Antagonistic effects of phorbol esters on insulin regulation of insulin-like growth factor-binding protein-1 (IGFBP-1) but not glucose-6-phosphatase gene expression

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Glucose-6-phosphatase (G6Pase) and insulin-like growth factor-binding protein-1 (IGFBP-1) genes contain a homologous promoter sequence that is required for gene repression by insulin. Interestingly, this element interacts with members of the forkhead family of transcription factors [e.g. HNF3 (hepatic nuclear factor 3), FKHR (forkhead in rhabdomyosarcoma)] *in vitro*, while insulin promotes the phosphorylation and inactivation of FKHR in a phosphatidylinositol 3-kinase- and protein kinase B (PKB)-dependent manner. This mechanism has been proposed to underlie insulin action on G6Pase and IGFBP-1 gene transcription. However, we find that treatment of cells with phorbol esters mimics the effect of insulin on G6Pase, but not IGFBP-1, gene expression. Indeed, phorbol ester treatment actually blocks

the ability of insulin to repress IGFBP-1 gene expression. In addition, the action of phorbol esters is significantly reduced by inhibition of the p42/p44 mitogen-activated protein (MAP) kinase pathway. However insulin-induced phosphorylation of PKB or FKHR is not affected by the presence of phorbol esters. Therefore we suggest that activation of p42/p44 MAP kinases will reduce the sensitivity of the IGFBP-1 gene promoter, but not the G6Pase gene promoter, to insulin. Importantly, the activation of PKB and phosphorylation of FKHR is not, in itself, sufficient to reduce IGFBP-1 gene expression in the presence of phorbol esters.

Key words: forkhead, MAP kinase, PKB, transcription.

INTRODUCTION

Multiple differences in gene-expression patterns are found in people with type 2 diabetes mellitus compared with healthy individuals, especially in insulin-responsive tissues (e.g. liver, adipose tissue and muscle) [1]. Whether these differences underlie the progression of this disorder or are a consequence of some other defect (e.g. insulin resistance) is not yet clear [2]. Insulin is known to regulate the expression of over 100 gene products, but as yet no 'consensus' insulin-response element (IRE) has been established that could account for the regulation of more than a few genes [1,3]. Recently however, a DNA sequence has been characterized that can bind members of the forkhead family of transcription factors and is required for the insulin inhibition of expression of at least three hepatic genes [1]. This sequence (TRTTTKK) is relatively short and degenerate and is therefore found in promoters that are not repressed (or even regulated) by insulin. However, when multimerized and placed upstream of normally insulin-unresponsive promoters it permits around 50 % repression in the presence of insulin or insulin-like growth factor (IGF)-1 [4–9].

Glucose-6-phosphatase (G6Pase), IGF-binding protein-1 (IGFBP-1) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK), three important metabolic genes in the liver, all contain the IRE within the insulin-responsive regions of their promoters. G6Pase and PEPCK are key rate-controlling enzymes required for hepatic gluconeogenesis [10] while IGFBP-1 binds

to and inhibits IGF-I and IGF-II with high affinity (for review see [11]). The expression of all three genes is induced by glucocorticoids, repressed in a dominant fashion by insulin [3,12–14] and is increased in the diabetic state [15–17]. Much work is now focused on identifying the signalling pathway that links the insulin receptor to each gene promoter (and to this IRE). Insulin induces multiple signalling cascades including the activation of phosphoinositide 3-kinase (PI 3-kinase) with a resultant stimulation of protein kinase B (PKB) and p70 ribosomal S6 kinase. In addition, insulin induces the small G-protein Ras to activate a protein-kinase cascade including Raf, mitogen-activated protein (MAP) kinase kinase-1, p42/p44 MAP kinase and p90 ribosomal S6 kinase (p90RSK), although this pathway is relatively weakly induced by insulin in liver cells. As the PEPCK, IGFBP-1 and G6Pase gene promoters contain a similar IRE, it is suggested that the same insulin-regulated mechanism is invoked in repressing each gene. To date this hypothesis has been strengthened by the finding that the action of insulin on all three gene promoters is blocked by inhibitors of PI 3-kinase [4,18–20], whereas PEPCK and G6Pase regulation is unaffected by agents that inhibit activation of either p42/p44 MAP kinases or p70 ribosomal S6 kinase ([20,21] and P. A. Lochhead, unpublished work). Furthermore, forkhead transcription factors that bind to this IRE *in vitro* [14,22] induce transcription of promoters containing two or more copies of the IRE [6–9]. Insulin treatment reduces this transcription and promotes nuclear exclusion of FKHR (forkhead in rhabdomyosarcoma)/FKHR-L1 (FKHR-

Abbreviations used: G6Pase, glucose-6-phosphatase; IGF, insulin-like growth factor; IGFBP-1, IGF-binding protein-1; IRE, insulin-response element; PKB, protein kinase B; PEPCK, phosphoenolpyruvate carboxykinase; PI 3-kinase, phosphoinositide 3-kinase; MAP, mitogen-activated protein; PKC, protein kinase C; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; p90RSK, p90 ribosomal S6 kinase; FKHR, forkhead in rhabdomyosarcoma; FKHR-L1, FKHR-like 1; AFX, ALL1 fused gene from chromosome X; GSK3, glycogen synthase kinase 3.

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like 1) and AFX (ALL1 fused gene from chromosome X) by a PI 3-kinase-dependent mechanism [6,23,24]. One such mechanism involves the PKB-catalysed phosphorylation of Ser-256, which is sufficient to cause nuclear exclusion of FKHR [23,24]. Therefore, the available data suggest a pathway from the insulin receptor through PI 3-kinase and PKB to FKHR, resulting in nuclear exclusion of FKHR and a loss of G6Pase and IGFBP-1 gene-promoter activity (for review see [25]). In contrast, the PEPCK promoter contains only one copy of the IRE and expression of a dominant-negative form of PKB does not affect insulin regulation of PEPCK gene expression [26]. These data argue that this mechanism does not underlie regulation of the PEPCK gene by insulin.

Many of the cellular actions of insulin can be mimicked by phorbol esters [via activation of specific isoforms of protein kinase C (PKC)], although most of insulin's actions do not appear to require activation of PKC. For example, phorbol ester activation of classical PKCs results in repression of PEPCK gene expression, although the ability of insulin to repress this gene is unaffected by down-regulation of PKC [27]. PKB and PKC have somewhat similar substrate specificities *in vitro*, suggesting that phorbol ester signalling may converge with insulin signalling at targets directly downstream of PKB. Potential common targets include glycogen synthase kinase 3 (GSK3) [28,29] and FKHR/FKHR-L1/AFX. For this reason, we have examined the effect of phorbol esters on G6Pase and IGFBP-1 gene expression concomitantly with regulation of PKB, GSK3 and FKHR-L1. We find that although G6Pase expression is reduced by phorbol ester treatment of H4IIE cells, IGFBP-1 expression is not. Indeed, the effect of insulin on IGFBP-1 expression is actually lost in the presence of phorbol esters. Importantly, phosphorylation of FKHR by PKB is not sufficient to reduce the activity of the IGFBP-1 gene promoter.

EXPERIMENTAL

Materials

Radioisotopes were obtained from Amersham, Little Chalfont, Bucks., U.K. ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$) and ICN, Thame, Oxon, U.K. ($[\alpha\text{-}^{32}\text{P}]\text{UTP}$). Insulin was purchased from Novo Nordisk (Crawley, West Sussex, U.K.), 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP) from Boehringer Mannheim/Roche Diagnostics (Lewes, East Sussex, U.K.), PMA from Sigma and RNase Protection Assay Kit II from AMS Biotech/Ambion (Austin, TX, U.S.A.). All other chemicals were of the highest grade available.

Cell culture

The rat hepatoma cell line H4IIE was maintained in Dulbecco's modified Eagle's medium containing 1000 mg/l glucose and 5% (v/v) foetal calf serum, as described previously [30]. Cells were incubated with hormones, inhibitors or DMSO carrier as indicated in the absence of serum at 37 °C.

RNA isolation and RNase-protection assay

H4IIE cells were serum-starved overnight and treated with hormone/inhibitor for the times and at the concentrations indicated in the figure legends. Cell stimulations were performed in duplicate and assayed in duplicate. Total cellular RNA (approx. 100 $\mu\text{g}/10^6$ cells) was isolated using TriReagent™ (Sigma) following the manufacturers instructions. An RNase-protection assay was performed in order to determine the relative proportions of G6Pase, IGFBP-1 and cyclophilin mRNA in each sample. The synthesis of a G6Pase-specific RNA probe has been described previously [30]. The sequence of the rat IGFBP-1

cDNA from nucleotides +253 to +480 (in exon 1) was amplified from total rat liver RNA by reverse transcriptase PCR and the resultant products cloned into the plasmid pCR2.1-TOPO (Invitrogen). The plasmid was cut with *Bam*HI to produce a linear DNA template, while pTRI-cyclophilin linear plasmid (Ambion) was used as a control DNA template. G6Pase, IGFBP-1 and cyclophilin RNA probes were synthesized by transcription *in vitro* in the presence of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (0.33 $\mu\text{Ci}/\text{nmol}$) using T7 RNA polymerase as directed by the MaxiScript Kit (Ambion). Each probe (3000 c.p.m.) was hybridized overnight at 42 °C with 10 μg of total RNA, as directed by the RNase Protection Assay Kit II. The probes and mRNA of interest (in the total RNA sample) hybridized to form double-stranded RNA complexes and single-stranded RNA was removed by digestion with RNase. Samples were analysed on an 8 M urea/5% polyacrylamide gel and the intensity of the band was proportional to the amount of mRNA. Band intensity was quantified on a PhosphorImager (Fuji) and data calculated as a ratio of G6Pase/cyclophilin or IGFBP-1/cyclophilin and presented as fold activation (for induced samples), where the intensities of control samples (no serum) were set at 1, or as percentage gene expression (for non-induced samples) relative to expression of the test gene in untreated cells.

Preparation of cell extract for kinase assays and Western blotting

H4IIE cells were incubated in serum-free medium with hormones and inhibitors for the times and at the concentrations indicated in the figure legends. Cells were then scraped into ice-cold lysis buffer [25 mM Tris/HCl, pH 7.4, 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 1 mM benzamide, 0.1 mM PMSF, 0.27 M sucrose, 2 μM microcystin and 0.1% (v/v) 2-mercaptoethanol]. Cell debris was removed by centrifugation at 13000 *g* for 5 min and the protein concentration determined, using BSA as an internal standard, by the method of Bradford [30a].

Immunoprecipitation and assay of protein kinases

Cell extract (0.1 mg) was incubated for 1 h on a shaking platform with protein-G-Sepharose conjugated to the appropriate antibody. The immunocomplexes were pelleted and washed twice with 1.0 ml of buffer A [50 mM Tris/HCl, pH 7.5, 50 mM NaF, 500 mM NaCl, 1 mM sodium vanadate, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 5 mM sodium pyrophosphate, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol], and twice with 1.0 ml of buffer B [50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol]. The immunoprecipitated kinase activities were assayed at 30 °C, in a total volume of 50 μl containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 2.5 μM protein kinase A-inhibitor peptide, 10 mM MgCl_2 , 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^6 c.p.m./nmol) and 30 μM crosstide [29]. One unit of kinase activity was defined as the amount which catalyses the phosphorylation of 1 nmol of substrate in 1 min. Immunoprecipitated FKHR was resuspended in SDS sample buffer (Novex) and analysed by Western blotting as described previously [31].

Antibodies and Western-blot analysis

An antibody to p90RSK was raised in sheep by injection of the peptide RNQSPVLEPVGRSTLAQ, equivalent to residues 712–728 of human p90RSK (accession no. NP004577). Phospho-specific Thr-32 FKHR-L1, FKHR, PKB α and GSK3 α antibodies were all purchased from Upstate Biotechnology, and phospho-

specific Ser-9/Ser-21 GSK3, Thr-308 PKB, Thr-202/Tyr-204 p42/p44 MAPK and Ser-256 FKHR antibodies and p42/p44 MAPK antibodies were purchased from New England Biolabs. Following incubation with 10% (w/v) non-fat milk, primary antibodies were incubated at 4 °C overnight prior to incubation for 1 h at room temperature with secondary antibody, followed by development using the ECL kit (Amersham) as per the manufacturer's instructions.

RESULTS

Differential effects of phorbol esters on the regulation of expression of G6Pase and IGFBP-1 genes

Phorbol esters reduced basal as well as cAMP- and glucocorticoid-induced G6Pase gene expression within 3 h (Figure 1), identical with their effect on PEPCK gene expression [27], and suggestive of a common phorbol ester-regulated mechanism for inhibition of both gene promoters. However, there was no

Figure 1 Effects of phorbol esters on G6Pase gene expression

H4IIE cells were serum-starved overnight prior to a 3 h incubation with hormones as indicated, concentrations as follows: dexamethasone (Dex), 500 nM; 8CPT-cAMP (cAMP), 0.01 mM; insulin, 10 nM and PMA (TPA), 400 ng/ml, or the equivalent volume of DMSO. Total cellular RNA was isolated, and an RNase-protection assay performed as described in the Experimental section. (Top panel) PMA repressed basal G6Pase gene expression. (Bottom panel) PMA repressed G6Pase expression induced by glucocorticoids and cAMP. Representative experiments are shown for the gels whereas the histograms show the means $\pm$ S.E.M. from three experiments carried out in duplicate.

Figure 2 Effects of phorbol esters on IGFBP-1 gene expression

H4IIE cells were serum-starved overnight prior to a 3 h incubation with hormones as indicated, with concentrations as given for Figure 1. Total cellular RNA was isolated, and an RNase-protection assay performed as described in the Experimental section. PMA (TPA) had little effect on basal (top panel) or induced (bottom panel) IGFBP-1 expression but blocked the ability of insulin to repress the gene. The lower panels are representative experiments, the histograms display means $\pm$ S.E.M. from two experiments carried out in duplicate. Dex, dexamethasone.

inhibition of IGFBP-1 gene expression by phorbol esters (Figure 2). Indeed we observed a slight induction of basal gene expression (Figure 2, top panel), although the effect of phorbol ester and glucocorticoid was not additive (Figure 2, bottom panel). Most interestingly, the ability of insulin to block IGFBP-1 gene expression did not occur in the presence of phorbol esters (Figure 2). This effect of phorbol ester was seen in the presence and absence of dexamethasone (Figure 2) and with dexamethasone plus 8CPT-cAMP (results not shown). These data demonstrate a major difference between the mechanisms underlying the insulin regulation of G6Pase and PEPCK gene expression and those responsible for insulin action on IGFBP-1 expression.

Figure 3 Pre-incubation of H4IIE with phorbol ester reduces the effects of PMA (TPA) on G6Pase and IGFBP-1 gene expression

Serum-starved H4IIE cells were pre-incubated with 400 ng/ml PMA or the equivalent volume of DMSO (control) for 18–20 h and then incubated with hormones/PMA (as indicated) for 3 h. Total cellular RNA was isolated, and G6Pase and IGFBP-1 mRNA was analysed by RNase-protection assay as described in the Experimental section. The ability of PMA to repress basal (top left panel) and induced (bottom left panel) G6Pase gene expression was lost by prolonged exposure of H4IIE to phorbol esters. Similarly, the ability of phorbol esters to block insulin regulation of both basal (top right panel) and induced (bottom right panel) levels of IGFBP-1 gene expression was lost following pre-treatment with PMA. The regulation of either gene by insulin was unaffected by this treatment. An additional lane (—) compares the effect of PMA on IGFBP-1 and G6Pase gene expression in the absence of PKC down-regulation. Results are the means $\pm$ S.E.M. from two (IGFBP-1) or three (G6Pase) independent experiments carried out in duplicate. Dex, dexamethasone; cAMP, 8CPT-cAMP.

Phorbol ester effects are mostly mediated through activation of various PKC isoforms (mainly PKCs α , β , δ and ϵ). Prolonged exposure of cells to phorbol esters promotes degradation of these PKC isoforms and thus renders cells refractive to further phorbol ester treatment [32]. The ability of PMA to inhibit G6Pase expression (Figure 3, left-hand panels), or block insulin regulation of IGFBP-1 gene expression (Figure 3, right-hand panels), was almost completely lost following exposure of H4IIE cells to phorbol ester for 24 h. Therefore PKC activation underlies the response of these gene promoters to phorbol esters. However,

insulin regulation of either gene was not affected under these conditions (Figure 3), demonstrating that activation of phorbol ester-sensitive isoforms of PKC is not required for insulin regulation of G6Pase or IGFBP-1 expression.

Activation of PKB and phosphorylation of FKHR-L1 is not sufficient to reduce IGFBP-1 gene expression

To identify candidate signalling pathways that could mediate the effects of PMA on G6Pase or IGFBP-1 gene expression, we

Figure 4 Insulin regulation of PKB, GSK3 and FKHR/FKHR-L1 is not affected by phorbol esters

H4IIE cells were serum-starved overnight prior to incubation with hormones [10 nM insulin and 400 ng/ml PMA (TPA), or the equivalent volume of DMSO]. At the times indicated, cells were lysed and PKB was immunoprecipitated and assayed (top left panel) as described in the Experimental section. Results are the means $\pm$ S.E.M. from three experiments performed in triplicate. Alternatively, after 30 min (bottom panels) or 3 h (top right panel), cells were lysed and the lysates subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibodies as labelled. Similar results were obtained from two experiments carried out in duplicate. In the bottom right panel FKHR was immunoprecipitated prior to electrophoresis, transfer and immunoblotting with antibodies as labelled (phospho, phospho-specific antibody).

compared the effect of this agent on two major insulin-regulated pathways (Figure 4). Insulin activated PKB 10–15-fold within 15 min in H4IIE cells and this activity was maintained for at least 3 h (Figure 4, top left panel). Surprisingly, PMA treatment also activated PKB in these cells but to a much lower level than insulin (Figure 4, top left panel). In addition, PMA activation occurred transiently with maximal activation at 1 h and almost complete loss of induction after 3 h of treatment. Importantly, the activation of PKB by insulin was not antagonized by simultaneous PMA treatment (Figure 4, top left panel).

Phorbol ester, like insulin, stimulated the phosphorylation of GSK3 and FKHR-L1, two well-characterized substrates of PKB (Figure 4, bottom left panel). Phosphorylation of Thr-308 (PKB) was correlated with activation of PKB whereas phosphorylation of Ser-9 (GSK3 β), Ser-21 (GSK3 α), Ser-256 (FKHR) and Thr-

32 (FKHR-L1) was indicative of inhibition of these proteins. Using phospho-specific antibodies we observed PMA stimulation of phosphorylation of these residues on endogenous PKB, GSK3 α , GSK3 β and FKHR-L1 in H4IIE cells (Figure 4, bottom left and both right-hand panels). PMA produced an equivalent intensity of staining, with antibodies specific for phospho-GSK3 α and β , and phospho-Thr-32 of FKHR-L1, to that seen with insulin treatment. Meanwhile, PMA had a lesser effect on Thr-308 (PKB) phosphorylation. Simultaneous insulin and PMA treatment of H4IIE cells resulted in a similar level of phosphorylation of these residues to that observed with insulin alone. Cells treated with these agents for 3 h (Figure 4, top right panel), exhibited reduced PMA-mediated phosphorylation of Thr-308, Ser-21 and Thr-32, suggesting that PKB underlies the effect of PMA on GSK3 α and FKHR-L1. However, PMA-mediated

Figure 5 Phorbol esters activate p90RSK and MAP kinase to a much greater extent than insulin in H4IIE cells

H4IIE cells were serum-starved overnight prior to incubation with hormones $\pm$ PD 184352 [$2 \mu\text{M}$ PD 184352 (PD), 10 nM insulin and 400 ng/ml PMA (TPA)]. (Top left panel) At the times indicated, cells were lysed and p90RSK was immunoprecipitated and assayed as described in the Experimental section. Results are the means $\pm$ S.E.M. from two experiments performed in triplicate. Alternatively (bottom left panel), after 30 min or 3 h, cells were lysed and the lysates were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibodies raised against p42/p44 MAP kinase (MAPK) or p42/p44 MAP kinase phosphorylated at Thr-202 and Tyr-204 (Phospho MAPK). In addition, cells pre-incubated $\pm$ PD 184352 for 30 min prior to addition of hormones for 30 min (top right panel) or 3 h (bottom right panel), were lysed and p90RSK was immunoprecipitated and assayed as described in the Experimental section. Results are the means $\pm$ S.E.M. from three experiments performed in triplicate.

phosphorylation of GSK3 β remained high at 3 h, suggesting a role for a different protein kinase in this effect. The residue most associated with nuclear exclusion and inhibition of FKHR was Ser-256. Phosphorylation of this residue of endogenous FKHR was assessed by immunoprecipitating FKHR prior to Western-blot analysis and detection with phospho-specific antibodies raised against the sequence surrounding Ser-256 (Figure 4, bottom right panel and [23]). The insulin stimulation of phosphorylation of Ser-256 in H4IIE cells was not affected by PMA, at either 30 min or 3 h (Figure 4, bottom right panel).

Phorbol ester antagonism of insulin regulation of IGFBP-1 gene expression is significantly reduced by PD 184352

p90RSK (also known as MAP kinase-activated protein kinase 1) and p42/p44 MAP kinase were activated by both insulin and PMA treatment of H4IIE cells (Figure 5). However, the activation of this pathway by PMA was much stronger than that observed with insulin (Figure 5). The PMA-induced activation of p90RSK (almost 30-fold) occurred within 15 min and was maintained for up to 3 h before its activity declined over a

Figure 6 Phorbol ester- but not insulin-regulated IGFBP-1 and G6Pase gene expression is sensitive to an inhibitor of MAP kinase kinase activation

H4IIE cells were serum-starved overnight and pre-incubated with or without PD184352 (PD) for 30 min prior to a 3 h incubation with hormones $\pm$ PD 184352 [$2 \mu\text{M}$ PD 184352, 500 nM

further 20 h period (Figure 5, top left panel). Insulin activated this kinase almost 10-fold, but the activation was gone within 3 h (Figure 5, top left panel). Similarly, the activation of MAP kinases by PMA was much greater than that observed with insulin (Figure 5, bottom left panel), although the activation of this kinase by either agent was not maintained during 3 h of phorbol ester stimulation of H4IIE cells (Figure 5, bottom left panel). The presence of an inhibitor of MAP kinase kinase activation, PD 184352 [33,34], completely blocked the activation of the MAP kinase pathway by insulin or PMA at 30 min (Figure 5, top right panel) or 3 h (Figure 5, bottom right panel). Meanwhile, this compound had no effect on the ability of insulin to regulate the gene expression of either G6Pase (Figure 6, top panel) or IGFBP-1 (Figure 6, middle and bottom panels), demonstrating that MAP kinase was not required for insulin action on these genes. However the presence of PD 184352 reduced the effect of PMA on IGFBP-1 expression by over 50%. In addition, the effect of PMA on G6Pase expression was also reduced (by between 20 and 50%) in the presence of PMA (Figure 6). This suggests that strong activation of the MAP kinase pathway mediates part of the antagonistic action of PMA on insulin regulation of IGFBP-1 expression and also the insulin-mimetic action of PMA on G6Pase expression.

DISCUSSION

Differential effects of phorbol esters on the insulin-regulation of three gene promoters that contain related IRES

The fact that phorbol ester treatment of H4IIE cells overcomes the insulin regulation of IGFBP-1, but not G6Pase, gene expression is the first direct demonstration of a difference in the regulation of endogenous G6Pase and IGFBP-1 gene expression by insulin. Previously, a difference between phorbol ester effects on PEPCK and IGFBP-1 gene expression has been reported [35]. The data suggest either that this differential action is due to a positive-acting PMA-responsive element found in the IGFBP-1 but not G6Pase promoter, or alternatively that PMA blocks the signalling pathway between the insulin receptor and the IGFBP-1 but not the G6Pase gene promoter. We cannot conclusively distinguish between these two hypotheses. Two reports have suggested a stimulatory effect of phorbol ester on basal IGFBP-1 expression [35,36], therefore indicating the presence of a phorbol ester-sensitive element within the IGFBP-1 promoter, or alternatively a basal level of insulin signalling that is blocked when PMA is added. Indeed, the level of induction varies between reports and is always less than 3-fold. One of these studies (on IGFBP-1 promoter constructs in transient transfection) found insulin action to be dominant over the slight phorbol ester induction of the IGFBP-1 promoter construct [36], whereas the other found the effect of phorbol ester to be dominant over insulin action, but in a time-dependent manner [35]. We also observed a slight (and variable) induction of basal IGFBP-1 gene expression by PMA (1–3-fold) in H4IIE cells. However addition of PMA completely blocked insulin repression of the gene. Also, PMA treatment of cells did not induce the gene in the presence of dexamethasone, whereas PMA completely blocked the insulin

dexamethasone (Dex), 0.1 mM 8CPT-cAMP (cAMP), 10 nM insulin and 400 ng/ml PMA (TPA)]. Total cellular RNA was isolated, and an RNase-protection assay performed to assess the level of G6Pase (top panel), IGFBP-1 (middle panel) or dexamethasone-induced IGFBP-1 (bottom panel) mRNA. The effect of PD 184352 on the regulation of G6Pase (top panel) was statistically significant ($P < 0.01$, * versus **). Representative experiments are displayed in the lower sections of each panel, and means $\pm$ S.E.M. from two experiments carried out in duplicate are displayed in the histograms.

repression of dexamethasone-induced gene expression (Figure 2). These data clearly demonstrate that phorbol esters are dominant over insulin regulation of IGFBP-1 gene expression. We favour the hypothesis that at least part of the action of phorbol ester involves inhibition of insulin action on this gene promoter, rather than activation of a phorbol ester-sensitive promoter element. However, it is also possible that a PMA-regulated factor induces, or maintains in an active conformation, the IGFBP-1 promoter through interaction with a promoter element distinct from the IRE. This factor may only be active in the presence of both PMA and insulin or alternatively may interfere with the function of an IRE-binding protein when PMA is present.

Phorbol ester activation of PKB and inhibition of FKHR/FKHR-L1 does not repress IGFBP-1 gene expression

Mapping the signalling connection between the insulin receptor and the nucleus has proved very difficult. Insulin regulates the expression of a multitude of gene products (both positively and negatively) with varying intensity. The effect on transcription is achieved through regulation of the localization and/or activity of specific DNA-binding proteins, such as FKHR. A relatively simple mechanism has been identified to underlie insulin regulation of FKHR, involving activation of PI 3-kinase and PKB, with direct phosphorylation of FKHR on three or more sites (for review see [25]). However, the data linking FKHR to the insulin regulation of endogenous gene transcription is weak. Recombinant FKHR can bind to the IGFBP-1 IRE *in vitro*, and when co-expressed transiently in hepatoma cells will induce transcription from synthetic promoter constructs containing multiple copies of the IRE [6–9]. However, recent work has shown that the ability of insulin to regulate a variety of IRE-containing promoters does not correlate with the ability of FKHR-L1 to bind to IRE sequences *in vitro* [37]. Therefore it has been proposed that endogenous FKHR-L1 is not involved in the regulation of this IRE by insulin *in vivo* [37]. We now provide evidence that activation of PKB and inhibition of FKHR or FKHR-L1 are not sufficient to repress IGFBP-1 expression in the presence of PMA. Insulin regulation of PKB and two of its substrates (GSK3 and FKHR/FKHR-L1) is unaffected by the presence of phorbol esters. Indeed, phorbol esters, like insulin, induced phosphorylation of the 14-3-3 binding motif surrounding Thr-32 of FKHR-L1 (Figure 4). Under the same experimental conditions insulin regulation of IGFBP-1 was not observed (Figure 2). It will take a more detailed characterization of the mechanism of PMA action on the insulin signalling cascade or the IGFBP-1 gene promoter to determine whether there is a distinct FKHR- and PKB-independent mechanism involved in the insulin regulation of the IGFBP-1 gene.

Our data do not exclude the PKB/FKHR pathway from a role in the regulation of G6Pase gene expression by insulin and PMA, but suggest that activation of PKB and removal of this transcription factor from the nucleus is not sufficient to repress the IGFBP-1 gene promoter (at least in the presence of PMA).

At least part of the signal used by phorbol esters to antagonize insulin action involves the strong activation of MAP kinases

The activation of p90RSK by phorbol esters is blocked by PD 184352 (Figure 5, right-hand panels). Interestingly, only half of the action of phorbol esters on IGFBP-1 regulation was lost in the presence of PD 184352 (Figure 6). This suggests that more than one signalling pathway is initiated by phorbol esters to block the action of insulin on this gene, and that activation of

p42/p44 MAP kinase plays a major role. This pathway is also activated by insulin, thus the inhibitory effect of MAP kinase activation on insulin action must be due to the much stronger activation observed with PMA (Figure 5), compared with the 1.5-fold obtained with insulin in these cells [21]. Clearly, it is not sufficient to assign function to a protein kinase simply due to an increase in its activity, as the magnitude and duration [38] of activation of p42/p44 MAP kinase has profound implications on its function. Other antagonistic effects of the MAP kinase pathway over PI 3-kinase-mediated responses have been reported [39,40]. Interestingly, agents that avidly activate this pathway may reduce the insulin sensitivity of the IGFBP-1 but not the G6Pase or PEPCK promoters. Indeed this may underlie the loss of the dominant effect of insulin on IGFBP-1 expression seen during abdominal surgery [41] and chronic liver disease [42].

The data presented herein demonstrate that PMA reduces the insulin regulation of IGFBP-1 expression. This appears contradictory to several reports that have proposed a role for PKC in repression [43,44] or induction [35,36,45] of IGFBP-1 gene expression. These discrepancies may be related to experimental protocol, the interpretation of the effect of phorbol esters on PKC activity, or the lack of specificity of the PKC inhibitors employed. For example, phorbol esters activate PKC (within minutes); however, they also lead to PKC degradation in cells after prolonged exposure (2–24 h) [32]. In addition, many commonly used PKC inhibitors potentially affect a variety of protein kinases [34], adding a further complication to interpretation of data obtained with these reagents.

In summary, we demonstrate a major difference in the molecular mechanisms used by insulin to regulate G6Pase and IGFBP-1 gene expression. We demonstrate that insulin action on IGFBP-1 (but not G6Pase) expression may be antagonized when p42/p44 MAP kinase is strongly activated. Therefore mitogenic signals potentially override the effects of insulin on expression of a growth factor yet mimic the effects of insulin on a gene product involved in glucose homeostasis. It is likely that there are many agents that will antagonize the action of insulin on IGFBP-1 expression, since the p42/p44MAP kinase pathway is activated by multiple stimuli. Finally, we demonstrate that the loss of insulin action on IGFBP-1 gene expression is not accompanied by a loss of regulation of PKB or FKHR by insulin. We therefore propose the existence of a distinct signalling pathway, antagonized by phorbol esters, that is required for appropriate regulation of IGFBP-1 gene expression by insulin. We are currently mapping other phorbol ester-regulated signalling pathways in an attempt to determine all of the molecules required for this ability to block insulin action. Hopefully this will lead to the identification of insulin-regulated molecules that are involved in the repression of IGFBP-1 but not G6Pase gene expression.

This work was supported by Wellcome Trust Career Development Award 051792, a Biotechnology and Biological Sciences Research Council CASE award (to S.P., industrial partner Glaxo SmithKline) and the Medical Research Council (G.R.). We thank Professor Sir Philip Cohen for critical review of the manuscript.

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