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

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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-2-kinase activity. Activation of glycolysis involves the increase of fructose 2,6-bisphosphate (Fru-2,6-P$_2$) 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$_2$-ase; 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$_2$-ase 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 $V_{\text{max}}$ 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$_2$-ase mRNA levels and transcription rate were not successful because the cDNA probes available did not recognize Swiss 3T3 6PF2K/Fru-2,6-P$_2$-ase mRNA.

The aim of this work is to study the signaling pathways that control the transcription of the 6PF2K/Fru-2,6-P$_2$-ase 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$_2$-ase gene expression in Rat-1 fibroblasts and the role of mitogen-activated kinase (MAPK) in this regulation.

EXPERIMENTAL PROCEDURES

Chemicals—[α-32P]dCTP (3000 Ci/mmol), [α-32P]UTP (3000 Ci/mmol), [6-3H]thymidine (5 Ci/mmol), [3H]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 Boehring 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 Laboratories.
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-P2 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-P2 content | 6 h | 12 h | 24 h |
|--------------------|-----|------|------|
| Control            | 97 ± 13 | 119 ± 17 | 81 ± 7 |
| + FCS              | 154 ± 17** | 211 ± 15** | 233 ± 16** |

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.

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.
and LPA at 70 μM increased the levels of 6PF2K/Fru-2,6-P2ase mRNA 4- and 7-fold, respectively. No significant change in 6PF2K/Fru-2,6-P2ase 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-P2ase mRNA. In contrast, CTx at 10 ng/ml decreased the 6PF2K/Fru-2,6-P2ase 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-P2 levels. In contrast, TPA or endothelin did not modify the concentration of this metabolite (Table III).

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-P2ase 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-P2ase 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-P2ase 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-P2ase expression, we examined the effect of this compound on 6PF2K/Fru-2,6-P2ase mRNA levels. As shown in Fig. 5, PD 098059 is able to prevent the increase on 6PF2K/Fru-2,6-P2ase 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-P2ase mRNA levels was correlated with its ability to prevent the activation of 6PF2K/Fru-2,6-P2ase 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-
The activity 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 concentrations of the inhibitor (Fig. 6).

**DISCUSSION**

Several reports have pointed out the essential role of Fru-2,6-P2 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-P2ase are still unclear. The results presented here demonstrate that MAPK is involved in the regulation of 6PF2K/Fru-2,6-P2ase gene transcription in response to mitogenic signals.

The modulation of the expression of 6PF2K/Fru-2,6-P2ase 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-P2ase and Fru-2,6-P2 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-P2ase 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-P2ase transcription. Previous studies reported a different effect of cAMP on Fru-2,6-P2 levels in fibroblasts. In Swiss 3T3 (21) and human fibroblasts (16), cAMP induces an increase in the concentration of Fru-2,6-P2, 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 adenylyl cyclase via a pertussis toxin-sensitive G protein (G_iα), and activation of Ras via a pertussis toxin-sensitive G protein (G_iβγ) (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 Rap-1 by PKA, reducing the binding affinity of Rap-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 enhancement of the protein expression. 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 p62TEC/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_s, 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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