Insulin inhibits glucocorticoid-stimulated L-type 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression by activation of the c-Jun N-terminal kinase pathway

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The hepatic isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PF2K/Fru-2,6-BPase) is transcriptionally stimulated by glucocorticoids, whereas insulin blocks this stimulatory effect. Although this inhibitory effect has been extensively reported, nothing is known about the signalling pathway responsible. We have used well-characterized inhibitors for proteins involved in different signalling cascades to assess the involvement of these pathways on the transcriptional regulation of glucocorticoid-stimulated PF2K/Fru-2,6-BPase by insulin. Our results demonstrate that the phosphoinositide 3-kinase, p70/p85 ribosomal S6 kinase, extracellular signal-regulated protein kinase (ERK)1/2 and p38 mitogen-activated protein (MAP) kinase pathways are not involved in the inhibitory effect of insulin on glucocorticoid-stimulated PF2K/Fru-2,6-BPase. To evaluate the implication of the MAP kinase/ERK kinase (MEK)-4–stress-activated protein kinase–c-Jun-N-terminal protein kinase ('JNK–SAPK') pathway we overexpressed the N-terminal JNK-binding domain of the JNK-interacting protein 1 ('JIP-1'), demonstrating that activation of JNK is necessary for the insulin inhibitory effect. Moreover, overexpression of MEK kinase 1 and JNK–haemagglutinin resulted in the inhibition of the glucocorticoid-stimulated PF2K/Fru-2,6-BPase. These results provide clear and specific evidence for the role of JNK in the insulin inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression. In addition, we performed experiments with a mutant of the glucocorticoid receptor in which the JNK phosphorylation target Ser-246 had been mutated to Ala. Our results demonstrate that the phosphorylation of the glucocorticoid receptor on Ser-246 is not responsible for the JNK repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression.

Key words: dexamethasone, hepatic carbohydrate metabolism, signal transduction.

INTRODUCTION

Insulin and glucocorticoids are potent metabolic hormones that have opposite effects in hepatic carbohydrate metabolism. Glycolysis is induced by high concentrations of insulin and low concentrations of glucocorticoids; conversely, low insulin and high glucocorticoid levels induce the gluconeogenic flux. The metabolic actions of these hormones are mediated by modulation of the activities of key regulatory glycolytic and gluconeogenic enzymes; one of these is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PF2K/Fru-2,6-BPase; EC 2.7.1.105/3.1.3.46). This is a bifunctional enzyme that catalyses both the synthesis and the degradation of fructose-2,6-bisphosphate [Fru(2,6)P_2], a potent stimulator of 6-phosphofructo-1-kinase and inhibitor of fructose 1,6-bisphosphatase. PF2K/Fru-2,6-BPase can be considered to be either a glycolytic or a gluconeogenic enzyme; it acts as a switch between glycolysis and gluconeogenesis in mammalian liver by regulating the level of Fru(2,6)P_2 [1]. Different PF2K/Fru-2,6-BPase isoenzymes have been described depending on the tissue and cell specificity. Hepatic PF2K/Fru-2,6-BPase is subjected to a complex pattern of hormonal regulation in which insulin exerts an opposite transcriptional effect depending on the hormonal context. Insulin weakly stimulates transcription from the L-type PF2K/Fru-2,6-BPase promoter when used alone [2,3]; in contrast, in the presence of glucocorticoids, insulin inhibits the glucocorticoid-induced stimulation of PF2K/Fru-2,6-BPase gene expression [4,5]. This pattern of hormonal regulation resembles that of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) [6,7]. However, changes in the phosphorylation state of the enzyme induced by insulin need to be considered for an understanding of the effect of this hormone on the activity of the bifunctional enzyme.

Insulin regulates the expression of several genes through the activation of distinct signalling pathways [8]. There is evidence for a direct role of mitogen-activated protein (MAP) kinase cascades in the regulation of expression of certain immediate early genes such as c-*fos* [9]. Insulin might mediate its mitogenic effects via this pathway. In contrast, phosphoinositide 3-kinase (PI-3K) is a major conduit for the signals that regulate the metabolic responses to insulin [10]. In adipocytes, PI-3K inhibitors such as wortmannin and LY 294002 have been used to indicate the importance of this pathway in GLUT 4 translocation to the surface membrane [11] and system A amino acid transport [12]. The effects of insulin on glycogen synthase kinase 3 (GSK-3) [13,14], PEPCK [15,16], glucose-6-phosphatase [17] and gene 33 [18] are also blocked by inhibiting PI-3K activity. The activation of p70/p85 ribosomal S6 kinase (p70 S6 kinase) was not involved in the regulation of these genes by insulin. However,

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; Fru(2,6)P_2, fructose-2,6-bisphosphate; GSK-3, glycogen synthase kinase 3; JNK, c-Jun-N-terminal kinase; JBD, JNK-binding domain; JIP-1, JNK-interacting protein 1; JNK–SAPK, MEK-4–stress-activated protein kinase–c-Jun-N-terminal protein kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; p70 S6 kinase, p70/p85 ribosomal S6 kinase; PEPCK, phosphoenolpyruvate carboxykinase; PF2K/Fru-2,6-BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PI-3K, phosphoinositide 3-kinase.

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a PI-3K/p70 S6 kinase-dependent pathway is required for the regulation of glucose-6-phosphate dehydrogenase [19] and hexokinase II [20] by insulin.

Recently, several studies have been conducted to explain the molecular mechanism involved in the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression [21]. Although this previous work seems to eliminate the involvement of the PI-3K and ERK1/2 cascades, nothing is known about the transductional pathway responsible for this effect.

Our results show clearly that the MEK-4–stress-activated protein kinase–c-Jun-N-terminal protein kinase (JNK–SAPK) pathway is responsible for mediating the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression. We further demonstrate that this effect is independent from the phosphorylation of the glucocorticoid receptor on Ser-246.

EXPERIMENTAL

Materials

[α-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and D-threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol (40–60 μCi/mmol) were from Amersham Pharmacia Biotech. The random primer labelling kit, restriction enzymes and insulin were from Boerhinger Mannheim. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and antibiotics were from Bio-Whitakker. LY 294002, SB 203580 and PD 098050 were from Calbiochem. Rapamycin and dexamethasone were purchased from Sigma Chemical Co. Luciferase assay systems were obtained from Promega. TLC sheets were from Merck. Anti-ERK2 antibody was from Santa Cruz Biotechnology. Other materials and chemicals were of the highest quality available.

Tissue culture

H4IIE cells and CV1 cells were routinely grown in DMEM supplemented with 10% (v/v) FCS, 100 i.u./ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified air/CO_2 (19:1). Cells were serum-starved overnight and treated with hormones and chemical inhibitors for the durations and in the concentrations indicated in the figure legends. The inhibitors were added 30 min before the hormones. Because the inhibitors used were dissolved in DMSO, an equal amount of DMSO carrier was added to the cells with all treatments described here.

Plasmid constructs

The reporter gene construct L-pBLCAT (in which CAT stands for chloramphenicol acetyltransferase) containing a 500 bp *Hin*fI–*Hin*fI 5′ flanking region of the L isoform of PF2K/Fru-2,6-BPase cloned into a pBLCAT3 plasmid was provided by Dr A. J. Lange (University of Minnesota, Minneapolis, MN, U.S.A.) [22]. Plasmids coding for the JNK-binding domain (JBD) of JIP-1 and GAL 4-luciferase were provided by Dr R. J. Davis (University of Massachusetts, Worcester, MA, U.S.A.) [23] and GAL 4–ATF-2 was provided by Dr N. Jones (Imperial Cancer Research Fundation, London, U.K.) [24]. Expression vectors coding for the MEK kinase (MEKK)-1 protein and the epitope-tagged JNK–haemagglutinin (JNK–HA) were provided by Dr C. Caelles (Universitat de Barcelona, Barcelona, Spain); the wild-type glucocorticoid receptor and the S246A glucocorticoid receptor mutant were provided by Dr J. M. Garabedian (University of New York, New York, U.S.A.) [25]; and pPCK10 plasmid coding for the PEPCK was provided by Dr R. W. Hanson (Case Western Reserve University, Cleveland, OH, U.S.A.) [26].

Total RNA extraction

Total cellular RNA was extracted by the LiCl/urea method [27]. The RNA was dissolved in sterile water; the concentration and purity of each sample were assessed by A_{260} and by the ratio of A_{260} to A_{280} respectively. The abundance of PF2K/Fru-2,6-BPase mRNA was measured by Northern blot analysis with standard procedures [28]. The following probes were used: a 1 Kb *Eco*RI fragment isolated from the pKBplasmid [29] to detect PF2K/Fru-2,6-BPase abundance and a 2.6 kb *Pst*-1 fragment isolated from the pPCK10 plasmid [26] for PEPCK. The integrity of the RNA was verified by observing the rRNA bands in the ethidium bromide gel under UV irradiation. The levels of mRNA were evaluated by densitometric scanning of the autoradiogram and corrected by the amount of 18 S rRNA with the use of a ribosomic cDNA probe [30].

Transfection and CAT assay

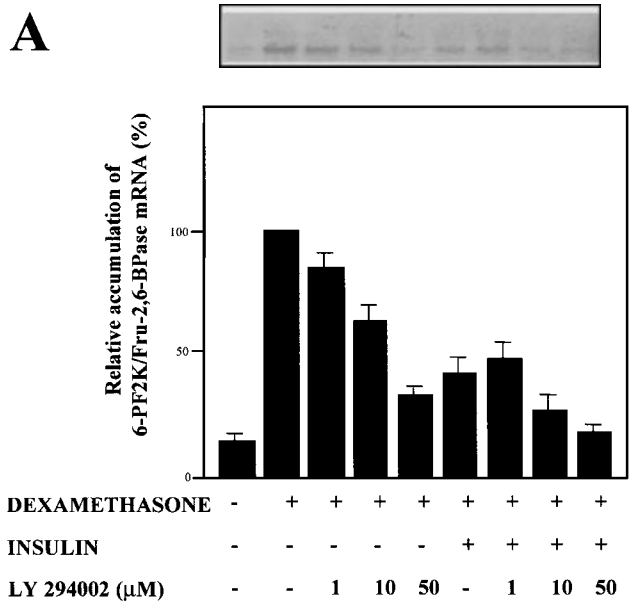
CsCl double-gradient centrifugation was used to prepare all plasmids for transfections. H4IIE cells and CV1 cells were transfected with the different combinations of the indicated plasmids with 30 μg DNA per 100 mm tissue culture dish by the calcium phosphate method [31,32]. After transfection, cells were harvested by tryptic digestion, split into 60 mm tissue culture dishes and then incubated in DMEM supplemented with 10% (v/v) FCS for 24 h. Cells were further washed and incubated in serum-free DMEM for 12 h and placed in serum-free DMEM containing the agents indicated in the figure legends for 12 h. Cell extracts were prepared and assayed for CAT [28], luciferase and *Renilla* activities (in accordance with the manufacturers' instructions). The values were normalized by the amount of protein measured by the Bio-Rad method. The statistical significance of the differences was assessed by Student's unpaired test with STATVIEW II™ software for Apple Macintosh computers.

Kinase assays

Cells were harvested in 500 μl of cell lysis buffer [20 mM Hepes (pH 7.5)/1% (v/v) Nonidet P40/10 mM EGTA/2.5 mM $MgCl_2$/40 mM β-glycerol phosphate/2 mM sodium vanadate/1 mM dithiothreitol/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptin], kept on ice for 30 min and centrifuged at 6000 ***g*** for 10 min at 4 °C. For endogenous ERK activity, the resulting supernatants were incubated for 1 h at 4 °C with 40 μl of anti-(ERK)–Protein A–Sepharose beads. For transfected JNK–HA activity, the resulting supernatants were incubated for 2 h at 4 °C with 40 μl of anti-(HA)–Protein G–Sepharose beads. The beads were washed three times in ice-cold PBS in the presence of 0.1% (v/v) Nonidet P40 and 2 mM Na_3VO_4 and once in kinase buffer [12.5 mM MOPS (pH 7.5)/12.5 mM β-glycerol phosphate/7.5 mM $MgCl_2$/0.5 mM EGTA/0.5 mM NaF/0.5 mM Na_3VO_4/1 mM dithiothreitol] resuspended in 30 μl of the kinase buffer; the kinase reaction was initiated by adding the substrates (40 μg of myelin basic protein for ERK activity and 1 μg of glutathione S-transferase–c-Jun for JNK activity) along with 20 μM ATP and 100 μCi of [γ-^{32}P]ATP. The reaction proceeded for 30 min at 37 °C and was terminated by the addition of 15 μl of 4 × Laemmli buffer. Samples were subjected to SDS/PAGE [12% (w/v) gel]; results were quantified by autoradiography.

RESULTS AND DISCUSSION

The molecular pathways leading to the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression were studied in H4IIE rat hepatoma cells. This cell line was



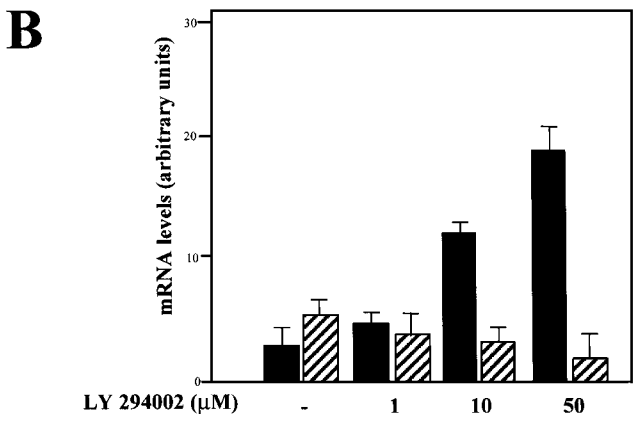


Figure 1 Effect of LY 294002 on the insulin repression of glucocorticoid-stimulated 6PF2k/Fru-2,6-BPase mRNA

(**A**) H4IIE cells deprived of serum for 24 h were preincubated with LY 294002 for 10 min before the addition of 1 μM dexamethasone and 100 nM insulin, as indicated, for 3 h. Total mRNA was isolated and Northern blot analysis was performed as described in the Experimental section. The intensity of the autoradiographic signal was quantified by densitometric scanning and corrected by the amount of RNA obtained with the 18 S rRNA probe. Values are expressed relative to the values of cells treated with dexamethasone alone, which was taken as 100 arbitrary units. Results are means $\pm$ S.E.M. for three independent experiments. Upper panel: a representative Northern blot. (**B**) H4IIE cells deprived of serum for 24 h were preincubated with the indicated LY294002 concentrations for 10 min before the addition of both 1 μM dexamethasone and 100 nM insulin. Northern blot analysis was performed as described above with PEPCK (filled bars) and PF2K/Fru-2,6-BPase (hatched bars) as probes.

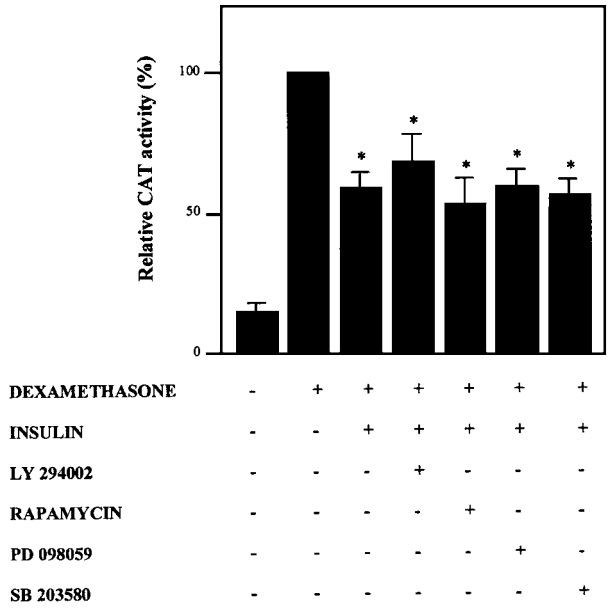


Figure 2 Effect of LY 294002, rapamycin, PD 098059 and SB 203580 on insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase expression

H4IIE cells were transiently transfected with the reporter gene construct L-pBLCAT as described in the Experimental section. Cells deprived of serum for 24 h were preincubated with 50 μM LY 294002, 100 nM rapamycin or 50 μM PD 098059 for 10 min before the addition of 1 μM dexamethasone and 100 nM insulin. Cell extracts for CAT assays were obtained 12 h after the addition of hormone. Results are means $\pm$ S.E.M. for five independent experiments. Statistical significance: $^*P < 0.05$ in comparison with dexamethasone-treated cells.

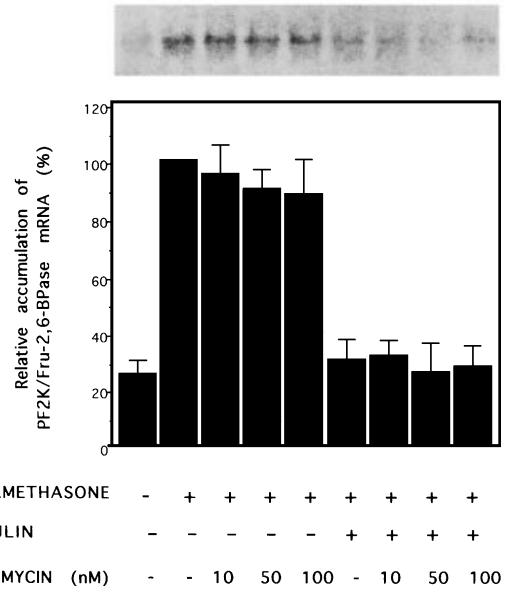


Figure 3 Effect of rapamycin on the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase mRNA

H4IIE cells deprived of serum for 24 h were preincubated with rapamycin for 10 min before the addition of 1 μM dexamethasone and 100 nM insulin, as indicated, for 3 h and then processed as described in the legend to Figure 1. Values are expressed relative to those for cells treated with dexamethasone, which was taken as 100 arbitrary units. Results are means $\pm$ S.E.M. for three independent experiments. Upper panel: a representative Northern blot.

chosen because much information is available on PF2K/Fru-2,6-BPase regulation and the insulin signal transduction pathway. It has been reported that insulin activates the PI-3K pathway in H4IIE cells [15]. To assess the role of PI-3K in the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase mRNA levels, we determined the effects of LY 294002, the highly specific inhibitor of the p110 catalytic subunit of PI-3K [33]. Treatment of H4IIE cells with insulin for 3 h decreased PF2K/Fru-2,6-BPase mRNA levels by 50%. The addition of LY 294002 did not block this repression (Figure 1A). In addition, we analysed the content of PEPCK mRNA to ensure that LY

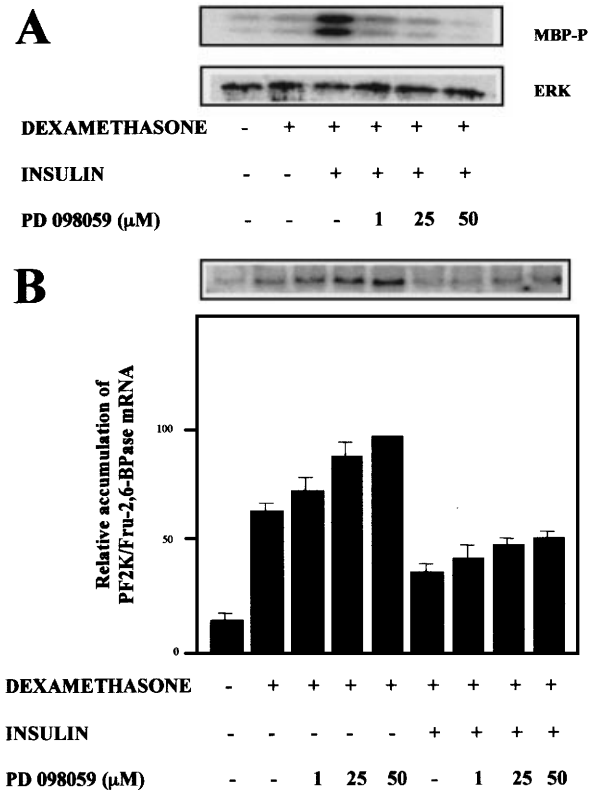


Figure 4 Effect of PD 098059 on the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase mRNA

(**A**) H4IIE cells deprived of serum for 24 h were preincubated with the indicated PD 098059 concentrations for 10 min before the addition of 1 μM dexamethasone and 100 nM insulin for 15 min. Cell extracts were obtained and ERK activity was measured by ^{32}P incorporation into myelin basic protein (MBP) as described in the Experimental section. (**B**) H4IIE cells deprived of serum for 24 h were preincubated with the indicated PD 098059 concentrations for 10 min before the addition of 1 μM dexamethasone and 100 nM insulin, as indicated, for 3 h and then processed as described in the legend to Figure 1. Values are expressed relative to those for cells treated with dexamethasone and 50 μM PD 098059, which was taken as 100 arbitrary units. Results are means $\pm$ S.E.M. for three independent experiments. Upper panel: a representative Northern blot.

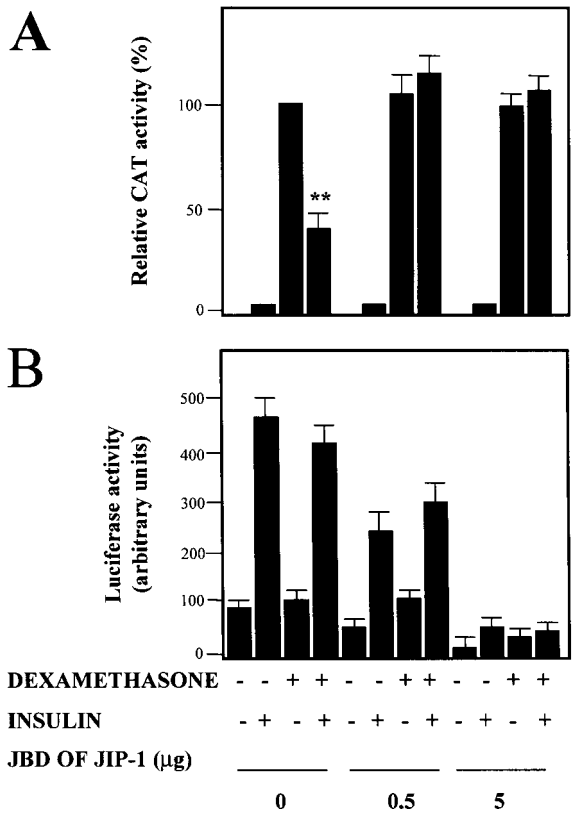


Figure 5 Effect of overexpression of the JBD of JIP-1 on the insulin repression of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression

H4IIE cells were transiently transfected with 15 μg of the reporter gene construct L-pBLCAT, 2.5 μg of GAL 4–ATF-2 plasmid, 2.5 μg of GAL 4–luciferase plasmid and the indicated concentrations of the plasmid coding for the JBD of JIP-1 as described in the Experimental section. After transfection, cells were serum-starved for 24 h, then treated with 100 nM insulin and 1 μM dexamethasone separately or together for 12 h. (**A**) CAT activities are expressed relative to that for cells treated with dexamethasone in the absence of the JBD of JIP-1; this was taken as 100 arbitrary units. (**B**) Cell extracts were assayed for luciferase activity; values are expressed as arbitrary units. Results in both panels are means $\pm$ S.E.M. for four independent experiments. Statistical significance: $^{**}P < 0.01$ in comparison with dexamethasone-treated cells.

294002 inhibited the PI-3K pathway under our experimental conditions. Previous reports have demonstrated that PEPCK gene expression is inhibited by insulin through the PI 3-K pathway [15,34]. As expected, LY 294002 blocked the inhibitory effect of insulin on glucocorticoid-stimulated PEPCK gene expression (Figure 1B). Indeed, LY 294002, both in the absence and in the presence of insulin, produced concentration-dependent decreases in glucocorticoid-stimulated PF2K/Fru-2,6-BPase mRNA levels. The finding that inhibition of PI 3-K represses the stimulatory action of glucocorticoids on PF2K/Fru-2,6-BPase expression clearly indicates that activation of PI 3-K is not required for insulin to inhibit the stimulation of PF2K/Fru-2,6-BPase gene expression by glucocorticoids. Very recently it has been reported that rat glucocorticoid receptor is phosphorylated by GSK-3 *in vitro* and in cultured mammalian cells. This phosphorylation inhibits the transcriptional enhancement of the glucocorticoid receptor [35]. GSK-3 is one of the final targets of the PI-3K pathway, so the inhibition of the glucocorticoid stimulatory effect on PF2K/Fru-2,6-BPase mRNA levels caused by LY 294002 could be due to a phosphorylation of the glucocorticoid receptor by GSK-3. To determine further whether the regulation of PF2K/Fru-2,6-BPase mRNA levels described above was due to a regulation of its transcriptional rate, H4IIE cells were transiently transfected with the reporter gene construct L-pBLCAT, a CAT reporter gene under the transcriptional control of the L-type PF2K/Fru-2,6-BPase promoter. The results of the CAT analysis show that LY 294002 was not able to revert the insulin inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression (Figure 2). These results strongly suggest the independence of the PI-3K pathway in insulin-mediated repression.

To test further the involvement of p70 S6 kinase, we analysed the effect of rapamycin on the insulin inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase mRNA levels. Rapamycin blocks the stimulation of p70 S6 kinase activity by insulin in a variety of cell types, without affecting PI-3K activity [36,37]. The addition of rapamycin did not block the ability of insulin to repress the stimulatory effect of glucocorticoids (Figure 3). The same results were found at the transcriptional level (Figure 2).

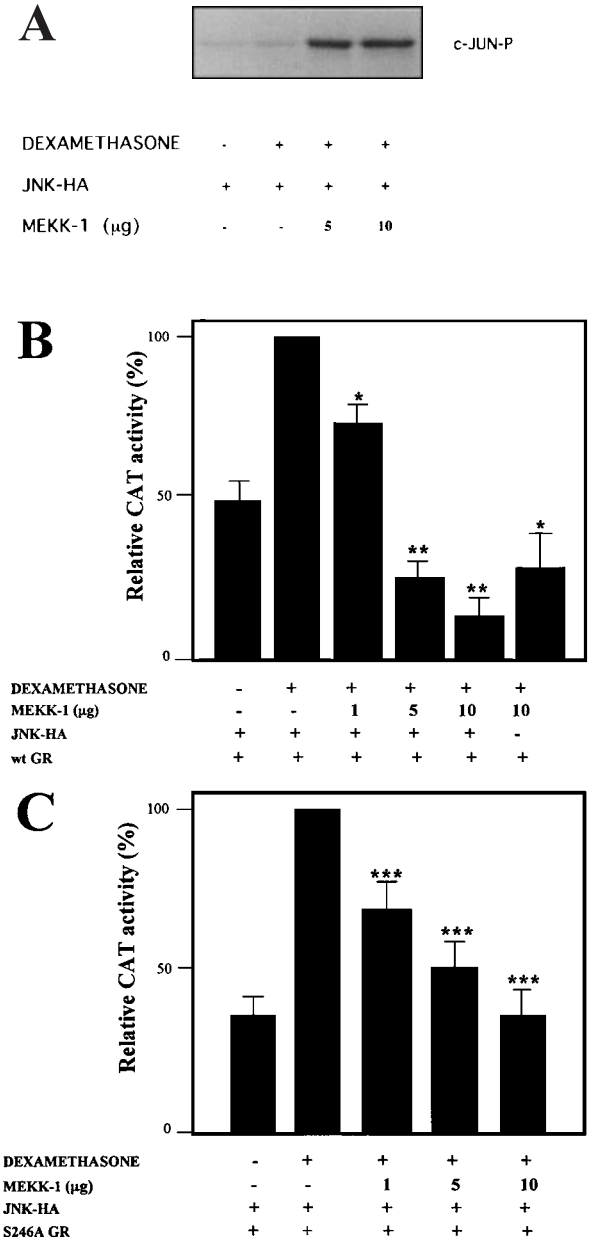


Figure 6 Effect of the overexpression of MEKK-1, JNK–HA and wild-type or S246A glucocorticoid receptor on glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression

(**A**) CV1 cells were transfected as described in the Experimental section with the indicated concentrations of the plasmid coding for MEKK-1 and 2.5 μg of the plasmid coding for JNK–HA, serum-starved for 24 h and treated with 1 μM dexamethasone for 15 min. Cell immunoprecipitates were obtained and JNK activity was measured by ^{32}P incorporation into glutathione S-transferase–c-Jun (1–233) as described in the Experimental section. (**B**) CV1 cells were transfected as described in the Experimental section with the indicated concentrations of the plasmid coding for MEKK-1, 2.5 μg of JNK–HA and 5 μg of the wild-type (wt) glucocorticoid receptor (GR), then treated with 1 μM dexamethasone for 12 h. Cell extracts were assayed for CAT activity and values are expressed relative to that for cells treated with dexamethasone in the absence of MEKK-1 overexpression, which was taken as 100. Results are means $\pm$ S.E.M. for three independent experiments. Statistical significances: $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with dexamethasone-treated cells. (**C**) CV1 cells were transfected as described in the Experimental section with the indicated concentrations of the plasmid coding for MEKK-1, 2.5 μg of JNK–HA and 5 μg of the plasmid coding for the mutant S246A GR and treated with 1 μM dexamethasone for 12 h. Cell extracts were assayed for CAT activity and values are expressed relative to that for cells treated with dexamethasone in the absence of MEKK-1 overexpression, which was taken as 100. Results are means $\pm$ S.E.M. for eight independent experiments. Statistical significance: $^{***}P < 0.001$ in comparison with dexamethasone-treated cells.

Thus our experiments indicate that p70 S6 kinase is not required for the insulin-mediated inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression.

Multiple members of the MAP kinase family are activated in response to insulin, including ERK1/2, p38 and JNK [38–42]. To evaluate the relative importance of these kinases on the inhibitory effect of insulin on glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression, we determined the effect of several specific inhibitors on this regulation. PD 098059 was used to determine the role of ERK1/2. This compound is a non-competitive inhibitor of MEK that blocks the activation of MEK by Raf-1 [43]. Northern blot analyses of cells show that insulin inhibits glucocorticoid-induced PF2K/Fru-2,6-BPase mRNA levels. When cells were pretreated with PD 098059, this inhibitor did not totally prevent the effect of insulin on PF2K/Fru-2,6-BPase mRNA levels; however, a small increase was observed in a dose-dependent manner (Figure 4B). The finding that PD 098059 increases PF2K/Fru-2,6-BPase mRNA levels in cells treated with dexamethasone, both in the presence and in the absence of insulin, indicates that the target of ERK1/2 regulation is the glucocorticoid receptor. The inhibitory effect of MAP kinase on the glucocorticoid receptor has been previously reported, although no direct phosphorylation of the glucocorticoid receptor by ERK1/2 has been found [44]. These results suggest the independence of the ERK1/2 pathway in the mediation of insulin repression of glucocorticoid-induced PF2K/Fru-2,6-BPase mRNA levels. Additionally, we performed a kinase assay *in vitro* to ensure that ERK1/2 was inactivated in the presence of PD 098059 under our experimental conditions (Figure 4A). To determine further whether the regulation of PF2K/Fru-2,6-BPase mRNA levels was due to a regulation of its transcriptional rate, H4IIE cells were transiently transfected with the reporter gene construct L-pBLCAT. The transcriptional analysis shows that the use of PD 098059 did not prevent the insulin inhibitory effect (Figure 2). These results show that the ERK1/2 cascade is not involved in the insulin repression of glucocorticoids in the regulation of the L-type PF2K/Fru-2,6-BPase.

The activation of p38 MAP kinase is induced by insulin in H4IIE cells [45]. We used the highly specific inhibitor of the p38 MAP kinase, SB 203580 [46], to determine whether this member of the MAP kinase family could be responsible for the effect of insulin on PF2K/Fru-2,6-BPase gene expression. The addition of this chemical inhibitor did not cause any significant change on mRNA levels (results not shown). The transcriptional analysis with SB 203580 did not modify the repressive effect of insulin (Figure 2). These results show that at least the α and β isoforms of the p38 family of MAP kinase do not seem to have an effect on the regulation of glucocorticoid-stimulated PF2K/Fru-2,6-BPase by insulin.

No specific chemical inhibitors are available for the JNK. However, the N-terminal JBD of the cytoplasmic protein JIP-1 has been recently described to cause a cytoplasmic retention of JNK, an inhibition of JNK-regulated gene expression and a suppression of the effects of the JNK signalling pathway [23]. JBD of JIP-1 competes with c-Jun and ATF-2, targets of the JNK signal transduction pathway, for interaction with JNK, leading to inhibition of the JNK signal transduction pathway. To study the involvement of JNK in insulin repression, the JBD of JIP-1 was co-transfected with the reporter gene construct L-pBLCAT and the transcriptional activity was analysed in the presence or absence of insulin and dexamethasone. In the absence of the JBD of JIP-1, insulin inhibits the stimulatory effect of dexamethasone on PF2K/Fru-2,6-BPase gene expression; however, the presence of the JBD of JIP-1 completely blocks this

effect (Figure 5A). To ensure the endogenous activation of JNK by insulin and its complete inhibition by the JBD of JIP-1, we additionally co-transfected a reporter system based on two plasmids, the first encoding the GAL 4–ATF-2 fusion gene and the second encoding the luciferase reporter gene under the control of a promoter with a GAL 4 DNA-binding domain. In the presence of insulin, JNK was endogenously activated and thus we obtained a large increase in luciferase activity. In contrast, in the presence of the JBD of JIP-1, luciferase activity was repressed in a manner dependent on dose concentration (Figure 5B). These results demonstrate that JNK is responsible for the insulin-mediated repression of glucocorticoid-stimulated L-type PF2K/Fru-2,6-BPase gene expression.

To identify the molecular mechanism for insulin-induced JNK inhibition of glucocorticoids in the regulation of PF2K/Fru-2,6-BPase gene expression, we performed a number of experiments with the CV1 cell line as a model of cells that do not express the glucocorticoid receptor endogenously and in which the L-type PF2K/Fru-2,6-BPase is efficiently expressed. To obtain evidence that activation of the JNK enzymic activity itself was responsible for insulin repression, we overexpressed MEKK-1 (a kinase believed to be further upstream in the JNK–SAPK signalling pathway and responsible for its activation in transfected cells [47]), JNK with a HA-tagged epitope and the wild-type glucocorticoid receptor. Our results show that overexpression of MEKK-1 and JNK–HA in CV1 cells ectopically expressing the wild-type glucocorticoid receptor totally inhibited glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression (Figure 6B). We performed a JNK assay *in vitro* to ensure the activation of transfected JNK–HA by MEKK-1 (Figure 6A). These results provide strong evidence for the direct role of JNK in the inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression.

The glucocorticoid receptor is phosphorylated at four major sites (Thr-171, Ser-224, Ser-232 and Ser-246) in its N-terminal transcriptional regulatory region [44]. It has recently been reported that JNK inhibits glucocorticoid receptor transcriptional activation by direct receptor phosphorylation at Ser-246 [25]. To determine whether JNK regulation was due to phosphorylation of the glucocorticoid receptor, we performed experiments with a mutant of the glucocorticoid receptor in which Ser-246 had been mutated to Ala, consequently losing its responsiveness to JNK-mediated phosphorylation. In these experiments we endogenously activated JNK by co-transfecting MEKK-1. JNK activation in cells expressing the mutant S246A glucocorticoid receptor resulted in the inhibition of the glucocorticoid-induced PF2K/Fru-2,6-BPase gene expression in the same way as when the wild-type glucocorticoid receptor was used (Figure 6C). These results demonstrate that JNK mediates the insulin inhibition of glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression through a mechanism independent of phosphorylation at Ser-246 in the glucocorticoid receptor. These findings provide evidence for a novel mechanism of JNK inhibition of glucocorticoid-stimulated gene expression independent of direct phosphorylation of the glucocorticoid receptor on Ser-246.

Insulin inhibition of glucocorticoid-activated gene expression is a common phenomenon in the regulation of gluconeogenic enzymes. It has been stated that insulin inhibits glucocorticoid-induced PEPCK and glucose-6-phosphatase gene expression through the PI-3K pathway independently of p70 S6 kinase [15,17]. Our results demonstrate that the signalling pathway responsible for the effects of insulin relative to the inhibition of the glucocorticoid-stimulated PF2K/Fru-2,6-BPase gene expression is the JNK–SAPK pathway.

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REFERENCES

1 Pilkis, S. J., Claus, T. H., Kurland, I. J. and Lange, J. A. (1995) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu. Rev. Biochem. **64**, 799–835
2 Colosia, A. D., Marker, A. J., Lange, J. A., El-Maghrabi, M. R., Granner, D. K., Tauler, A. and Pilkis, J. S. (1988) Induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA by refeeding and insulin. J. Biol. Chem. **263**, 18669–18677
3 Espinet, C., Vargas, M. A., El-Maghrabi, M. R., Lange, J. A. and Pilkis, J. S. (1993) Expression of the liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA in FAO-1 cells. Biochem. J. **293**, 173–179
4 Lemaigre, P. F., Lause, P. and Rousseau, G. G. (1994) Insulin inhibits glucocorticoid-induced stimulation of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene transcription. FEBS Lett. **340**, 221–225
5 Vargas, M. A., Sola, M. M., Lange, J. A., Poveda, G. and Pilkis, J. S. (1994) cAMP-independent synergistic effects of insulin and dexamethasone on fructose 2,6-bisphosphate metabolism in H4IIE cells. Diabetes **43**, 792–799
6 Granner, D. K., Andreone, T., Sasaki, K. and Beale, E. (1983) Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. Nature (London) **305**, 549–551
7 Sasaki, K. and Granner, D. K. (1988) Regulation of the phosphoenolpyruvate carboxykinase gene transcription by insulin and cAMP: reciprocal actions on initiation and elongation. Proc. Natl. Acad. Sci. U.S.A. **85**, 2954–2958
8 Avruch, J. (1998) Insulin signal transduction through protein kinase cascades. Mol. Cell. Biochem. **182**, 31–48
9 Lazar, F. D., Wiese, J. R., Brady, J. M., Mastick, C. C., Waters, B. S., Yamauchi, K., Pessin, E. J., Cuatrecasas, P. and Saltiel, R. A. (1995) Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J. Biol. Chem. **270**, 20801–20807
10 Shepherd, R. P., Withers, J. D. and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. **333**, 471–490
11 Summers, S. A., Garza, L. A., Zhou, H. and Birnbaum, J. M. (1998) Regulation of insulin-stimulated glucose transporter GLUT 4 translocation and Akt kinase activity by ceramide. Mol. Cell. Biol. **18**, 5457–5464
12 Su, T. Z., Wang, M., Li-Jyun, S., Saltiel, R. A. and Oxender, D. L. (1998) Regulation of system A amino acid transport in 3T3-L1 adipocytes by insulin. J. Biol. Chem. **273**, 3173–3179
13 Cross, D. A., Alessi, R. D., Vandenheede, J. R., McDowell, H. E., Hundal, H. S. and Cohen, P. (1994) The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem. J. **303**, 21–26
14 Cross, D. A., Alessi, R. D., Cohen, P., Andjelkovic, M. and Hemmings, B. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by PKB. Nature (London) **378**, 785–789
15 Sutherland, C., Waltner-Law, M., Gnudi, L., Kahn, B. B. and Granner, D. K. (1998) Activation of the ras mitogen-activated protein kinase ribosomal protein kinase pathway is not required for the repression of phosphoenolpyruvate carboxykinase gene transcription by insulin. J. Biol. Chem. **273**, 3198–3204
16 Sutherland, C., O'Brien, M. R. and Granner, D. K. (1995) Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is required for the regulation of phosphoenolpyruvate carboxykinase gene expression by insulin. Dissociation of signaling pathways for insulin and phorbol ester regulation of phosphoenolpyruvate carboxykinase gene expression. J. Biol. Chem. **270**, 15501–15506
17 Dickens, M., Svitek, A. C., Culbert, A. A., O'Brien, M. R. and Tavaré, M. J. (1998) Central role for phosphatidylinositide 3-kinase in the repression of glucose-6-phosphatase gene transcription by insulin. J. Biol. Chem. **273**, 20144–20149
18 Yang, S. and Dickson, A. J. (1995) Wortmannin influences insulin regulation of gene 33 expression in rat hepatoma cells. Biochem. J. **310**, 375–378
19 Wagle, A., Jivraj, S., Garlock, G. L. and Stapleton, S. R. (1998) Insulin regulation of glucose 6-phosphate dehydrogenase gene expression is rapamycin-sensitive and requires phosphatidylinositol 3-kinase. J. Biol. Chem. **273**, 14968–14974
20 Osawa, H., Sutherland, C., Robey, B. R., Printz, L. R. and Granner, D. K. (1996) Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. J. Biol. Chem. **271**, 16690–16694

21 Pierreux, E. C., Urso, B., De Meyts, P., Rousseau, G. G. and Lemaigre, P. F. (1998) Inhibition by insulin of glucose-induced gene transcription: involvement of the ligand-binding domain of the glucocorticoid receptor and independence from the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. Mol. Endocrinol. **12**, 1344–1354

22 Lange, J. A., Espinet, C., Hall, R., El-Maghrabi, M. R., Vargas, M. A., Miksicek, R. J., Granner, D. K. and Pilkis, J. S. (1992) Regulation of gene expression of rat skeletal muscle/liver 6-phosphofructo-2-kinase. Isolation and characterization of a glucocorticoid response element in the first intron of the gene. J. Biol. Chem. **267**, 15673–15680

23 Dickens, M., Rogers, S. J., Cavanagh, J., Raitano, A., Xia, Z., Halpern, R. J., Greenberg, E. M., Sawyers, L. C. and Davis, J. R. (1997) A cytoplasmic inhibitor of the c-Jun N-terminal kinase signal transduction pathway. Science **277**, 693–698

24 Livingstone, C., Patel, G. and Jones, N. (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. EMBO J. **14**, 1785–1797

25 Rogatsky, I., Logan, K. S. and Garabedian, J. M. (1998) Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. Proc. Natl. Acad. Sci. U.S.A. **95**, 2050–2055

26 Yoo-Warren, H., Monahan, J. E., Short, J., Short, H., Bruzel, A., Wynshaw-Boris, A., Meisner, H. M., Samols, D. and Hanson, W. R. (1983) Isolation and characterization of the gene coding for cytosolic phosphoenolpyruvate carboxykinase (GTP) from the rat. Proc. Natl. Acad. Sci. U.S.A. **80**, 3656–3660

27 Aufray, C. and Rougeon, F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. **107**, 303–314

28 Ausebel, F. M., Brent, R., Kington, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) Current Protocols in Molecular Biology, John Wiley, New York

29 Tauler, A., Rosenberg, H. A., Colosia, A., Studier, W. F. and Pilkis, J. S. (1988) Expression of the bisphosphatase domain of rat liver 6-PF2K in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **85**, 6642–6646

30 Gerlach, W. L. and Bedbrok, J. R. (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**, 1869–1885

31 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**, 1044–1051

32 O'Brien, M. R., Lucas, C. P., Yamasaki, T., Noisin, L. E. and Granner, D. K. (1994) Potential convergence of insulin and cAMP signal transduction systems of the phosphoenolpyruvate carboxykinase gene promoter through CCAAT/enhancer binding protein (C/EBP). J. Biol. Chem. **269**, 30419–30428

33 Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY 294002). J. Biol. Chem. **269**, 5241–5248

34 Agati, M. J., Yeagley, D. and Quinn, G. P. (1998) Assessment of the roles of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, PKB, and PKC in insulin inhibition of cAMP-induced phosphoenolpyruvate carboxykinase gene transcription. J. Biol. Chem. **273**, 18751–18759

35 Rogatsky, I., Waase, M. L. and Garabedian, J. M. (1998) Phosphorylation and inhibition of rat glucocorticoid receptor transcriptional activation by glycogen synthase kinase-3 (GSK-3). Species-specific differences between human and rat glucocorticoid receptor signaling as revealed through GSK-3 phosphorylation. J. Biol. Chem. **273**, 14315–14321

36 Ferrari, S., Pearson, B. R., Siegmann, M., Kozma, C. S. and Thomas, G. (1993) The immunosuppressant rapamycin induces inactivation of p70 S6K through dephosphorylation of a novel set of sites. J. Biol. Chem. **268**, 16091–16094

37 Jefferies, H. B., Reinhard, C., Kozma, C. S. and Thomas, G. (1994) Rapamycin selectively represses translation of the 'polypyrimidine tract' mRNA family. Proc. Natl. Acad. Sci. U.S.A. **91**, 4441–4445

38 Sale, M. E., Atkinson, P. G. P. and Sale, J. G. (1995) Requirement of MAP kinase for differentiation of fibroblasts to adipocytes for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J. **14**, 674–684

39 Porras, A., Maszynski, K., Rapp, U. R. and Santos, E. (1994) Dissociation between activation of Raf-1 kinase and the 42-kDa mitogen activated protein kinase/90-kDa S6 kinase (MAPK/RSK) cascade in the insulin/Ras pathway of adipocytic differentiation of 3T3 L1 cells. J. Biol. Chem. **269**, 12741–12748

40 Moxham, C. M., Tabrizchi, A., Davis, J. R. and Malbon, C. C. (1996) Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo. J. Biol. Chem. **271**, 30765–30773

41 Desbois-Mouthon, C., Blivet-Van Eggelpoel, M. J., Auclair, M., Cherqui, G., Capeau, J. and Caron, M. (1998) Insulin differentially regulates SAPKs/JNKs and ERKs in CHO cells overexpressing human insulin receptors. Biochem. Biophys. Res. Commun. **243**, 765–770

42 Tsakiridis, T., Taha, C., Grinstein, S. and Klip, A. (1996) Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. J. Biol. Chem. **271**, 19664–19667

43 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, R. A. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689

44 Krstic, D. M., Rogatsky, I., Yamamoto, R. K. and Garabedian, J. M. (1997) Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement. Mol. Cell. Biol. **17**, 3947–3954

45 Schliess, F., Heinrich, S. and Haussinger, D. (1998) Hyperosmotic induction of the mitogen-activated protein kinase phosphatase MKP-1 in H4IIE rat hepatoma cells. Arch. Biochem. Biophys. **351**, 35–40

46 Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R. and Landvatter, S. W. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature (London) **372**, 739–745

47 Xu, S. and Cobb, H. M. (1997) MEKK-1 binds directly to the c-Jun N-terminal kinase/stress-activated protein kinases. J. Biol. Chem. **272**, 32056–32060

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