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Effect of Growth Factors on the Expression of 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase in Rat-1 Fibroblasts*

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Manel Joaquin‡§, Cristina Salvado§¶, Beatriz Bellosillo¶, Alex J. Lange‖, Joan Gil, and Albert Tauler**

From the Unitat de Bioquímica, Departament de Ciències Fisiològiques Humanes i de la Nutriciò, Universitat de Barcelona, Avinguda Diagonal 643, Barcelona 08028, Catalunya, Spain and the ‖Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455-0347

The activation of glycolytic flux is a biochemical characteristic of growing cells. Several reports have demonstrated the role of fructose 2,6-bisphosphate in this process. In this paper we show that the levels of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-P_2ase) mRNA are modulated in response to serum and growth factors and this effect is due to regulation of its transcription rate. The modulation of the expression of this enzyme by growth factors differs according their mitogenic effect; both lysophosphatidic acid and epidermal growth factor, when added alone, increased the mRNA levels, but endothelin had no effect. Furthermore, cAMP, which acts as an antimitogenic signal in Rat-1 fibroblasts, produced a decrease in 6PF2K/Fru-2,6-P_2ase mRNA and inhibited the effects of lysophosphatidic acid and epidermal growth factor on 6PF2K/Fru-2,6-P_2ase expression. PD 098059, a specific inhibitor of the activation of the mitogen-activated protein kinase, was able to prevent the effect of EGF on 6PF2K/Fru-2,6-P_2ase gene expression. These results imply that activation of mitogen-activated protein kinase is required for the stimulation of the transcription of 6PF2K/Fru-2,6-P_2ase by EGF.

Quiescent cells can be stimulated to recommence DNA synthesis by distinct and interactive signal transduction pathways. These signaling pathways trigger a cascade of molecular events required for progression of DNA replication (1, 2). One of the essential processes for this progression is the activation of the glycolytic flux (3, 4). It is known that glycolysis is stimulated both when growth promoting agents are added to quiescent cells and in many types of transformed cells (5–7). The major regulatory step in the glycolytic pathway is 6-phosphofructo-1-kinase activity. Activation of glycolysis involves the increase of fructose 2,6-bisphosphate (Fru-2,6-P_2)[1] levels, a potent allosteric stimulator of 6-phosphofructo-1-kinase (reviewed in Refs. 8 and 9). Fru-2,6-P_2 content is regulated by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-P_2ase; EC 2.7.1.105/3.1.3.46), which catalyzes both its synthesis and its degradation (8, 9). Different 6PF2K/Fru-2,6-P_2ase isoenzymes have been described depending on the tissues and cell specificity. In rat, the liver, the muscle, and the F-type isoforms are encoded by the same gene arising from distinct promoters (10, 11). These isoenzymes differ with respect to their N-terminal sequence, regulation by phosphorylation, and kinetic properties. It has been shown that the F-type is the isoform expressed in several proliferative tissues and in serum-stimulated Rat-1 fibroblasts (10). Although extensive studies have been performed on the regulation of liver and muscle isoforms, this is not the case of F-type.

Different reports have demonstrated that Fru-2,6-P_2 levels increase after stimulation of fibroblasts with fetal bovine serum, insulin, epidermal growth factor (EGF), or tumor promoter phorbol esters (12–21). In some cases, the increase in this metabolite has been correlated with an increase in the V_{max} of 6-phosphofructo-2-kinase activity (19–24). Although this metabolic effect is clear, the mechanism by which it is achieved is not known. Recently, a study of the regulation of Fru-2,6-P_2 metabolism through different signal transduction pathways has been performed using murine Swiss 3T3 fibroblasts as a model (21). However, the attempts to measure regulation of 6PF2K/Fru-2,6-P_2ase mRNA levels and transcription rate were not successful because the cDNA probes available did not recognize Swiss 3T3 6PF2K/Fru-2,6-P_2ase mRNA.

The aim of this work is to study the signaling pathways that control the transcription of the 6PF2K/Fru-2,6-P_2ase gene during cell proliferation. For this propose, we have investigated the effect of EGF, lysophosphatidic acid (LPA), endothelin, and cholera toxin (CTx) on 6PF2K/Fru-2,6-P_2ase gene expression in Rat-1 fibroblasts and the role of mitogen-activated kinase (MAPK) in this regulation.

EXPERIMENTAL PROCEDURES

Chemicals—[α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), [6-^{3}H]thymidine (5 Ci/mmol), ECL Western kit, and Hybond-N membranes were from Amersham International (Amersham, Bucks., United Kingdom (UK)). The random-primer DNA-labeling kit was from Boehringer Mannheim. Fetal calf serum, minimum essential medium, and penicillin/streptomycin were from Bio-Whittaker. EGF, LPA, insulin, and endothelin-1 were from Sigma. Anti-phosphotyrosine monoclonal antibody PY20 was from Affiniti Research Products Ltd. (Nottingham, UK), and anti-MAPK monoclonal antibody was from Zymed Laborato-

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§ These authors made equal contributions to this work.

¶ Recipient of a research fellowship from the Generalitat de Catalunya.

** To whom correspondence should be addressed. Fax: 34-3-402-1896; E-mail: tauler@farmacia.far.ub.es.

[1] The abbreviations used are: Fru-2,6-P_2, fructose 2,6-bisphosphate; 6PF2K/Fru-2,6-P_2ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MEK, MAPK or ERK kinase; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CTx, cholera toxin; MEM, minimum essential medium.

TABLE I
Effect of serum addition on fructose 2,6-bisphosphate levels

Quiescent Rat-1 fibroblasts were stimulated with 10% FCS (v/v). At the indicated incubation time, extracts for Fru-2,6-P_2 quantification were obtained. Data are means ± S.E. from three independent experiments. Statistically significant differences with respect to control cells are indicated: *, $p < 0.05$; **, $p < 0.01$.

	Fru-2,6-P_2 content		
	6 h	12 h	24 h
	pmol/mg of protein		
Control	97 ± 13	119 ± 17	81 ± 7
+ FCS	154 ± 17*	211 ± 15**	233 ± 16**

ries, Inc. (San Francisco, CA). PD 098059 was a gift from Dr. A. Saltiel, Parke-Davis Pharmaceutical Division (Detroit, MI). Other materials and chemicals were of the highest quality available.

Cell Cultures—Rat-1 fibroblasts were routinely grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37 °C in a 5% CO_2 atmosphere. For experimental purposes, cells were subcultured in six-well plates or 100-mm dishes and their quiescence was induced by maintaining the cells in serum-free medium during 24 h. After this, cells were stimulated with either FCS or different growth factors.

Measurement of DNA Synthesis—Quiescent Rat-1 cells were activated with 10% FCS and different growth factors. At the indicated times, cells were exposed to [6-^{3}H]thymidine (0.5 μCi/ml) for the last 1 h of stimulation. Trichloroacetic acid-precipitable material was dissolved in 0.1 M NaOH and quantified by liquid scintillation counting.

Fructose 2,6-Bisphosphate Determination—To measure Fru-2,6-P_2 levels, Rat-1 cells were homogenized in 0.1 M NaOH, heated at 80 °C for 15 min, and centrifuged at 12,000 × *g* for 5 min. Fru-2,6-P_2 was determined in supernatants by its ability to activate pyrophosphate-dependent 6-phosphofructo-1-kinase from potato tubers as described by Van Schaftingen *et al.* (25).

Western Blot Analysis—Immunoblot analysis were performed essentially as described by Burnette (26). For 6PF2K/Fru-2,6-P_2ase, we used a 1:1000 dilution of a polyclonal antibody raised against the rat liver protein (27). Western blot analysis of MAPK phosphorylation was performed as described previously (28) using anti-phosphotyrosine or anti-MAPK monoclonal antibodies. Bound antibodies were detected by enhanced chemiluminescence method. The amount of protein was determined by densitometric scanning of the autoradiograms.

RNA Isolation and Analysis—Total RNA was extracted from Rat-1 cells by the LiCl/urea method (29). Northern blot analyses were carried out by standard procedures (30). To detect 6PF2K/Fru-2,6-P_2ase mRNA, a 1.4-kilobase *Eco*RI fragment from pPKB plasmid was used as a probe (31). The integrity of the RNA was verified by observing the rRNA bands in the ethidium bromide gel under UV irradiation. The level of mRNA was evaluated by densitometric scanning of the autoradiograms and corrected for the amount of 18 S rRNA using a ribosomic cDNA probe (32).

Transfection and CAT Assays—Stable transfections were performed by the $CaPO_4$ method as described by Sambrook *et al.* (30). Rat-1 cells were plated at 10^6 cells/100-mm culture dish 24 h before transfection. The DNA precipitate (50 μg of the *Hin*dIII/*Bsp*mI 5′ flanking region of the F isoform of 6PF2K/Fru-2,6-P_2ase cloned into pBLCAT3 plasmid as described by Lange (11) and 5 μg of pSV2Neo) were left overnight. After 24 h, the cells were trypsinized and replated at 1:4 dilution. G418 (400 μg/ml) was added 24 h later. Transfections containing resistant colonies were pooled for experimentation. CAT activity determinations were carried out as described by Ausebel *et al.* (33).

Other Methods—Protein was determined as described by Bradford (34) with bovine serum albumin as a standard. Statistical significance of difference was assessed by Student's unpaired *t* test.

RESULTS

Regulation of Fru-2,6-P_2 Metabolism by Serum—Confluent Rat-1 fibroblasts were made quiescent by maintaining them in serum-free medium for 24 h and then shifted to medium supplemented with 10% fetal-calf serum. After serum addition, the cells were harvested and samples were taken to be analyzed at different times. The data reported in Table I show that, after being exposed to serum, an increase of the intracellular Fru-2,6-P_2 content was detected. During this process, the amount of 6PF2K/Fru-2,6-P_2ase protein also increased. This was measured by immunobloting with an antibody obtained against the liver 6PF2K/Fru-2,6-P_2ase (Fig. 1). To determine whether the abundance of 6PF2K/Fru-2,6-P_2ase protein was correlated with the amount of 6PF2K/Fru-2,6-P_2ase mRNA, Northern blot analysis was done using a 1.4-kilobase cDNA probe (31). As shown in Fig. 2, there was a transient increase in the level of 6PF2K/Fru-2,6-P_2ase mRNA with a maximum at 12–15 h (5-fold increase over serum deprivation mRNA levels) and a gradual decline to serum-free mRNA levels by 30 h. The analysis of the incorporation of [6-^{3}H]thymidine into DNA during the course of this experiment revealed that the peak in mRNA accumulation was coincident with the onset of DNA synthesis (Fig. 2).

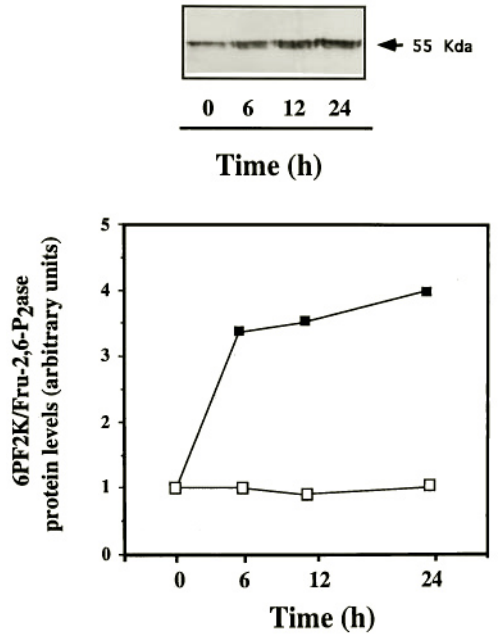

FIG. 1. **Effect of fetal calf serum on the levels of 6PF2K/Fru-2,6-P_2ase protein.** Quiescent Rat-1 fibroblasts were stimulated with MEM supplemented with 10% FCS (v/v). At the indicated incubation time, 100 μg of total protein was extracted from cultured cells in the presence (■) or in the absence (□) of FCS and analyzed by Western blot using a polyclonal antibody raised against purified adult liver 6PF2K/Fru-2,6-P_2ase. A representative Western blot obtained with preparations including FCS is shown. The intensity of the autoradiographic signal was quantified and is presented with respect to time zero, which was considered as 1 arbitrary unit.

To determine whether the regulation of 6PF2K/Fru-2,6-P_2ase mRNA levels was due to a regulation of its transcription rate, Rat-1 fibroblasts were stable transfected with a CAT reporter gene under transcriptional control of a 1.7-kilobase genomic DNA fragment that contain the F-type promoter (11). In preliminary RNase protection experiments, we observed that the F-type isoform is the only one expressed in these cells. Quiescent transfected cells were stimulated with 10% of serum, and CAT activity was measured at different times. As shown in Fig. 3, up to 4-fold induction of CAT activity was found at 4 and 8 h after addition of serum. In contrast, CAT activity did not change in cells incubated in the absence of serum during this period.

Effect of Growth Factors on 6PF2K/Fru-2,6-P_2ase mRNA Levels—In order to study the signal transduction pathways that control the expression of 6PF2K/Fru-2,6-P_2ase in Rat-1 fibroblasts during proliferation, we determined the effect of EGF, LPA, endothelin, and cholera toxin (CTx) on 6PF2K/Fru-2,6-P_2ase mRNA levels. As shown in Table II, EGF at 10 ng/ml

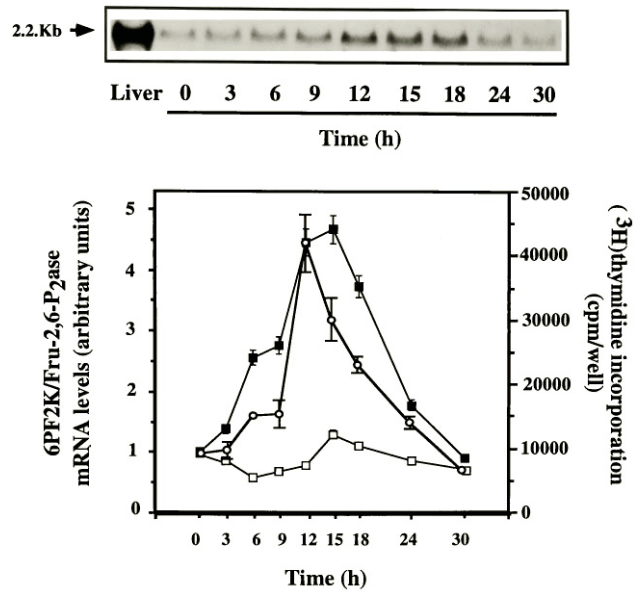

FIG. 2. **Expression of 6PF2K/Fru-2,6-P_2ase mRNA during the cell cycle of Rat-1 fibroblasts.** Quiescent Rat-1 fibroblasts were stimulated with MEM supplemented with 10% FCS (v/v). At the indicated incubation times, total RNA (20 μg/lane) was extracted from cultured cells in the presence (■) or absence (□) of FCS and analyzed by Northern blot. *Upper*, a representative Northern blot obtained with preparations including FCS is shown. *Lower*, 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 control cells, which was considered as 1 arbitrary unit. Data are means ± S.E. of three independent experiments. To measure thymidine incorporation, at the indicated incubation times, serum-stimulated cells were exposed to [6-^{3}H]thymidine (0.5 μCi) during the last 1 h of stimulation, and incorporation was assayed by the trichloroacetic acid standard procedure. Each point (○) represents the mean ± S.E. of three independent experiments, each one performed by triplicate.

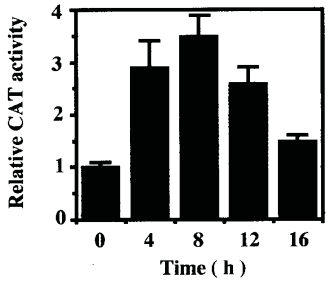

FIG. 3. **Transcriptional activity of 6PF2K/Fru-2,6-P_2ase gene during the cell cycle of Rat-1 fibroblasts Rat-1 fibroblasts stably transfected with a CAT reporter gene under control of F-type promoter were stimulated with MEM supplemented with 10% FCS (v/v).** At the indicated incubation times, CAT activity was measured from cultured cells. The intensity of the autoradiographic signal was quantified by densitometric scanning. Each point represents the mean ± S.E. of three independent experiments.

and LPA at 70 μM increased the levels of 6PF2K/Fru-2,6-P_2ase mRNA 4- and 7-fold, respectively. No significant change in 6PF2K/Fru-2,6-P_2ase mRNA levels was detected after endothelin addition. Furthermore, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (PKC) also failed to increase 6PF2K/Fru-2,6-P_2ase mRNA. In contrast, CTx at 10 ng/ml decreased the 6PF2K/Fru-2,6-P_2ase mRNA levels by approximately 50%. In agreement with the results from measurement of mRNA levels, addition of EGF and LPA to quiescent Rat-1 fibroblasts induced an increase in Fru-2,6-P_2 levels. In contrast, TPA or endothelin did not modify the concentration of this metabolite (Table III).

TABLE II

Effect of growth factors on 6PF2K/Fru-2,6-P_2ase mRNA levels

Quiescent Rat-1 fibroblasts were treated with different growth factors for 15 h at the following concentrations: FCS, 10% (v/v); EGF (10 ng/ml), LPA (70 μM), TPA (100 ng/ml), endothelin (100 nM), and cholera toxin (10 ng/ml). Total RNA (20 μg/lane) was extracted and processed by Northern-blot. 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 ± S.E. from three to five independent experiments.

Relative amount of 6PF2K/Fru-2,6-P_2ase mRNA	
	Arbitrary units
Control	1.0
+ FCS	6.2 ± 0.1
+ EGF	3.8 ± 0.3
+ LPA	7.1 ± 0.8
+ TPA	1.5 ± 0.1
+ ET-1	1.4 ± 0.2
+ CTx	0.5 ± 0.1

TABLE III

Effect of growth factors on fructose 2,6-bisphosphate levels

Quiescent Rat-1 fibroblasts were stimulated with EGF (10 ng/ml), LPA (70 μM), TPA (100 ng/ml), and cholera toxin (10 ng/ml). At the indicated incubation time, extracts for Fru-2,6-P_2 quantification were obtained. Data are means ± S.E. from three independent experiments. Statistically significant differences with respect to control cells are indicated: *, $p < 0.05$.

	Fructose 2,6-P_2 content
	pmol/mg of protein
Control	119 ± 17
+ EGF	167 ± 3*
+ LPA	188 ± 17*
+ TPA	111 ± 25
+ CTx	110 ± 11

Both EGF and LPA induce the activation of the mitogen-activated protein kinases ERK1 and ERK2 (MAPKs) through the Ras-Raf-MEK pathway (35, 36). In Rat-1 fibroblasts, cAMP can inhibit the activation of MAPKs by inhibiting Raf activation (37). To examine the correlation between MAPK activation and the increase in 6PF2K/Fru-2,6-P_2ase mRNA found after mitogenic responses, quiescent Rat-1 cells were preincubated with CTx for 90 min before stimulation with EGF and the 6PF2K/Fru-2,6-P_2ase mRNA levels were then measured. The phosphorylation state of ERK2 is shown in Fig. 4. As we expected, a phosphotyrosine protein that comigrated with ERK2 was detected after EGF and serum addition, but it was almost undetectable with TPA. Preincubation with CTx blocked the effect of EGF on ERK2 phosphorylation (*panel B*). Under these experimental conditions, EGF produced a 4-fold increase on the levels of 6PF2K/Fru-2,6-P_2ase mRNA and preincubation with CTx completely inhibited this effect (*panel C*). The same results were obtained with LPA (data not shown).

Recently, PD 098059 has been identified as a specific cell-permeant inhibitor of MEK (38). To prove that MAPK activation is involved in mediating the effect of EGF on 6PF2K/Fru-2,6-P_2ase expression, we examined the effect of this compound on 6PF2K/Fru-2,6-P_2ase mRNA levels. As shown in Fig. 5, PD 098059 is able to prevent the increase on 6PF2K/Fru-2,6-P_2ase mRNA by EGF. The concentration of inhibitor required to avoid this activation was similar to the one reported to inhibit MAPK (38).

The ability of PD 098059 to inhibit the effect EGF on 6PF2K/Fru-2,6-P_2ase mRNA levels was correlated with its ability to prevent the activation of 6PF2K/Fru-2,6-P_2ase transcription. Cells stably transfected with a CAT reporter gene under transcriptional control of 1.7-kilobase F-type promoter, were incubated at different concentrations of PD 098059 and CAT activ-

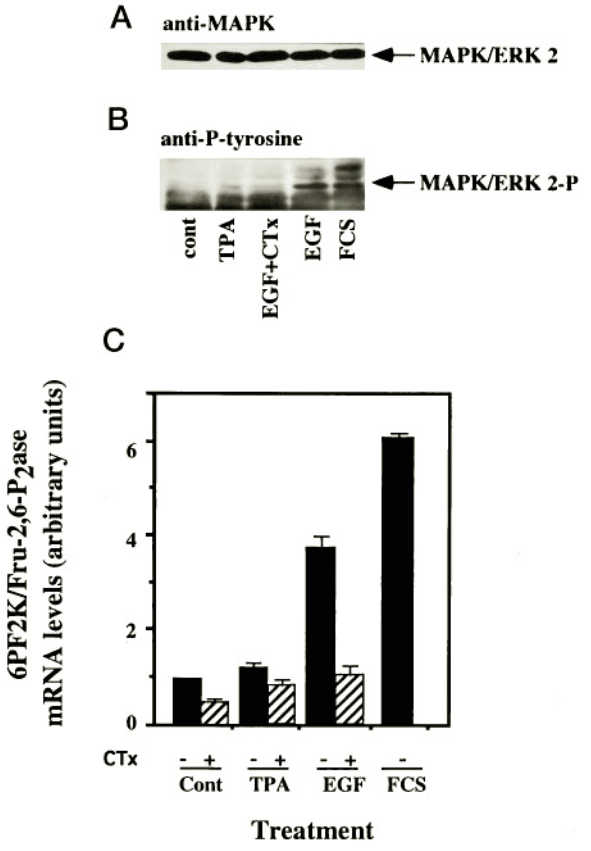

FIG. 4. **Effect of cAMP on MAPK activation and 6PF2K/Fru-2,6-P_2ase mRNA levels.** For MAPK determination, quiescent Rat-1 fibroblasts were subjected to treatment: FCS (10% v/v), EGF (10 ng/ml), TPA (100 ng/ml) for 5 min. In the case of EGF + CTx, cells were preincubated with CTx (10 ng/ml) for 90 min before EGF treatment. After the treatment, protein extracts were obtained and processed by Western blot and MAPK. *Panel A*, MAPK was detected by Western blotting using an anti-MAPK monoclonal antibody. *Panel B*, activated MAPK was detected using the PY20 anti-phosphotyrosine antibody. *Panel C*, quiescent Rat-1 fibroblasts were treated with the different agonists for 15 h. Total RNA (20 μg/lane) were extracted and processed by Northern blot. 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 ± S.E. from three to five independent experiments.

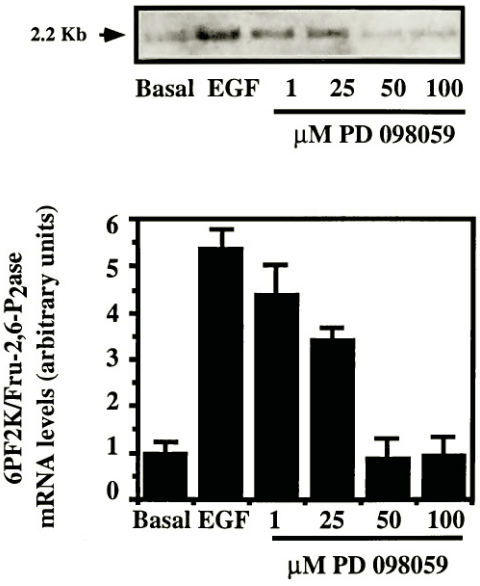

FIG. 5. **Effect of MAPK inhibitor PD 098059 on 6PF2K/Fru-2,6-P_2ase mRNA levels.** Quiescent Rat-1 fibroblasts were cultured for 15 h with EGF (10 ng/ml) with the indicated concentrations of PD 098059. Total RNA (20 μg/lane) was extracted from cultured cells and analyzed by Northern blot. *Upper*, a representative Northern blot is shown. *Lower*, 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 ± S.E. from three to five independent experiments.

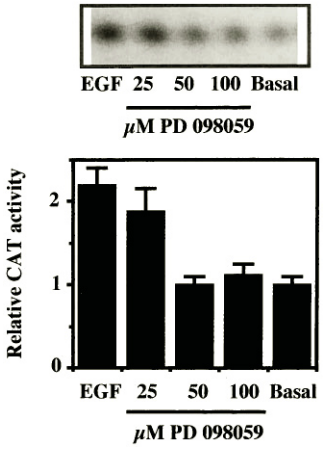

FIG. 6. **Effect of MAPK inhibitor PD 098059 on the stimulation of 6PF2K/Fru-2,6-P_2ase transcription by EGF.** Rat-1 fibroblasts stably transfected with a CAT reporter gene under control of F-type promoter were incubated for 1 h in the absence or in the presence of the indicated concentration of PD 098059, then stimulated for another 8 h in the absence (basal) or in the presence of EGF (10 ng/ml). *Upper*, a autoradiography of a thin-layer chromatography plate from a representative CAT assay is shown. *Lower*, the intensity of the autoradiographic signal was quantified by densitometric scanning. Values are expressed relative to the value of untreated cells which was considered as 1 arbitrary unit. Data are means ± S.E. from three independent experiments.

ity was assayed 8 h after addition of EGF. As expected, PD 098059 suppressed the increase of CAT activity induced by EGF and the activity remaining at basal levels at high concentration of the inhibitor (Fig. 6).

DISCUSSION

Several reports have pointed out the essential role of Fru-2,6-P_2 in the activation of glycolytic flux in growing cells (3, 4). The levels of this metabolite increase in different proliferative states (12–24, 39), and this change has been correlated with an increase in 6-phosphofructo-2-kinase activity (18–24) and recently with an increase in mRNA levels (40, 41). However, the mechanisms by which growth factors regulate 6PF2K/Fru-2,6-P_2ase are still unclear. The results presented here demonstrate that MAPK is involved in the regulation of 6PF2K/Fru-2,6-P_2ase gene transcription in response to mitogenic signals.

The modulation of the expression of 6PF2K/Fru-2,6-P_2ase by growth factors is concomitant with their mitogenic response. Serum, LPA, and EGF, complete mitogens for Rat-1 fibroblasts (42), were able to increase the mRNA levels of 6PF2K/Fru-2,6-P_2ase and Fru-2,6-P_2 content. In contrast, endothelin, which has a low mitogenic activity (43), had no significant effect on the expression of this enzyme. Indeed, cAMP produced a decrease in 6PF2K/Fru-2,6-P_2ase mRNA levels and inhibited the effect of LPA and EGF. In agreement with these results, Darville *et al.* (41) have demonstrated that cAMP was also able to block the effect of serum on 6PF2K/Fru-2,6-P_2ase transcription. Previous studies reported a different effect of cAMP on Fru-2,6-P_2 levels in fibroblasts. In Swiss 3T3 (21) and human fibroblasts (16), cAMP induces an increase in the concentration of Fru-2,6-P_2, while in chick-embryo fibroblast (19), the pres-

ence of dibutyryl cAMP did not change the levels of this metabolite. It is very likely that the discrepancy between these results is due to the different mitogenic role of cAMP in these cells; cAMP is a mitogenic signal in Swiss 3T3 cells (44), but it is a strong antimitogen in Rat-1 fibroblasts (37).

With regard to the signaling pathways that control the transcription of the 6PF2K/Fru-2,6-P_2ase gene during cell proliferation, our results suggest that agents or growth factors (LPA, EGF) that activate the Ras-Raf-MEK-ERK pathway also increase the levels of 6PF2K/Fru-2,6-P_2ase mRNA. In Rat-1 fibroblasts, EGF promotes the activation of ERK1 and ERK2 proteins through a Ras-dependent pathway without affecting inositol phosphate levels (42, 45). In the same cells, LPA activates at least three independent signaling cascades by coupling its receptor to G_i and G_q proteins: stimulation of phospholipases C and D via a pertussis toxin-insensitive G protein (G_q), inhibition of adenylate cyclase via a pertussis toxin-sensitive G protein ($G_i\alpha$), and activation of Ras via a pertussis toxin-sensitive G_i protein ($G\beta\gamma$) (46). The activation of the Ras cascade by LPA produces the stimulation of MAPK (36). In Rat-1 fibroblasts, Ras is a point of convergence of EGF and LPA signaling pathways in the activation of MAPK. It has been shown that raising the cAMP levels in the cell induces the hyperphosphorylation of Raf-1 by PKA, reducing the binding affinity of Raf-1 to Ras and consequently inhibiting the Ras-Raf-MEK-ERK pathway (47). Our results show that the inhibition of this pathway by cAMP also leads to an inhibition of the stimulatory effect of EGF and LPA on 6PF2K/Fru-2,6-P_2ase expression. The effect of cAMP on 6PF2K/Fru-2,6-P_2ase expression is probably due to inhibition of MAPK activation. The implication of MAPK cascade on the expression of this enzyme has been suggested previously (41). However, definitive experiments were necessary to support this tentative hypothesis. Our finding that a specific inhibitor of MEK can prevent the effect of EGF on 6PF2K/Fru-2,6-P_2ase mRNA levels and on the transcriptional levels provides further evidence to confirm the idea that MAPK is involved in regulating 6PF2K/Fru-2,6-P_2ase expression during proliferative processes.

Activation of PKC increases Fru-2,6-P_2 levels in different cells (16–24). Although there is no direct evidence, several reports suggest that PKC may regulate 6PF2K/Fru-2,6-P_2ase gene expression. In chick embryo fibroblasts, incubation with TPA produced an increase in 6PF2K/Fru-2,6-P_2ase activity and this effect was abolished in cells incubated with actinomycin D, cycloheximide, or puromycin (19). In fetal hepatocytes (22) and Swiss 3T3 cells (21), the rise in 6PF2K/Fru-2,6-P_2ase activity produced by phorbol esters was inhibited by cycloheximide, suggesting that it is due to enhanced expression of the protein. Our results demonstrate that, in Rat-1 fibroblasts, endothelin, a strong stimulator of PKC, and TPA, had no detectable effect on 6PF2K/Fru-2,6-P_2ase mRNA levels and Fru-2,6-P_2, suggesting that activation of PKC pathway alone is not enough to stimulate 6PF2K/Fru-2,6-P_2ase expression in these cells. It has been reported that, in Rat-1 fibroblasts, endothelin fails to stimulate ERK phosphorylation, despite its potent coupling to the phospholipase C-PKC second messenger cascade (36). The different contribution of PKC to MAPK activation could explain the fact that, in Swiss 3T3 cells, bombesin, another phospholipase C-activating agonist, was able to increase 6PF2K/Fru-2,6-P_2ase activity (21). In these cells, bombesin stimulates MAPK activity via a pathway that is almost entirely dependent on PKC (48).

Sequence analysis of the F promoter of 6PF2K/Fru-2,6-P_2ase reveals the presence of several DNA-binding sites that could directly or indirectly regulate the transcription of the 6PF2K/Fru-2,6-P_2ase gene by MAPK activation. A region containing three binding motifs for transcription factors of the *ets* oncogene family has been found in the F promoter (10). The transcriptional activity of various Ets-related transcription factors is regulated by MAPK. Thus, c-Ets-2 is both transcriptionally activated and phosphorylated by MAPK (49). Furthermore, phosphorylation of the Ets-related transcription factor $p62^{TCF}$/Elk-1 by MAPK stimulates ternary complex formation at c-*fos* promoter, thus activating the *fos* transcription and consequently, the AP-1 DNA binding activity (50–52). Darville *et al.* (53) reported an AP-1 DNA-binding site about 250 bp downstream of the F promoter, and several reports show that an intronic AP-1 site could regulate gene expression (54–56). Indeed, in the same 6PF2K/Fru-2,6-P_2ase gene, a potent glucocorticoid response element was identified in the first intron of liver transcript (11).

Recently, an E2F binding site localized at the 5′ end of the first exon has been suggested to be involved in the serum regulation of F promoter (41). E2F transcriptional activity is blocked by its binding to hypophosphorylated retinoblastoma protein (pRB). G_1 cyclins, including cyclin D1, forms complexes with cyclin-dependent kinases which can phosphorylate pRB, causing to release E2F (57). Although there is no evidence, MAPK pathway could regulate the dissociation of E2F from retinoblastoma protein (pRB) leading to activation of E2F target genes. In this sense, EGF induction of cyclin D1 transcription was antagonized by either dominant negative MAPK or dominant negatives of Ets-2 activation, suggesting that MAPK and Ets-2 function downstream of EGF in the context of cyclin D1 induction (58). It would be very interesting to study whether this putative MAPK-Ets-cyclin D1-pRB-E2F pathway controls 6PF2K/Fru-2,6-P_2ase expression.

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