assay system were from Promega. ExpressHyb Hybridization Solution was from Clontech. Phospho-specific PKB antibody was from New England Biolabs. Anti-Myc monoclonal antibody 9E10 and anti-p70^{S6K} polyclonal antibody were from Santa Cruz Biotechnology. p70^{S6K} substrate peptide was from Upstate Biotechnology. Other materials and chemicals were of the highest quality available.

Plasmid constructs

The reporter-gene construct FpBLCAT3 [17] contains the *HindIII/BspMI* 5'-flanking region of the F isoform of 6PF2K/*Fru-2,6-BPase* cloned into pBLCAT3 plasmid and was provided by Dr A. Lange (University of Minnesota, Minneapolis, MN, U.S.A.). Expression vectors for the catalytic subunit of PI 3-kinase, including constitutively active p110* and kinase-dead p110KR [18] were provided by Dr A. Klippel (Chiron Corporation, Emeryville, CA, U.S.A.). The expression vector for the constitutively active PKB/Akt, PKBgag [19], was provided by Dr G. Evan (Imperial Cancer Research Fund Laboratories, London, U.K.). Expression vector for Myc-tagged wild-type PDK1 [20] was provided by Dr B. Hemmings, and expression vectors for Myc-tagged wild-type p70^{S6K} and Myc-tagged constitutively active p70^{S6K}E389D₃E [20] were provided by Dr G. Thomas, both at the Friedrich-Miescher-Institut, Basel, Switzerland.

Cell cultures

Rat-1 fibroblasts and FTO-2B hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 100 units/ml penicillin and 100 mg/ml streptomycin, in a 5% CO₂ humidified atmosphere at 37 °C. For experimental purposes, cells were subcultured in 100-mm dishes and made quiescent by maintaining them in medium supplemented with 0.1% FCS for 48 h. After this, cells were preincubated for 30 min in the presence of specific inhibitors, and were subsequently treated with EGF-supplemented medium plus DMSO vehicle for 6–8 h.

Transfection and chloramphenicol acetyltransferase (CAT) assays

CsCl double-gradient centrifugation was used to prepare all plasmids for transfection. Rat-1 transient transfections were performed using either the Tfx-10 or the Transfast Reagent (liposome-mediated transfections). Cells were plated at 5.8×10^5 cells/60-mm culture dish or 1.45×10^6 /100-mm culture dish on the day before transfection. Transfections were performed by following the manufacturer's protocol. FTO-2B hepatoma cells were transfected using the calcium phosphate/DNA co-precipitation method as described previously [6]. Each plate contained pCMV5 luciferase expression vector as an internal control and the total amount of plasmid was adjusted with empty pCMV5 expression vector. Quiescence was achieved as described above, and the same treatment was performed before harvesting.

CAT activity determinations were carried out as described by Ausebel et al. [21].

RNA isolation and analysis

Total RNA was extracted from Rat-1 cells by the LiCl/urea method [22]. The RNA was dissolved in sterile water, and the concentration and purity of each sample were assessed by absorbance at 260 nm and by the 260 nm/280 nm ratio, respectively. The abundance of 6PF2K/*Fru-2,6-BPase* mRNA was measured by Northern-blot analysis using ExpressHyb solution according to the instructions of the manufacturer (Clontech), and a 1-kb *EcoRI* fragment isolated from pKB plasmid [23] was used as a probe. The integrity of the RNA was verified by observing the rRNA bands in ethidium bromide-stained gel under UV irradiation. The level of mRNA was evaluated by densitometric scanning of the autoradiogram and corrected for the amount of 18 S rRNA using a ribosomal cDNA probe.

Kinase assays

The cells were harvested in ice-cold lysis buffer (50 mM Tris/HCl, pH 8.0, 120 mM NaCl, 20 mM NaF, 10 mM β -glycerol phosphate, 1% Nonidet P40, 5 mM EGTA, 1 mM benzamidine, 0.1 mM PMSF, 15 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄, and 1 μ g/ml each of aprotinin, leupeptin and pepstatin), and kept on ice for 30 min. Endogenous or Myc-tagged p70^{S6K} was precipitated from 200 μ g of cell extract by incubation for 2 h at 4 °C with 2 μ g of rabbit polyclonal anti-p70^{S6K} (Santa Cruz Biotechnology) followed by 1 h at 4 °C with 40 μ l of 50% Protein A-Sepharose, or with 3 μ g of Myc monoclonal antibody (9E10) and 40 μ l of 50% Protein G-Sepharose. Samples were washed three times in ice-cold PBS containing 0.1% Nonidet P-40 and 2 mM Na₃VO₄, and once in assay buffer (20 mM Mops, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM Na₃VO₄ and 1 mM dithiothreitol). Immune-complex kinase assays were performed for 30 min at 37 °C in 40 μ l of assay buffer containing 50 μ M p70^{S6K} substrate peptide and 50 μ M [γ - 32 P]ATP (5 mCi/assay). Substrate-bound reactivity was collected by binding to p81 phosphocellulose paper and quantified by scintillation counting after rinsing with 0.75% cold phosphoric acid.

Immunoblotting

Cell lysates were prepared as described above, and proteins were subjected to SDS/PAGE (10% gels). Proteins were transferred on to PVDF membranes. The blots were blocked with blocking solution (5% non-fat dried milk/Tris-buffered saline/Tween 20, pH. 7.5), washed with Tris-buffered saline/Tween 20 and then incubated with phospho-specific Akt antibody (1:1000 dilution) overnight at 4 °C. After extensive washing, the blots were incubated with secondary antibody (1:5000 dilution) coupled to horseradish peroxidase, and proteins were visualized with the ECL[®] detection kit (NEN Life Science Products). Equal loading of each lane was confirmed by immunoblotting with actin antibody.

Other methods

Protein was determined as described by Bradford [24] with BSA as standard. The statistical significance of differences was assessed by Student's unpaired *t* test.

Figure 1 Effect of PI 3-kinase inhibitor LY 294002 on the stimulation of *6PF2K/Fru-2,6-BPase* gene expression by EGF

(A) Quiescent Rat-1 fibroblasts were pretreated for 30 min with the indicated concentrations of LY 294002 and then cultured for 6 h with EGF (20 ng/ml). The carrier for the inhibitors (DMSO) was added as a control. Total RNA (20 μ g/lane) was extracted from cultured cells and analysed by Northern blot (a representative Northern blot is shown above the graph). The intensity of the autoradiographic signal was quantified by densitometric scanning and corrected for the amount of RNA obtained with the 18 S rRNA probe. Values are expressed relative to the value of untreated cells, which was considered as 1 arbitrary unit. Data are means $\pm$ S.E.M. from three

RESULTS

The role of PI 3-kinase in the regulation of *6PF2K/Fru-2,6-BPase* expression by growth factors was studied by measuring the effect of LY 294002 on the EGF-dependent stimulation of *6PF2K/Fru-2,6-BPase* mRNA levels. LY 294002 is a highly specific PI 3-kinase inhibitor that directly inhibits the activity of the p110 catalytic subunit of PI 3-kinase by competing with the ATP-binding site [25]. Rat-1 fibroblasts were made quiescent by maintaining them in 0.1 % FCS medium for 48 h and were then shifted to medium supplemented with EGF at 20 ng/ml. As shown in Figure 1(A), EGF produced a 2-fold increase in the levels of *6PF2K/Fru-2,6-BPase* mRNA. The addition of LY 294002 prior to EGF blocked this effect in a dose-dependent manner. To determine whether the regulation of *6PF2K/Fru-2,6-BPase* mRNA levels was due to a regulation of its transcriptional rate, Rat-1 cells were transiently transfected with a *CAT* reporter gene under the transcriptional control of a 1.7-kb genomic DNA fragment that contains the F-type promoter. As shown in Figure 1(B), EGF induced up to 2-fold basal *CAT* activity and LY 294002 repressed this induction. In the absence of EGF, the basal levels of *6PF2K/Fru-2,6-BPase* transcriptional activity were also inhibited by treatment with LY 294002. These results indicate that PI 3-kinase contributes positively to the activation of *6PF2K/Fru-2,6-BPase* transcription. As a measure of the effect of LY 294002, we determined the phosphorylation state of PKB. We used Western blots probed with an antibody that specifically recognizes phosphorylation of Ser-473. As shown in Figure 1(C), EGF stimulated the phosphorylation of PKB in Ser-473 compared with non-treated cells. In contrast, cells pretreated with LY 294002 at 10 and 50 μ M in the presence or absence of EGF contained no detectable phosphoprotein.

A second strategy to investigate whether PI 3-kinase is both necessary and sufficient for EGF to activate *6PF2K/Fru-2,6-BPase* gene expression involved the use of expression vectors for both the constitutively active (p110*) and the kinase-dead (p110KR) catalytic subunit of this enzyme. p110* was constructed by joining the SH2 (Src homology 2) region of p85 to its N-terminus so that it no longer requires the interaction/activation by p85, whereas p110KR carries a point mutation in the ATP-binding site [18]. Co-transfection experiments with these forms and the F-type *6PF2K/Fru-2,6-BPase* promoter were performed in Rat-1 cells and *CAT* activity was determined in the presence or in the absence of EGF. Expression of p110* increased the transcriptional activity of F-type *6PF2K/Fru-2,6-BPase* gene in both the absence and the presence of EGF (Figure 2A). In contrast, the dominant-negative form of PI 3-kinase, p110KR, reduced both basal and EGF-induced *6PF2K/Fru-2,6-BPase* promoter activity. To rule out the possibility that this

independent experiments. Statistically significant differences are indicated by * $P < 0.05$ compared with EGF-treated cells. (B) Quiescent Rat-1 fibroblasts transiently transfected with a *CAT* reporter gene under control of the F-type promoter were incubated for 30 min with the indicated concentrations of LY 294002 or rapamycin and then stimulated for a further 8 h with EGF (20 ng/ml)-supplemented medium carrying DMSO as a vehicle. The intensity of the autoradiographic signal was quantified by densitometric scanning. Values are normalized to luciferase activity and expressed relative to the value of untreated cells, which was considered as 1 arbitrary unit. Data are means $\pm$ S.E.M. from five independent experiments. Statistically significant differences are indicated by *** $P < 0.001$ and * $P < 0.05$ compared with EGF-treated cells, and ++ $P < 0.01$ and + $P < 0.05$ compared with untreated cells. (C) Samples were obtained from cells pretreated for 30 min with the indicated concentrations of LY 294002 and activated for 10 min with EGF. Equal amounts of cell-lysate protein were subjected to SDS/PAGE followed by Western blotting as described in the Experimental section. The phosphorylated site in PKB was detected with an antibody specific to phospho-Ser-473. The control lane corresponds to Swiss 3T3 cells stimulated with platelet-derived growth factor. Equal loading was confirmed by immunoblotting with actin antibody.

Figure 2 Effect of PI 3-kinase on EGF-stimulated 6PF2K/Fru-2,6-BPase transcriptional activity

Rat-1 cells (**A**) and FTO-2B cells (**B**) were transiently transfected in 100-mm culture dishes with 9 μ g of F-type 6PF2K/Fru-2,6-BPase promoter-CAT fusion gene, and 2.4 μ g of p110* or p110KR expression vector as described in the Experimental section. Following transfection, cells were maintained in 0.1% FCS medium for 48 h and treated with 20 ng/ml EGF. Cells were harvested 8 h after growth-factor addition. CAT activity was assayed and values corrected for luciferase activity from the co-transfected pCMV5luciferase plasmid, and expressed as arbitrary units. Data are means $\pm$ S.E.M. from three independent experiments. Statistically significant differences are indicated by *** P < 0.001 and * P < 0.05 compared with untreated cells, and *** P < 0.001 and + P < 0.05 compared with EGF-treated cells.

effect was cell-specific, the same experiment was performed in the FTO-2B hepatoma cell line with similar results (Figure 2B). However, the activation of p110* on PF2K/Fru-2,6-BPase was lower and, in contrast, the inhibition of p110KR was higher.

To explore the link between the activation of PI 3-kinase and the observed up-regulation of 6PF2K/Fru-2,6-BPase gene expression, we examined the role of PKB in this stimulation. PKB is a direct target of 3'-phosphorylated phosphoinositides, the products of PI 3-kinase activity. Once targeted to the membrane via its pleckstrin homology domain, PKB becomes phosphorylated and activated by PDK1 [26]. The dominant-negative form of PI 3-kinase, p110KR, was co-transfected with F-type 6PF2K/Fru-2,6-BPase promoter, PDK1 expression vector and a constitutively active form of PKB (PKBgag), which is myristoylated and predominantly found at the plasma membrane

Figure 3 Effect of PKB/Akt on 6PF2K/Fru-2,6-BPase gene expression inhibited by PI 3-kinase

Expression vectors for the constitutively active p110* or the kinase-dead p110KR (0.8 μ g), wild-type (wt) PDK1 (0.8 μ g) and the indicated amounts of the constitutively active PKBgag were co-transfected in Rat-1 fibroblasts (60-mm culture dish) with 3 μ g of a CAT reporter gene under the control of the F-type promoter as described in the Experimental section. (**A**) Cell extracts for assaying CAT activity were obtained 48 h after maintaining the cells in 0.1% FCS medium. Values were normalized to luciferase activity from co-transfected pCMV5luciferase plasmid. Data are means $\pm$ S.E.M. from three independent experiments. Statistically significant differences are indicated by ** P < 0.01 and * P < 0.05 compared with cells transfected with FpBLCAT3 plasmid alone. (**B**) Cell extracts for assaying CAT activity were obtained from quiescent transfected cells treated with the indicated amounts of LY 294002 for 8 h. Values are normalized to luciferase activity from co-transfected pCMV5luciferase plasmid. Data are means $\pm$ S.E.M. from three independent experiments. Statistically significant differences are indicated by ** P < 0.01 and * P < 0.05 compared with untreated cells transfected with FpBLCAT3 plasmid alone, and + P < 0.01 compared with untreated cells transfected with PDK1 wt plasmid.

Figure 4 Effect of transfected p70^{S6K} on 6PF2K/Fru-2,6-BPase gene expression

(A) Rat-1 fibroblasts (60-mm culture dish) were transiently transfected with the expression plasmids for PDK1 wild-type (wt; 0.8 μ g), p70^{S6K}wt (0.8 μ g), and the indicated amounts of the constitutively active p70^{S6K}E389D₃E, together with 3 μ g of a CAT reporter gene under the control of F-type promoter as described in the Experimental section. CAT activity was determined 48 h after maintaining the cells in 0.1% FCS medium. Values are corrected for luciferase activity from co-transfected pCMV5luciferase plasmid. Data are means $\pm$ S.E.M. from three independent experiments. (B) Samples were obtained from cells transfected and treated as described above, and analysed for p70^{S6K} activity as described in the Experimental section. Values are corrected by subtracting the value for control cells.

[19]. The expression of PKBgag repressed the inhibitory effect of p110KR (Figure 3A) and LY 294002 (Figure 3B). Indeed, the rate of activation of F-type 6PF2K/Fru-2,6-BPase transcriptional activity obtained in the presence of both PKB and PDK1 was the same as with p110*. PKB and PDK1 alone were also able to increase CAT activity but to a lesser extent than when added together. Thus PDK1 and PKB can be considered as downstream effectors of PI 3-kinase directly involved in the regulation of 6PF2K/Fru-2,6-BPase gene expression.

The ribosomal protein p70^{S6K} has also been identified as a downstream effector of the PI 3-kinase signalling pathway [27]. The precise mechanism by which p70^{S6K} is regulated by PI 3-kinase is not fully understood, but it involves at least phosphorylation by PDK1, the same kinase involved in PKB activation. To determine whether p70^{S6K} regulates 6PF2K/Fru-2,6-BPase gene expression, the effect of the over-expression of

Figure 5 Effect of p70^{S6K} inhibitor rapamycin on the stimulation of 6PF2K/Fru-2,6-BPase gene expression by EGF

(A) Quiescent Rat-1 fibroblasts were preincubated for 30 min with the indicated concentrations of rapamycin and then treated for 6 h with EGF (20 ng/ml). The carrier for the inhibitors (DMSO) was added as a control. Total RNA (20 μ g/lane) was extracted from cultured cells and analysed by Northern blotting (a representative Northern blot is shown above the graph). The intensity of the autoradiographic signal was quantified by densitometric scanning and corrected for the amount of RNA obtained with the 18 S rRNA probe. Data are means $\pm$ S.E.M. from three independent experiments. (B) Samples were obtained from cells pretreated for 30 min with the indicated concentrations of rapamycin and activated for 10 min with EGF (20 ng/ml), and analysed for endogenous p70^{S6K} activity as described in the Experimental section. For both panels, values are expressed relative to the value of untreated cells which was considered as 1 arbitrary unit.

this kinase was evaluated by co-transfection experiments. For this purpose, expression vectors for the wild-type form of p70^{S6K}, p70^{S6K}wt, and for the variant with acidic residues placed at Thr-389 and at the four (Ser/Thr)-Pro sites, p70^{S6K}E389D₃E, were used. The p70^{S6K}E389D₃E variant has high basal kinase activity and is more efficiently phosphorylated by PDK1 than the wild-type enzyme, further increasing its activity. Co-transfection with PDK1 and either the wild-type form of p70^{S6K} or the p70^{S6K}E389D₃E variant did not increase the transcriptional activity of the F-type promoter more than did PDK1 alone

(Figure 4A). Kinase assays *in vitro* confirm that expression of increasing amounts of p70^{S6K}E389D₃E increases p70^{S6K} activity in a dose-dependent fashion (Figure 4B).

A separate pathway contributing to p70^{S6K} activation involves the FKBP (FK506-binding protein) 12-*rapamycin*-associated protein [FRAP/mammalian target of rapamycin (mTOR)]. In order to confirm the previous results, we measured the effect of the FRAP inhibitor rapamycin on the EGF-dependent stimulation of 6PF2K/Fru-2,6-BPase mRNA levels. Quiescent Rat-1 fibroblasts were preincubated for 30 min with increasing concentrations of rapamycin and then treated with medium supplemented with EGF at 20 ng/ml. EGF increased the levels of 6PF2K/Fru-2,6-BPase mRNA 2-fold (Figure 5A). The addition of rapamycin did not affect the enhancement of transcription by EGF at any of the concentrations tested. Kinase assays confirmed that rapamycin was able to block the activation of p70^{S6K} by EGF (Figure 5B). The same result was obtained in Rat-1 fibroblasts transiently transfected with a *CAT* reporter gene under the transcriptional control of the F-type promoter; the EGF-dependent stimulation of 6PF2K/Fru-2,6-BPase transcriptional activity was insensitive to 100 nM rapamycin (Figure 1B). These results suggest that the serine/threonine kinase p70^{S6K} is not involved in the regulation of 6PF2K/Fru-2,6-BPase gene expression.

DISCUSSION

In order to activate the glycolytic flux, transcriptional activity of the F-type isoform of 6PF2K/Fru-2,6-BPase enzyme increases during cell proliferation [5,6]. The signal-transduction pathways that lead to this activation are still unclear. Previously we reported the involvement of the MAPK cascade in this regulation [6]; however, this result does not rule out the involvement of other pathways. Here we examined the ability of PI 3-kinase pathway to regulate the transcriptional activity of the specific proliferative isoform of 6PF2K/Fru-2,6-BPase enzyme.

By using a PI 3-kinase-specific inhibitor, LY 294002, we have demonstrated that activation of this kinase was essential for the EGF-stimulated increase in 6PF2K/Fru-2,6-BPase mRNA levels. Transient transfection of a constitutively active PI 3-kinase was sufficient to activate transcription from the F-type 6PF2K/Fru-2,6-BPase promoter. In contrast, co-transfection with a dominant-negative form of PI 3-kinase completely abrogated the stimulatory effect of EGF and down-regulated the basal promoter activity. These results indicate that activation of PI 3-kinase is necessary and sufficient for activation of 6PF2K/Fru-2,6-BPase gene expression, irrespective of the cell type. The small differences found in response to PI 3-kinase in the different cell lines could be due to the different levels of endogenous PI 3-kinase activity either in the basal or in the EGF-stimulated conditions.

In an attempt to obtain further insights into specific pathways that lie between PI 3-kinase and 6PF2K/Fru-2,6-BPase gene expression, we evaluated the effect of p70^{S6K} and PKB, both serine/threonine kinases that are activated by PI3-kinase. Our results demonstrate that over-expression of p70^{S6K} does not affect 6PF2K/Fru-2,6-BPase gene expression. In contrast, a constitutively active form of PKB was sufficient to activate 6PF2K/Fru-2,6-BPase gene expression, even in the presence of a dominant-negative form of PI 3-kinase. There is controversy as to the mechanism by which PKB is activated. The generation of phosphatidylinositides containing a 3'-phosphate by PI 3-kinase has been implicated not only in the translocation of PKB to the membrane, but also in the modulation of its activity [29,30]. However, other reports suggest that this modulation occurs

through a phosphorylation-dependent mechanism involving a specific kinase, PDK1 [26]. In order to ensure full PKB activity, in some cases PKB was co-transfected together with PDK1. Interestingly, the finding that overexpression of PDK1 alone significantly activated the 6PF2K/Fru-2,6-BPase promoter is consistent with the role of this kinase in the activation of endogenous PKB. However, other possibilities should be considered. In fact, PDK1 can phosphorylate and activate p70^{S6K} *in vivo*, although we have shown that this kinase is not involved in the regulation of 6PF2K/Fru-2,6-BPase expression, but it could also mediate the activity of members of the AGC (protein kinases A, G and C) family of Ser/Thr kinases [20]. The effect of PDK1 is partially abolished by LY 294002, suggesting that changes in the concentration of basal levels of phosphatidylinositides 3'-phosphate could regulate PDK1 activity, as has been suggested by others [31].

PI 3-kinase has recently been implicated in the regulation of enzymes that control metabolic processes. In some of them, PKB is involved as a downstream effector; this is the case for fatty acid synthase [11], phosphoenolpyruvate carboxykinase [12], GLUT4 transporter [7] and glycogen synthase kinase-3 [9]. In contrast, the p70^{S6K}-dependent pathway is required for the regulation of glucose-6-phosphate dehydrogenase [14] and hexokinase II [15]. Thus PI 3-kinase is a point of divergence in metabolic signalling. The direction of divergence depends on the nature of the signal, the function of the enzyme and the cell type. From the results presented here, we have demonstrated that PI 3-kinase and PKB are involved in the transcriptional regulation of the proliferation-specific isoform of the 6PF2K/Fru-2,6-BPase enzyme. Further work is needed on the identification of proteins downstream of PKB, including transcription factors that could bind to regulatory elements of these metabolic enzymes. It has been reported that activated forms of PKB and glycogen synthase kinase-3 are translocated to the nucleus, where they may modify the activity of nuclear factors [32–34]. In concordance with this, it has been established that PKB can substitute for interleukin-2 or PI 3-kinase and induce the transcriptional activity of E2F transcription factors in Kit225 cells by regulating the cellular levels of the cyclin/cdk inhibitor p27^{kip1} [35]. In addition, expression of cyclin D1, which can modulate E2F release from the retinoblastoma protein pRB, is mediated by PI 3-kinase through mTOR-p70^{S6K}-independent signalling in growth-factor-stimulated NIH-3T3 fibroblasts [36]. Enhancement of transcriptional activity of activator protein 1 (AP-1) is also mediated by PI 3-kinase in JB6P⁺ cells [37]. Interestingly, sequence analysis of F-type 6PF2K/Fru-2,6-BPase promoter reveals the presence of DNA-binding sites for E2F and AP-1, suggesting a hypothetical role for these sequences.

The fact that PI 3-kinase and MAPK pathways are both implicated in F-type 6PF2K/Fru-2,6-BPase transcriptional regulation is not surprising. The transcriptional activities of E2F and AP-1 are regulated by both pathways [36–40]. In addition, there is good evidence of interaction of these pathways upstream of the transcriptional response. For example, it has been shown that Ras can interact and stimulate the PI 3-kinase catalytic subunit in a GTP-dependent manner and is required for its optimal activation in response to growth factors [41]. Furthermore, the kinase activity of PI 3-kinase γ is necessary for the activation of Erk2/MAPK through MEK1 (extracellular-signal-regulated kinase/MAPK kinase) [42]. At present, it is not clear how important the synergetic interaction of PI 3-kinase and MAPK pathways is in the regulation of F-type 6PF2K/Fru-2,6-BPase enzyme. However, we cannot rule out the possibility that both pathways could act independently on the F-type 6PF2K/Fru-2,6-BPase promoter.

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