# Phorbol ester-induced activation of mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase and extracellular-signal-regulated protein kinase decreases glucose-6-phosphatase gene expression

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Glucose-6-phosphatase (G6Pase) plays a central role in blood glucose homoeostasis, and insulin suppresses *G6Pase* gene expression by the activation of phosphoinositide 3-kinase (PI 3-kinase). Here, we show that the phorbol ester PMA decreases both basal and dexamethasone/cAMP-induced expression of a luciferase gene under the control of the G6Pase promoter in transiently transfected H4IIE hepatoma cells. This regulation was suppressed by the inhibitors of the mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), PD98059 and U0126, but not by the inhibitor of PI 3-kinase, LY294002. The co-expression of a constitutively active mutant of MEK mimicked the regulation of G6Pase promoter activity by PMA. The effect of PMA on both basal and induced *G6Pase* gene transcription was impaired by the overexpression of a dominant negative MEK construct, as well as by the expression of mitogen-activated protein kinase phosphatase-1. The mutation of the forkhead-binding sites within the insulin-response unit of the G6Pase promoter, which decreases the effect of insulin on *G6Pase* gene expression, did not alter the regulation of gene expression by PMA. The data show that PMA decreases *G6Pase* gene expression by the activation of MEK and extracellular-signal regulated protein kinase. With that, PMA mimics the effect of insulin on *G6Pase* gene expression by a different signalling pathway.

Key words: diabetes, gluconeogenesis, insulin, liver, protein kinase B.

## INTRODUCTION

Glucose-6-phosphatase (G6Pase) catalyses the hydrolysis of glucose 6-phosphate to glucose, which is the terminal step of both hepatic gluconeogenesis and glycogen breakdown [1]. *G6Pase* gene expression is induced by cAMP and glucocorticoids and is reduced by insulin. In Type II diabetes *G6Pase* gene expression is increased, which contributes to elevated hepatic glucose production [2]. Furthermore, the adenovirus-mediated overexpression of G6Pase in rat liver causes a disturbance of glucose and lipid homoeostasis, which is similar to early stage Type II diabetes [3]. Because of the potential involvement of an increased *G6Pase* gene expression in the development of metabolic changes associated with Type II diabetes [1–3], the molecular pathways leading to a decrease in *G6Pase* gene expression are of particular interest. Most studies have focused on the signalling pathways involved in the regulation of *G6Pase* gene transcription by insulin. *In vitro* models have shown that insulin decreases both basal and glucocorticoid/cAMP-induced *G6Pase* gene transcription by the activation of phosphoinositide 3-kinase (PI 3-kinase) [4,5] and not the mitogen-activated protein kinase (MAPK) cascade Raf/MAPK kinase (MEK)/extracellular-signal-regulated protein kinase (ERK) [4]. The activation of PI 3-kinase leads via 3-phosphoinositide-dependent kinase 1 to the stimulation of protein kinase B (PKB) [6,7]. PKB is partially responsible for the regulation of *G6Pase* gene transcription by insulin [5]. PKB regulates *G6Pase* gene transcription by the insulin-response unit (IRU) within the G6Pase promoter. The IRU is located between nt $-196$ and $-156$ in the human promoter [4,8,9]. It contains three forkhead-binding sites, with sequences similar to the insulin-response sequence of the phosphoenolpyruvate carboxykinase (PEPCK) promoter [8,9]. The forkhead in rhabdosarcoma transcription factor (FKHR) stimulates the *G6Pase* gene expression by binding to the IRU. PKB suppresses the FKHR-transactivated *G6Pase* gene transcription by the phosphorylation of FKHR [5]. However, other, as yet unknown, mechanisms that are independent of PKB, forkhead proteins and the IRU also account for the effect of insulin on *G6Pase* gene transcription.

Phorbol esters, such as PMA, initiate signalling processes that are different from those activated by insulin. PMA imitates stimuli that act via the generation of diacylglycerol and the subsequent activation of conventional and novel isoforms of the protein kinase C family [10]. This leads to a stimulation of the MAPK cascade Raf/MEK/ERK, presumably by the direct phosphorylation of Raf [11,12]. Raf phosphorylates and activates both isoenzymes MEK1 and MEK2. MEK stimulates the two isoenzymes ERK1 and ERK2 with high specificity. PMA imitates the effect of insulin on the eukaryotic initiation factor 4F assembly and general protein synthesis by the activation of the Raf/MAPK/ERK module [13]. Like insulin, PMA is also able to inhibit *PEPCK* gene expression, but this effect is independent of MEK and ERK [14,15]. However, PMA can also exhibit effects opposite to insulin, e.g. the gene expression of the insulin-like-growth-factor-binding protein-1 is suppressed by insulin, but induced by PMA [16].

Abbreviations used: $Bt_2$cAMP, $N^6$,2′-*O*-dibutyryl cAMP; ERK, extracellular-signal-regulated protein kinase; FKHR, forkhead in rhabdosarcoma; G6Pase, glucose-6-phosphatase; IRU, insulin-response unit; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKP-1, MAPK phosphatase-1; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B.

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In the present study we report that PMA suppresses *G6Pase* gene transcription; however, in contrast with insulin, PMA decreases *G6Pase* gene expression by the activation of MEK and ERK.

## EXPERIMENTAL

### Materials

LY294002, PD98059, U0126 and PMA were obtained from Calbiochem. Myelin basic protein and Protein A–Sepharose were from Sigma. Anti-ERK2 and anti-PKB$\alpha$ antibodies were from Santa Cruz Biotechnology. Radioisotopes were purchased from Amersham Pharmacia Biotech. All other chemicals were purchased in the highest grade available from either Merck or Roche.

### Plasmids

The G6Pase reporter gene construct G6Pase($-1227/+57$) was created by cloning the human G6Pase promoter fragment $-1227$ to $+57$ into the promoterless luciferase reporter gene vector pGL3 basic (Promega) [5]. To generate the promoter gene construct G6Pase($-1227/+57$/IRUmut) the forkhead-binding sites of the IRU were mutated within the vector G6Pase($-1227/+57$) from $-196$ 5′-CGATCAGGCTGTTT-TTGTGTGCCTGTTTTTCTATTTTACG-3′ $-156$ into 5′-CGATCAGGCTCGAGTTGTGTGCCTCTTTTTCTCTTT-TACG-3′ (mutated nucleotides are underlined), as described in [5]. This prevents the regulation of G6Pase promoter activity by PKB and FKHR and also impairs the insulin effect [5]. The expression vectors for the wild-type MAP kinase phosphatase-1 (pSG-MKP-1) and the catalytically inactive mutant (pSG-MKP-1-CS) were kindly provided by Dr N. Tonks [17]. The vectors expressing catalytically inactive, dominant negative MEK-SA222 and constitutively active MEK-SS/DD were generously provided by Dr G. Pages and Dr J. Pouyssegur [18].

### Transfections

H4IIE cells were transfected with 8.5 $\mu$g/dish of the reporter vector, 0.5 $\mu$g/dish of pRL-TK control plasmid (Promega) and, where indicated, with 2 $\mu$g/dish of the expression vectors for MEK or MKP-1 using the calcium phosphate/DNA co-precipitation method, essentially as described in [5]. After the glycerol shock, the cells were serum starved for 1 h and then incubated in the presence or absence of dexamethasone (1 $\mu$M), $N^6$,2′-*O*-dibutyryl cAMP ($Bt_2$cAMP; 500 $\mu$M) and PMA (1 $\mu$M). In some experiments transfected cells were incubated for 10 min with the PI 3-kinase inhibitor LY294002 (100 $\mu$M) and for 60 min with the MEK inhibitors PD98059 (50 $\mu$M) or U0126 (20 $\mu$M) before the other hormones were added. After 20 h cell extracts were prepared and luciferase activities were determined using the dual luciferase assay reagent (Promega), according to the manufacturer's instructions. All experiments were performed at least three times each in triplicate with at least two different DNA preparations. Statistical analysis was performed using the *t* test for unpaired data.

### Immunoprecipitation and *in vitro* kinase assay

H4IIE cells were serum starved for 16 h and incubated in the presence or absence of LY294002 (100 $\mu$M), PD98059 (50 $\mu$M) and U0126 (20 $\mu$M) as described above, prior to the addition of PMA (1 $\mu$M), insulin (5 nM), dexamethasone (1 $\mu$M) and $Bt_2$cAMP (500 $\mu$M), as indicated. At the indicated times the cells were lysed in buffer A [50 mM Tris/HCl, pH 7.5, 0.1 % (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium $\beta$-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM dithiothreitol and protease inhibitor tablets (Roche)], and cell debris was removed by centrifugation. Supernatants were incubated with 2 $\mu$g of anti-ERK2 antibody protein–agarose complex or 2 $\mu$g of anti-PKB antibody protein–agarose complex as indicated, then washed three times with buffer A containing 500 mM NaCl and twice with buffer B [50 mM Tris/HCl, pH 7.5, 0.03 % (w/v) Brij 35, 0.1 mM EGTA and 1 mM dithiothreitol]. Immunoprecipitates were used for *in vitro* kinase assays. ERK2 assays were performed as described [19] in 50 $\mu$l incubations {25 mM Tris/HCl, pH 7.5, [$\gamma$-$^{32}$P]ATP (100 $\mu$M, 5000000 c.p.m.), 10 mM magnesium acetate, 100 $\mu$M EGTA and 0.33 mg/ml myelin basic protein} for 10 min at 30 °C. PKB assays were performed in the same buffer with the peptide Crosstide (30 $\mu$M, GRPRTSSFAEG; provided by Dr D. Alessi) instead of myelin basic protein as substrate [7]. In control assays the respective substrates, myelin basic protein or Crosstide peptide, were omitted. The assays were terminated by spotting on phosphocellulose paper. All papers were washed five times with 0.5 % (v/v) phosphoric acid to remove ATP and once in acetone. Radioactivity was measured by liquid-scintillation counting. One unit of protein kinase activity was the amount that led to the incorporation of 1 nmol phosphate into the substrate in 1 min.

### Western-blot analysis

H4IIE cell lysates were prepared as described above. Equal amounts of cell lysate protein were separated by SDS/PAGE (12.5 %) and transferred on to nitrocellulose membranes. Even transfer was verified by Ponceau staining. Membranes were blocked in 3 % BSA in TBS-T [15 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1 % (v/v) Triton X-100] for 30 min. The blots were incubated with anti-phospho ERK1/2 antibodies (Cell Signaling, Beverly, MA, U.S.A.) in blocking buffer overnight at 4 °C. After washing the blots three times with TBS-T, they were incubated with peroxidase-conjugated anti-rabbit Ig for 1 h and then washed again five times with TBS-T. Detection was performed by ECL® (Amersham Pharmacia). Subsequently, the blots were stripped and re-probed with an antibody specific for ERK1/2 (Cell Signaling).

### RNA isolation and Northern blots

H4IIE cells (800000) were serum starved for 16 h and incubated in the presence or absence of dexamethasone (1 $\mu$M), $Bt_2$cAMP (500 $\mu$M) and PMA (1 $\mu$M) for 5 h. In some experiments, transfected cells were incubated with the inhibitors PD98059 (50 $\mu$M) and U0126 (20 $\mu$M) as described, before the other hormones were added. RNA was isolated using the RNeasy Minikit (Qiagen). A 15 $\mu$g portion of total RNA was separated on a 1 % (w/v) agarose/6.6 % (v/v) formaldehyde denaturing gel and transferred on to nylon membranes by vacuum blotting. The transferred RNA was cross-linked by UV light. The membrane was prehybridized for 3 h at 42 °C in hybridization buffer [50 % (v/v) formamide, 100 $\mu$g/ml salmon sperm DNA, 1 M NaCl, 100 mg/ml dextran sulphate and 1 % (w/v) SDS]. It was then hybridized overnight at 42 °C using random primed $^{32}$P-labelled probe for G6Pase (Prime-a-gene; Promega) in hybridization buffer. The membrane was washed at 65 °C for 30 min twice with 300 mM NaCl, 35 mM sodium citrate, 0.1 % (w/v) SDS, pH 7.2, and twice with 30 mM NaCl, 3.5 mM sodium citrate, 0.1 % (w/v) SDS, pH 7.2. Transcripts were visualized and quantified by phosphorimaging. Subsequently, the blots

were rehybridized using a random primed $^{32}$P-labelled probe for actin.

## RESULTS

### PMA inhibits *G6Pase* gene expression

The transcription of the *G6Pase* gene is induced by dexamethasone and cAMP and decreased by insulin in hepatic cells [1,4,5,8]. In order to study the regulation of the G6Pase promoter by PMA, H4IIE hepatoma cells were transiently transfected with a luciferase expression vector under control of the G6Pase promoter fragment $-1227/+57$. The administration of PMA was able to decrease both basal and $Bt_2$cAMP/dexamethasone-induced G6Pase luciferase activity (Table 1). With that, PMA exhibited an insulin-mimetic effect. However, the inhibitory effect of PMA was less than that of insulin. PMA inhibited the basal and induced gene transcription by approx. $34\pm5\%$ and $67\pm6\%$ respectively, compared with the insulin effect of approx. $84\pm2\%$ and $90\pm5\%$ respectively. The PI 3-kinase inhibitor LY294002 did not significantly affect the inhibition of the basal G6Pase promoter activity by PMA, but partially prevented the suppression of induced gene transcription by the phorbol ester. In parallel incubations, LY294002 almost completely blocked the regulation of the G6Pase promoter by insulin, as has been described previously [5]. These results indicate that, in contrast with the regulation by insulin, PI 3-kinase does not have a central role in the regulation of *G6Pase* gene transcription by PMA.

### Inhibition of MEK blocks the PMA effect on *G6Pase* gene expression

We then analysed the potential involvement of the MEK/ERK cascade in the inhibition of *G6Pase* gene transcription by PMA. To do this, H4IIE cells were transiently transfected with the G6Pase promoter construct G6Pase($-1227/+57$) and pre-incubated with either PD98059 or U0126 prior to the application of the phorbol ester. As shown in Figure 1, both inhibitors of MEK blocked the regulation of basal (Figure 1A) as well as $Bt_2$cAMP/dexamethasone-induced (Figure 1B) G6Pase promoter activity. It has been previously reported [4] that PD98059 does not significantly block the regulation of induced *G6Pase* gene expression by insulin. We confirm this data and show, furthermore, that PD98059 also does not block the regulation of basal promoter activity by insulin. In addition, the structurally unrelated MEK inhibitor U0126 is also unable to alter the regulation of both basal and induced G6Pase promoter activity by insulin. The data suggest that the activation of MEK is not involved in the regulation of *G6Pase* gene expression by insulin, but is essential for the suppression of *G6Pase* gene transcription by PMA.

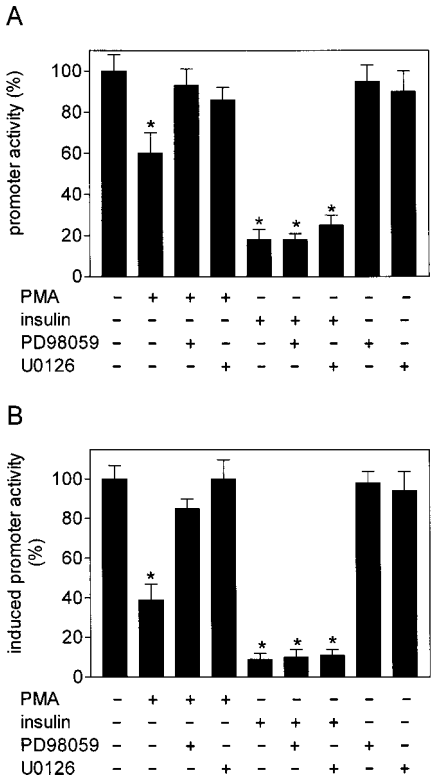


**Figure 1 MEK inhibitors PD98059 and U0126 block the suppression of basal (A) and induced (B) G6Pase promoter activity by PMA, but not by insulin**

H4IIE cells were co-transfected with 8.5 $\mu$g/dish of the promoter plasmid G6Pase($-1227/+57$) and 0.5 $\mu$g/dish of pRL control plasmid. The transfected cells were incubated with or without PMA (1 $\mu$M), insulin (5 nM) and the MEK inhibitors PD98059 (50 $\mu$M) and U0126 (20 $\mu$M), as indicated, and in the absence (**A**) or presence (**B**) of $Bt_2$cAMP (500 $\mu$M) dexamethasone (1 $\mu$M). Data are presented as means $\pm$ S.E.M. ($n = 3$) to either basal (**A**) or the induced (**B**) luciferase activities, which was set as 100. $Bt_2$cAMP/dexamethasone led to $6.8\pm0.6$-fold induction of the promoter activity compared with the basal activity. $^*P < 0.05$ compared with the basal or induced activity.

**Table 1 Suppression of basal and $Bt_2$cAMP/dexamethasone-induced G6Pase promoter activity by PMA and insulin**

H4IIE cells were co-transfected with 8.5 $\mu$g/dish of the promoter plasmid G6Pase($-1227/+57$) and 0.5 $\mu$g/dish of pRL control plasmid. Cells were incubated with or without PMA (1 $\mu$M), insulin (5 nM) and the PI 3-kinase inhibitor LY294002 (100 $\mu$M) as indicated, and in the absence (basal) or presence (induced) of the combination of $Bt_2$cAMP (500 $\mu$M)/dexamethasone (1 $\mu$M). Data are presented as means $\pm$ S.E.M. ($n = 3$) of fold-activation relative to either basal or the induced luciferase activity, which was set as 100. $Bt_2$cAMP/dexamethasone led to $8.0\pm0.9$-fold induction of the promoter activity. $^*P < 0.05$ compared with the basal or induced activity.

| | Relative promoter activity | |
|---|---|---|
| Treatment | Basal | Induced |
| None | $100\pm8$ | $100\pm10$ |
| PMA | $66\pm5^*$ | $33\pm7^*$ |
| PMA/LY294002 | $66\pm4^*$ | $56\pm8^*$ |
| Insulin | $16\pm2^*$ | $10\pm5^*$ |
| Insulin/LY294002 | $88\pm9$ | $80\pm8$ |
| LY294002 | $100\pm6$ | $85\pm7$ |

### PMA reduces G6Pase mRNA levels by the activation of MEK

We next studied whether, in H4IIE cells, the expression of endogenous G6Pase is also regulated by PMA via the activation of MEK and ERK. Northern-blot analysis (Figure 2) showed that, similar to the regulation of the G6Pase promoter construct, PMA decreased the amount of basal as well as $Bt_2$cAMP/dexamethasone-induced endogenous G6Pase transcript. Again, this suppression by PMA was impaired by the MEK inhibitors PD98059 and U0126. This indicates that MEK contributes to the regulation of the amount of endogenous G6Pase transcript by PMA. Furthermore, we observed an induction of basal and $Bt_2$cAMP/dexamethasone-induced *G6Pase* gene expression by PD98059 in the absence of PMA.

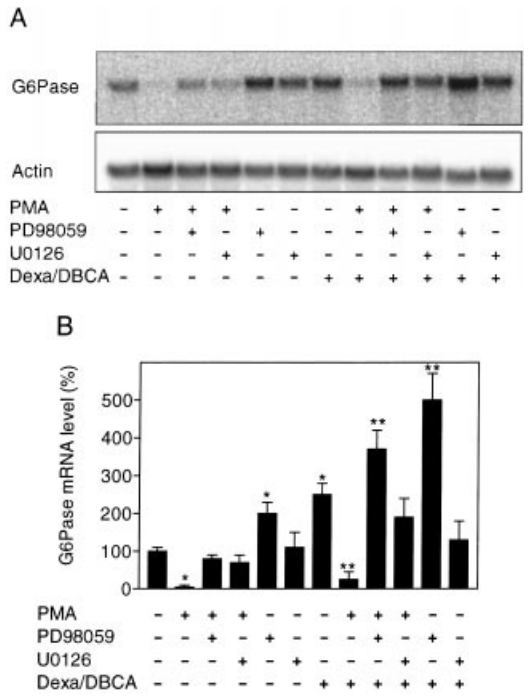


**Figure 2 MEK inhibitors PD98059 and U0126 suppress the PMA effect on the amount of basal and $Bt_2$cAMP/dexamethasone-induced G6Pase mRNA transcript in H4IIE cells**

H4IIE cells were incubated in the presence or absence of PMA (1 $\mu$M) and the MEK inhibitors PD98059 (50 $\mu$M) and U0126 (20 $\mu$M) and in the absence or presence of $Bt_2$cAMP (DBCA; 500 $\mu$M)/dexamethasone (Dexa; 1 $\mu$M). RNA (15 $\mu$g) was electrophoresed, transferred on to nylon membranes and hybridized using a random primed $^{32}$P-labelled probe for G6Pase. Actin was used as a normalization control. (**A**) Northern blot, (**B**) densitometric analysis of three Northern blots (means $\pm$ S.E.M.; $n = 3$). The G6Pase mRNA level is shown relative to that under basal conditions. $^{*}P < 0.05$ compared with basal expression, $^{**}P < 0.05$ compared with expression in the presence of only dexamethasone and $Bt_2$cAMP.

**Table 2 Regulation of basal and induced G6Pase promoter activity by a constitutively active MEK mutant and a dominant negative MEK mutant**

H4IIE cells were co-transfected with 8.5 $\mu$g/dish of the promoter plasmid G6Pase($-1227/+57$), 0.5 $\mu$g/dish of pRL control plasmid and 2 $\mu$g/dish of either dominant negative MEK (MEK-SA222), constitutively active MEK (MEK-SS/DD) or a vector control (pECE). Cells were incubated with or without PMA (1 $\mu$M) as indicated, and in the absence (basal) or presence (induced) of $Bt_2$cAMP (500 $\mu$M) and dexamethasone (1 $\mu$M). Data are presented as means $\pm$ S.E.M. ($n = 3$) of fold-activation relative to either basal or the induced luciferase activity after the co-expression of the vector control, which was set as 100. $^{*}P <$ 0.05 compared with the respective promoter activity in the absence of PMA, $^{**}P < 0.05$ compared with the basal or induced activity after the co-expression of the vector control in the absence of PMA, $^{***}P < 0.05$ compared with the PMA-inhibited activities after the co-expression of the constitutively active mutant and the vector control, $\dagger P < 0.05$ compared with the respective promoter activity without PMA and to the PMA-inhibited activities after the co-expression of the constitutively active mutant and the vector control.

| | | Relative promoter activity | |
|---|---|---|---|
| Co-expressed vector | Treatment | Basal | Induced |
| Vector control | None | $100 \pm 6$ | $100 \pm 7$ |
| Vector control | PMA | $63 \pm 5^{*}$ | $45 \pm 5^{*}$ |
| MEK-SA222 | None | $120 \pm 10$ | $116 \pm 10$ |
| MEK-SA222 | PMA | $113 \pm 8^{***}$ | $88 \pm 8\dagger$ |
| MEK-SS/DD | None | $69 \pm 5^{**}$ | $70 \pm 8^{**}$ |
| MEK-SS/DD | PMA | $61 \pm 5$ | $46 \pm 5^{*}$ |

**Table 3 Regulation of basal and induced G6Pase promoter activity by the overexpression of MKP-1**

H4IIE cells were co-transfected with 8.5 $\mu$g/dish of the promoter plasmid G6Pase($-1227/+57$), 0.5 $\mu$g/dish of pRL control plasmid and 2 $\mu$g/dish of either wild-type pSG5-MKP-1, the catalytically inactive MKP-1 mutant (pSG5-MKP-CS) or a vector control (pSG5). Cells were incubated with or without PMA (1 $\mu$M) as indicated, and in the absence (basal) or presence (induced) of $Bt_2$cAMP (500 $\mu$M) and dexamethasone (1 $\mu$M). Data are presented as means $\pm$ S.E.M. ($n = 3$) of fold-activation relative to either basal or the induced luciferase activity, after the co-expression of the vector control, which was set as 100. $^{*}P <$ 0.05 compared with the basal or induced activity after the co-expression of the respective vector in the absence of PMA, $^{**}P < 0.05$ compared with the PMA-inhibited activities after the co-expression of the constitutively active mutant and the vector control, $\dagger P < 0.05$ compared with the respective promoter activity without PMA and to the PMA-inhibited activities after the co-expression of the constitutively active mutant and the vector control.

| | | Relative promoter activity | |
|---|---|---|---|
| Co-expressed vector | Treatment | Basal | Induced |
| Vector control | None | $100 \pm 5$ | $100 \pm 7$ |
| Vector control | PMA | $79 \pm 4^{*}$ | $45 \pm 4^{*}$ |
| pSG5-MKP-1 | None | $100 \pm 8$ | $103 \pm 9$ |
| pSG5-MKP-1 | PMA | $98 \pm 4^{**}$ | $83 \pm 5\dagger$ |
| pSG5-MKP-CS | None | $100 \pm 8$ | $108 \pm 8$ |
| pSG5-MKP-CS | PMA | $75 \pm 4^{*}$ | $46 \pm 5$ |

## Overexpression of MEK inhibits *G6Pase* gene expression

In order to study the role of MEK in the suppression of G6Pase promoter activity further, H4IIE cells were co-transfected with plasmids expressing either a constitutively active or a catalytically inactive MEK [18] together with the G6Pase promoter vector. The co-expression of the active MEK construct decreased both basal and $Bt_2$cAMP/dexamethasone-induced luciferase expression compared with the co-expression of either the catalytically inactive MEK-SA222 construct or the vector control (Table 2). With that, the overexpression of MEK mimicked the PMA effect, indicating that MEK activation is sufficient to suppress *G6Pase* gene transcription. The co-expression of the construct MEK-SA222, which exhibits a dominant negative effect on the activation of endogenous MEK [18], completely blocked the regulation of basal *G6Pase* gene transcription by PMA. However, this construct did not fully suppress the effect of PMA on induced G6Pase promoter activity. Nevertheless, the results suggest that the activation of MEK is not only sufficient, but also contributes significantly to the suppression of *G6Pase* gene transcription by PMA, and support the data obtained with the MEK inhibitors.

## Overexpression of MKP-1 impairs the PMA effect on *G6Pase* gene expression

MEK activates ERK1/2 with high specificity [12]. MKP-1 dephosphorylates and thereby inactivates ERK, although other kinases can also be dephosphorylated by MKP-1 [12,17]. In order to support the hypothesis that PMA inhibits *G6Pase* gene transcription by the activation of the MEK/ERK cascade, H4IIE cells were co-transfected with the G6Pase promoter construct together with vectors expressing either wild-type MKP-1 or the catalytically inactive MKP-1-CS mutant [17]. Compared with the co-transfection of either the catalytically inactive mutant or the vector control, the transfection of wild-type MKP-1 completely blocked the PMA effect on basal promoter activity and suppressed the PMA effect on the induction of promoter activity by $Bt_2$cAMP/dexamethasone in part (Table 3). These results

**Table 4 ERK2 activities in H4IIE hepatoma cells**

Serum-starved cells were incubated in the presence of various combinations of PMA (1 $\mu$M), insulin (5 nM), $Bt_2$cAMP (500 $\mu$M), dexamethasone (Dexa; 1 $\mu$M) and the MEK inhibitors PD98059 (50 $\mu$M) and U0126 (20 $\mu$M). ERK2 activity was assayed in immunoprecipitates with basic myelin protein as substrate. Results are presented as means $\pm$ S.E.M. ($n = 4$), $^*P <$ 0.05 compared with basal activity.

| Mediator | Incubation time (min) | Specific activity (m-units/mg of protein) |
|---|---|---|
| None | | $1.5 \pm 0.6$ |
| PD98059 | | $1.0 \pm 0.7$ |
| U0126 | | $1.0 \pm 0.4$ |
| $Bt_2$cAMP/Dexa | 10 | $1.1 \pm 0.3$ |
| PMA | 10 | $25.5 \pm 5.9^*$ |
| PMA | 120 | $5.0 \pm 1.7^*$ |
| PMA | 1200 | $1.1 \pm 0.5$ |
| PMA/PD98059 | 10 | $2.3 \pm 0.7$ |
| PMA/U0126 | 10 | $1.0 \pm 0.4$ |
| PMA/$Bt_2$cAMP/Dexa | 10 | $14.5 \pm 1.4^*$ |
| PMA/$Bt_2$cAMP/Dexa/PD98059 | 10 | $1.9 \pm 0.4$ |
| PMA/$Bt_2$cAMP/Dexa/U0126 | 10 | $1.1 \pm 0.5$ |
| Insulin | 10 | $3.5 \pm 0.5^*$ |
| Insulin | 120 | $1.4 \pm 0.5$ |
| Insulin/PD98059 | 10 | $1.5 \pm 0.5$ |
| Insulin/U0126 | 10 | $1.4 \pm 0.5$ |
| Insulin/$Bt_2$cAMP/Dexa | 10 | $2.1 \pm 0.4$ |

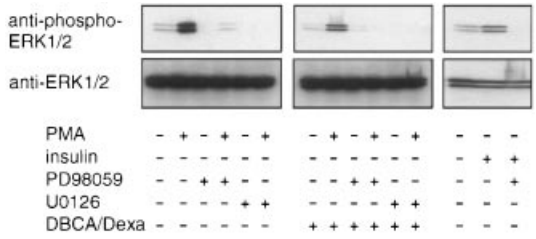


**Figure 3 Regulation of ERK1/2 phosphorylation by PMA and insulin**

Serum-starved H4IIE cells were incubated with or without the MEK inhibitors PD98059 (50 $\mu$M) and U0126 (20 $\mu$M) and for 10 min in the presence or absence of PMA (1 $\mu$M), insulin (5 nM), and the combination of $Bt_2$cAMP (DBCA; 500 $\mu$M) and dexamethasone (Dexa; 1 $\mu$M) as indicated. Cell lysates were electrophoresed by SDS/PAGE and transferred on to nylon membranes. Western blots were performed using an antibody specific for phosphorylated ERK1/2. To control for loading, blots were subsequently incubated with anti-ERK1/2 antibody.

provide further evidence for the involvement of the MEK/ERK cascade in the regulation of *G6Pase* gene expression by PMA.

### Regulation of ERK and PKB activities by PMA and insulin

The data presented so far indicate that PMA inhibits *G6Pase* gene expression in a different way to insulin by the activation of MEK and ERK, and not of PI 3-kinase. In order to further elucidate the molecular background of this differential regulation, we studied the effect of PMA and insulin on the activities of ERK and PKB in H4IIE cells. The activation of PKB is an event downstream of PI 3-kinase stimulation. We analysed ERK activities by an *in vitro* kinase assay using immunoprecipitated ERK2 with basic myelin protein as the substrate (Table 4), and in some cases by Western blotting of cell lysates using an antibody specific for phosphorylated ERK1/2 (Figure 3). Both approaches led to similar results. Under our experimental conditions, the incubation of H4IIE for 10 min with PMA increased the basal ERK2 activity of $1.5 \pm 0.6$ m-units/mg of protein by approx. 20-fold. This activation of ERK2 was almost completely blocked by the inhibitors of MEK PD98059 and U0126. This is consistent with the notion that activation of ERK1/2 is mediated by the activation of MEK-1/2. The addition of $Bt_2$cAMP and dexamethasone reduced the activation of ERK2 by PMA, but did not disrupt the ability of the MEK inhibitors to decrease ERK2 activation. Only an approx. 4-fold activation of ERK2 could be detected after the incubation with PMA for 120 min. After 1200 min no activation was detected. The incubation of H4IIE cells with insulin (5 nM) led to only an approx. 3-fold increase of ERK2 activity in H4IIE cells after 10 min, which correlates with previously published data [15]. The activation of ERK2 by insulin was suppressed by U0126 and PD98059. No elevated ERK2 activity could be detected after an incubation for 120 min with insulin. In H4IIE cells PMA activated PKB only slightly (Table 5). In parallel incubations insulin led to an approx. 7-fold increase of PKB activity after 120 min, which was blocked by the PI 3-kinase inhibitor LY294002, as described previously [5]. The administration of $Bt_2$cAMP and dexamethasone does not increase PKB activity in H4IIE cells [5]. These results indicate that in H4IIE cells PMA strongly activates the ERK pathway, but only slightly activates the PI 3-kinase pathway. However, insulin activates the PI 3-kinase pathway, but stimulates it to a much lesser extent than PMA stimulates the MEK/ERK pathway.

**Table 5 PKB$\alpha$ activity in H4IIE hepatoma cells**

Serum-starved cells were incubated in the presence of various combinations of PMA (1 $\mu$M), insulin (5 nM) and the PI 3-kinase inhibitor LY294002 (100 $\mu$M). PKB$\alpha$ activity was assayed in immunoprecipitates with the Crosstide peptide as substrate. Results are presented as means $\pm$ S.E.M. ($n = 4$), $^*P < 0.05$ compared with basal activity.

| Mediator | Incubation time (min) | Specific activity (m-units/mg of protein) |
|---|---|---|
| None | | $0.5 \pm 0.2$ |
| PMA | 10 | $0.9 \pm 0.1^*$ |
| PMA | 120 | $0.9 \pm 0.2$ |
| PMA/LY294002 | 120 | $0.2 \pm 0.2$ |
| Insulin | 10 | $2.4 \pm 0.2^*$ |
| Insulin | 120 | $3.4 \pm 0.4^*$ |
| Insulin/LY294002 | 120 | $0.6 \pm 0.3$ |

### IRU does not mediate the regulation of the G6Pase promoter by PMA

The effect of insulin on *G6Pase* gene expression is, in part, mediated by the IRU between nt $-196$ and $-156$ of the G6Pase promoter [5,8]. In order to study a potential involvement of this *cis*-active structure in the regulation via the activated MEK/ERK cascade, we compared the PMA effect on the wild-type G6Pase promoter with that on a construct with a mutated IRU. No significant difference of the regulation of basal (Figure 4A) and induced gene expression (Figure 4B) between the construct G6Pase($-1227/+57$) and the mutated construct G6Pase($-1227/+57$/IRUmut) was detected. In parallel experiments the mutation of the forkhead-binding sites impaired the insulin effect on the promoter activity by approx. 20–30 %, as has been shown previously [5]. The data indicate that the forkhead-binding sites within the IRU of the G6Pase promoter are not significantly involved in the regulation of the G6Pase promoter activity by PMA or the MEK/ERK cascade.

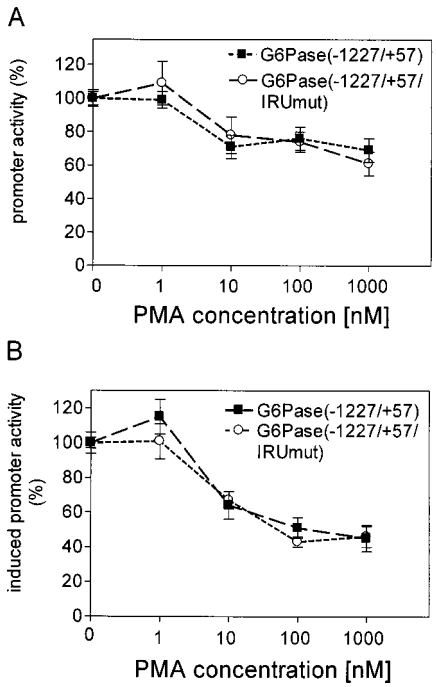


**Figure 4 Mutation of the forkhead-binding sites of the IRU does not impair the suppression of basal (A) and $Bt_2$cAMP/dexamethasone-induced (B) G6Pase promoter activity by PMA**

H4IIE cells were co-transfected with 0.5 $\mu$g/dish of pRL control plasmid together with either 8.5 $\mu$g/dish of either the promoter plasmids G6Pase(−1227/+57) or G6Pase(−1227/+57/IRUmut). Within the construct G6Pase(−1227/+57/IRUmut) the forkhead-binding sites of the IRU were mutated from −196 5′-CGATCAGGCTGTTTTTGTG-TGCCTGTTTTTCTATTTTACG-3′ −156 into 5′-CGATCAGGCTCGAGTTGTGTGCCTCTTTTTCT-CTTTTACG-3′ (mutated nucleotides are underlined) [5]. (**A**) The transfected cells were incubated with or without the indicated PMA concentration. Data are presented as means ± S.E.M. ($n = 3$) of the inhibition by PMA compared with the respective basal activities, which were set as 100. (**B**) Transfected cells were incubated in the presence or absence of PMA with the combination of $Bt_2$cAMP (500 $\mu$M) and dexamethasone (1 $\mu$M). The respective maximal inductions of the reporter gene expression compared with basal expression were set as 100. Data are presented as means ± S.E.M. ($n = 3$) of the relative inhibition of the $Bt_2$cAMP/dexamethasone-induced promoter activity by PMA.

## DISCUSSION

In the present study we have demonstrated that PMA and insulin decrease basal and induced *G6Pase* gene expression by different signalling pathways. PMA acts via the MEK/ERK cascade, whereas the effect of insulin is transferred by PI 3-kinase [4]. This assumption correlates with the different abilities of PMA and insulin to activate ERK and PKB. PMA strongly stimulated ERK, whereas insulin displayed only a weak effect on ERK activity. In addition, we show that in H4IIE cells PMA activates ERK transiently, as has been described previously for other cellular systems [20,21]. This indicates that a permanent stimulation of the MEK/ERK module is not required for the sustained inhibition of *G6Pase* gene expression by PMA. With that, the activation of MEK and ERK might be involved in initial steps of the gene regulation by PMA. Furthermore, we found a reduced stimulation of ERK2 activity by PMA in the presence of $Bt_2$cAMP and dexamethasone. This might be caused by the described inhibition of Raf and Ras function by cAMP-activated protein kinase A [11,12]. The activation of PI 3-kinase by PMA is cell-type dependent [13]. PI 3-kinase activation stimulates that of PKB via 3-phosphoinositide-dependent kinase 1 [6,7]. The observation, that PKB is only slightly stimulated by PMA, indicates that in H4IIE cells this compound is a much weaker stimulant of PI 3-kinase signalling than insulin.

The experiments with the structurally and mechanistically different MEK inhibitors PD98059 and U0126 indicate that the activation of MEK is indispensable for at least the major part of the PMA effect on *G6Pase* gene trancription. This assumption is supported by the overexpression of the dominant negative MEK-SA222 construct and of MKP-1 (Tables 2 and 3). However, these constructs were able to block the regulation of the basal promoter activity by PMA completely, but suppressed the effect of PMA on the induced gene transcription only in part. In contrast with this, the MEK inhibitors fully blocked the effect of PMA on both basal and induced promoter activity. The reason for this discrepancy is not clear. A possible explanation could be that the induction of promoter activity is partly inhibited by an additional pathway besides the MEK/ERK cascade, which is also activated by PMA. This unidentified pathway could be non-specifically suppressed by the MEK inhibitors, but not by the overexpression of MKP-1 and MEK-SA222. The observation that PMA inhibits the induced G6Pase promoter activity to a higher extent than basal level expression may support the speculation that an additional pathway may be involved. The activation of the MAPK cascade can be impaired by PI 3-kinase inhibitors [6]. This mechanism could account for the slight effect of LY294002 on the regulation of G6Pase promoter activity by PMA (Table 1).

PMA regulates not only the G6Pase promoter activity, but also the levels of endogenous G6Pase mRNA transcript by the activation of MEK. In addition, we observed that PD98059 increased the level of G6Pase mRNA transcript, but not the promoter activity. The reason for this is unclear. *G6Pase* gene expression is also regulated by post-transcriptional events [22]. We speculate that PD98059 interacts with a mechanism regulating G6Pase mRNA stability. The inability of U0126 to induce G6Pase expression may suggest that MEK is not involved in this mechanism.

The substrates of ERK, which lead to the suppression of *G6Pase* gene transcription, are unknown. ERK can stimulate kinases, such as MAPK signal integrating kinase-1 and mitogen- and stress-activated protein kinase-1 [12]. Activated ERK is translocated to the nucleus, where it phosphorylates and controls several transcription factors [11,12]. Further studies will have to establish if one of these proteins is involved in the regulation of *G6Pase* gene expression. The ability of PMA to decrease basal *G6Pase* gene expression indicates that the phorbol ester can act by means different from the modification of the glucocorticoid receptor by ERK, which has been reported in [23]. In addition, our results show that the forkhead-binding sites within the IRU, and therefore signalling via FKHR or a related transcription factor [24], do not play considerable roles in the regulation of *G6Pase* gene expression by PMA. This indicates that the slight activation of PKB by PMA does not significantly contribute to the inhibition of *G6Pase* gene expression and provides further confirmation of the use of different signalling pathways by PMA and insulin.

Although PMA has a similar effect on the expression of the key gluconeogenic enzymes G6Pase and PEPCK, the molecular basis of the *PEPCK* and *G6Pase* gene regulation by PMA differs. The MEK/ERK cascade is not involved in the PMA regulation of *PEPCK* gene transcription [15] in contrast with its effect on *G6Pase* gene expression. Furthermore, the PMA effect on *G6Pase* gene transcription is not mediated by the forkhead-binding sites of the IRU. The sequence of each of these forkhead-binding sites is very similar to that of the insulin-response sequence of

the PEPCK promoter [8], which mediates the suppression of the *PEPCK* gene expression by both insulin and PMA [14]. This suggests that the regulation via the forkhead-binding motif is modulated by other *cis*-active elements within the G6Pase and the PEPCK promoters.

In conclusion, we provide further evidence that multiple signalling pathways suppress *G6Pase* gene expression, and that these pathways act via multiple *cis*-active sequences.

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