A major action of insulin is to regulate the transcription rate of specific genes. The expression of these genes is dramatically altered in type 2 diabetes. For example, the expression of two hepatic genes, glucose-6-phosphatase and PEPCK, is normally inhibited by insulin, but in type 2 diabetes, their expression is insensitive to insulin. An agent that mimics the effect of insulin on the expression of these genes would reduce gluconeogenesis and hepatic glucose output, even in the presence of insulin resistance. The repressive actions of insulin on these genes are dependent on phosphatidylinositol (PI) 3-kinase. However, the molecules that lie between this lipid kinase and the two gene promoters are unknown. Glycogen synthase kinase-3 (GSK-3) is inhibited following activation of PI 3-kinase and protein kinase B. In hepatoma cells, we find that selectively reducing GSK-3 activity strongly reduces the expression of both gluconeogenic genes. The effect is at the level of transcription and is observed with induced or basal gene expression. In addition, GSK-3 inhibition does not result in the subsequent activation of protein kinase B or inhibition of the transcription factor FKHR, which are candidate regulatory molecules for these promoters. Thus, GSK-3 activity is required for basal activity of each promoter. Inhibitors of GSK-3 should therefore reduce hepatic glucose output, as well as increase the synthesis of glycogen from L-glucose. These findings indicate that GSK-3 inhibitors may have greater therapeutic potential for lowering blood glucose levels and treating type 2 diabetes than previously realized.

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The cellular actions of insulin include increased glucose transport, glycogen synthesis, and lipogenesis and decreased gluconeogenesis, glycogen, and fat breakdown. The result is reduced hepatic glucose output and increased peripheral glucose utilization. In type 2 diabetes, most of the intracellular actions of insulin are reduced or absent (1–3), yet the identity of the lesions underlying insulin resistance is not clear. To find ways to combat insulin resistance, much research focuses on the insulin-regulated signaling pathways that normally mediate glucose production and/or glucose utilization and that connect the insulin receptor to the proteins that directly mediate each action of insulin. The initial step in insulin signaling involves the binding of insulin to its specific cell surface receptor and activation of the tyrosine kinase activity of the receptor, followed by the phosphorylation of a small subset of proteins, including a group of proteins termed insulin receptor substrates (4). From this point, multiple signaling cascades are activated including the p42/p44 mitogen-activated protein (MAP) kinase cascade, the phosphatidylinositol (PI) 3-kinase–mTOR-p70 S6 kinase cascade, and the PI 3-kinase–PI 3,4,5-Tris phosphate–dependent kinase (PDK)-protein kinase B (PKB) cascade (rev. in 5). The activation of PKB by PDK-1 (5–8) leads to the phosphorylation of multiple cellular proteins including glycogen synthase kinase-3 (GSK-3) (9). Phosphorylation of GSK-3 at an NH2-terminal serine residue (Ser-21 and Ser-9 in GSK-3 (GSK-3) (9). Phosphorylation of GSK-3 at an NH2-terminal serine residue (Ser-21 and Ser-9 in GSK-3α and -3β, respectively) renders it inactive (10,11). As GSK-3 phosphorylates and inactivates glycogen synthase, insulin action on GSK-3 results in increased glycogen storage in muscle and liver. Indeed, glycogen synthesis can be induced by treatment of human liver cells with selective small molecule inhibitors of GSK-3 (SB-216763 and SB-415286) (12). This suggests that such molecules would aid glucose disposal in liver and probably muscle, reducing the hyperglycemia associated with diabetes.

Insulin also regulates the expression of >100 genes (rev. in 13,14). Two insulin-responsive genes vital for regulation of hepatic gluconeogenesis are PEPCK (15,16) and the glucose-6-phosphatase (G6Pase) catalytic subunit (17,18). In liver, dysfunctional regulation of these gene promoters is associated with the pathophysiology of type 2 diabetes (19–22). The transcription of each gene is enhanced by
glucagon and glucocorticoids and inhibited by insulin in a dominant manner (17,23–25). These actions of insulin are PI 3-kinase–dependent and not blocked by inhibition of the p70 S6 kinase or the p42/p44 MAP kinase pathways (26–29). Interestingly, these two genes share a common insulin-responsive element within their promoters; this suggests that a common regulatory mechanism may mediate the repression of these genes. This DNA element is located within the promoters of a number of insulin-responsive genes (13); thus, the signaling pathway that regulates PEPCK and G6Pase may represent a more widespread mechanism for the repression of multiple genes by insulin. Indeed, the IGF-1 binding protein (IGFBP-1) contains two such sequences. Much recent work has linked the repression of IGFBP1 gene expression to the phosphorylation and inactivation of certain members of the forkhead family of transcription factors (FKHR). These proteins are targets for PKB-mediated phosphorylation and are exported from the nucleus following PKB overexpression or insulin/IGF-1 treatment of cells (rev. in 30). Therefore, PKB and FKHR transcription factors are major candidates as mediators of insulin-regulated repression of PEPCK and G6Pase as well as IGFBP-1 gene transcription.

In this study, we demonstrate that the inhibition of GSK-3 leads to inhibition of PKB and G6Pase gene expression. Thus, inhibitors of GSK-3 are likely to have profound effects on glucose production in liver as well as glucose disposal in the periphery.

**RESEARCH DESIGN AND METHODS**

Radioisotopes [γ-32P]ATP, [α-32P]UTP, and [14C]sodium acetate were obtained from Amersham (Chalfont, Bucks, U.K.) and ICN (Thame, Oxfordshire, U.K.). Insulin was purchased from Novo Nordisk (Crawley, West Sussex, U.K.) and S-(4-chlorophenylthio)-cAMP (S-cAMP) from Boehringer-Mannheim (Lewes, East Sussex, U.K.). Dexamethasone was obtained from Sigma Chemical and RNA Protection Assay Kit II from AMS Biotech/Ambion (Austin, TX). All other chemicals were of the highest grade available.

**Antibodies.** The phospho-specific Thr32-Ser47 PKB antibody and PKB-α and GSK-3α antibodies were purchased from Upstate Biotech (Lake Placid, NY), and the phospho-specific Serb/Ser21 GSK-3 was purchased from New England Biolabs.

**Cell culture, hormone treatments, and clone acetyltansferase assay.** Isolation of the H4IIE rat hepatoma derived the stable transfectant HLIC, which contains the PEPCK promoter sequence from 42–67, relative to the transcription start-site in the PEPCK and G6Pase mRNA. Band intensity was quantitated on a phosphorimager (Fuji), and data was calculated as a ratio of G6Pase to cyclophilin and presented as fold activation, where the intensity of control (no sample) was set at one.

**Preparation of cell extract for kinase assays.** H4IIE cells were incubated in serum-free medium with hormones, viruses, and inhibitors for the times indicated and at the concentrations indicated in the figure legends. Cells were then harvested into ice-cold lysis buffer (25 mmol/l Tris/HCl, pH 7.5, 50 mmol/l NaF, 1 mmol/l sodium vanadate, 1 mmol/l EGTA, 1 mmol/l EDTA, 1% [vol/vol] Triton X-100, 5 mmol/l sodium pyrophosphate, protease inhibitors [complete minitab, 1 per 10 ml; Boehringer Mannheim], 0.27 mol/l sucrose, 2 mol/l microcystin, and 0.1% [vol/vol] 2-mercaptoethanol). Cell debris was removed by centrifugation at 13000g for 5 min and the protein concentration determined using bovine serum albumin (BSA) as an internal standard, by the method of Bradford.

**Preparation of adeno- and retroviruses and infection of H4IIE cells.** An adeno-virus expressing β-galactosidase was a gift from Dr. Chris Newgard (University of Texas). An adeno-virus expressing GSK-3α was generated using the AdEasy System, and the plasmids were a kind gift from Dr. B. Vogelstein (Howard Hughes Medical Institute, Baltimore, MD) (31). GSK-3α cDNA was amplified from rat adult liver Marathon cDNA library (Clontech) using gene-specific primers designed from the Genbank entries, accession numbers L40027 and L33801, respectively. Polymerase chain reaction (PCR) products were subcloned into PCR 2.1-TOPO vector (Invitrogen) for sequencing and then further subcloned into the pAAVTrack-CMV shuttle vector (33). Competent BJ5183 host cells were purchased from Quantum Biotechnologies. All virus stocks were produced by transient transfection of HEL293s cells (American Type Culture Collection). All cell culture reagents, including the transfection reagent lipofectamine plus, were purchased from Life Technologies. The GSK-3 and β-galactosidase adeno-viruses were functionally titrated to assess the amount required for 80–100% infection efficiency in H4IIE cells (as determined by GFP imaging and X-galactosidase staining, respectively). This was equivalent to 3–5 MOIs for each virus. H4IIE cells were infected in serum-free DMEM. After 36 h, cells were either lysed (as above) for GSK-3 assay, or horm gene-specific inhibitors were added for an additional 3 h (as described in the figure legend) and RNA prepared for PKB and G6Pase expression analysis (as described above).

**Immunoprecipitation and assay of protein kinases.** Cell extract (0.2 mg) was incubated for 1 h on a shaking platform with protein G Sepharose conjugated to anti-PKB. The immunocomplex was pelleted and washed twice with 1.0 ml buffer A (50 mmol/l Tris/HCl, pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EGTA, 1 mmol/l EDTA, 1% [vol/vol] Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mol/l sucrose, and 0.1% [vol/vol] 2-mercaptoethanol), and then washed with 1.0 ml of buffer B (50 mmol/l Tris/HCl, pH 7.5, 0.1 mmol/l EDTA, and 0.1% [vol/vol] 2-mercaptoethanol). Immunoprecipitated PKB was assayed at 30°C in a total volume of 50 μl containing 50 mmol/l Tris/HCl, pH 7.5, 0.1 mmol/l EGTA, 2.5 μmol/l protein kinase inhibitor (PKI), 10 mmol/l MgCl2, 0.1% [γ-32P]ATP (2 × 106 cpm/ml), and 30 μl/cell crosstide (9).

GSK-3 activity in 10 μg of total cell lysate was determined under assay conditions identical to those for PKB with crosstide replaced by 10 μmol/l GS2 phospho-peptide (10) in the presence or absence of 5 μmol/l SB216763. Dimethylsulphoxide carrier was added when no inhibitor was present. Units of GSK-3 activity were determined by subtracting the background activity (peptide absent and inhibitor present) from the activity with peptide present and no inhibitor present. Assays were performed in triplicate, and the experiments were carried out twice in duplicate. One unit of kinase activity catalyzes the phosphorylation of 1 mol of substrate in 1 min.

**Detection of phospho-GSK-3, phospho-PKB, or phospho-FKHR by Western blot.** A total of 30 μg cell extract (prepared as above) was subjected to SDS-PAGE (4–12% NuPAGE, Novex), then transferred to nitrocellulose and immunoblotted with 1 μg/ml of an antibody raised against GSK-3α (9) or with 1:1000 dilution of an antibody that specifically recognizes GSK-3α and -β only when phosphorylated at serine 21/0 or FKHR phosphorylated at Thr-32.

**RESULTS**

Lithium reduces the expression of two hepatic gluconeogenic genes. The induction of PEPCk or G6Pase gene expression by glucocorticoids and 8CPT-CAMP is inhibited by insulin (Fig. 1). Lithium chloride, a relatively specific inhibitor of GSK-3 in vitro at millimoles per liter
concentrations (34) mimics these effects of insulin in H4IIE cells (Fig. 1). However, the effect of lithium is not as potent as that of insulin, with a 50–80% reduction in expression of either gene observed at 20 mmol/l lithium (Fig. 1). The levels of β-actin or cyclophilin mRNA in these experiments remain unaltered (Fig. 1), thus demonstrating selectivity toward the PEPCK and G6Pase gene promoters.

In addition, basal expression of G6Pase is reduced by 50% in the presence of 20 mmol/l lithium chloride but is not affected by the same concentration of potassium chloride (Fig. 1C). This suggests a permissive role for GSK-3 in the regulation of these two gene promoters.

Structurally distinct inhibitors of GSK-3 repress G6Pase and PEPCK gene expression. SB-216763 and SB-415286 are relatively potent selective cell permeable inhibitors of GSK-3 (12). Treatment of H4IIE cells with either compound blocks the glucocorticoid/cAMP-induced expression of the PEPCK and G6Pase genes in a dose-dependent manner (Figs. 2 and 3). The level of inhibition of gene expression is similar to that seen with lithium (not as potent as insulin). The effect of SB-216763 on either gene is seen at lower concentrations than SB-415286 (consistent with its lower IC-50 toward GSK-3 in vitro [12]). However, maximal effects of SB-415286 are generally
greater than those of SB-216763. Most likely, this is caused by differences in the solubility of the two compounds in aqueous solution (12). Again, basal expression of G6Pase but not cyclophilin is reduced in the presence of SB-216763 (Fig. 2) or SB-415286 (Fig. 3).

**Inhibition of GSK-3 reduces PEPCK gene transcription.** The expression of CAT, under the control of the PEPCK gene promoter (in a stably transfected cell line, HL1C), is repressed by lithium, SB-216763, or SB-415286 treatment (Fig. 4). This demonstrates that inhibition of GSK-3 has a direct effect on PEPCK gene transcription, rather than decreasing the stability of PEPCK mRNA. The effects of all three agents are observed at similar concentrations to those required for inhibition of endogenous PEPCK (and G6Pase) mRNA production (Figs. 2 and 3). Thus, it is highly likely that the effect on the expression of both endogenous genes is at the level of gene transcription.

**Inhibition of GSK-3 does not induce PKB activity or FKHR phosphorylation.** Potential signaling molecules...
involved in the regulation of PEPCK and G6Pase expression by insulin include PKB and FKHR. However, treatment of H4IIE cells with lithium ions, SB-216763 or SB-415286, does not induce PKB activity (Fig. 5A). This protein kinase is induced 20- to 40-fold by insulin within 30 min, and 10- to 15-fold activation remains after 3-h stimulation with this hormone (Fig. 5A). Similarly, lithium ions SB-216763 or SB-415286 had no effect on the phosphorylation of FKHRL1 at Thr-32 (Fig. 5B and C), a residue targeted by insulin (through activation of PKB) in vivo and linked to the nuclear exclusion of this transcription factor. Therefore, inhibition of GSK-3 does not mimic the effects of insulin on PKB or FKHRL1. However, lithium ions promote a significant increase in the phosphorylation of Ser-21/Ser-9 of GSK-3α and -3β in H4IIE cells (Fig. 5B and C), although much less than insulin treatment of the same

FIG. 3. SB-415286 represses PEPCK and G6Pase expression in a dose-dependent manner. H4IIE cells were serum-starved for 20 h immediately before a 3-h incubation with dexamethasone (A and B) or without (C) dexamethasone (500 nmol/l) plus 8CPT-cAMP (0.1 mmol/l) and SB-415286 at the concentrations shown. Cells were then lysed, RNA isolated, and a primer extension (A) or RPA (B and C) carried out as previously described (see RESEARCH DESIGN AND METHODS). Quantitation (by phosphorimage analysis) of three experiments carried out in duplicate is shown (upper panels) along with a representative experiment (lower panels).
cells. Because there is a much less apparent effect on phosphorylation of these residues in the presence of either maleimide, it is likely that this action of lithium is unrelated to the inhibition of GSK-3 and does not require the activation of PKB (Fig. 5A).

The action of SB-216763 is prevented by over expression of GSK-3. Infection of H4IIE cells with an adenovirus expressing GSK-3α produces a 5- to 10-fold increase in GSK-3 activity (Fig. 6A). High efficiency (up to 100%) infection is achieved by adenovirus-mediated DNA delivery in H4IIE cells (33). The adenovirus expressing GSK-3α, but not a control virus expressing β-galactosidase, reduces repression of the G6Pase or PEPCK genes by SB-216763 (Fig. 6B and C). Interestingly, expression of GSK-3α does not affect basal or induced levels of gene expression in the absence of inhibitor (Fig. 6B and C), and this treatment does not affect the ability of insulin to inhibit G6Pase or PEPCK gene expression (data not shown).

DISCUSSION

GSK-3 inhibitors have therapeutic potential for the treatment of insulin resistance and diabetes. The gene products of PEPCK and G6Pase are key rate-controlling steps in hepatic gluconeogenesis. The regulation of PEPCK in vivo is solely at the level of gene expression (35), whereas the regulation of G6Pase gene transcription correlates with regulation of protein expression (24). Thus, agents that reduce the hepatic expression of PEPCK and G6Pase (in a similar manner to insulin) should reduce hepatic glucose production and output in vivo. Indeed, the intraperitoneal administration of lithium chloride to rats reduces hepatic PEPCK mRNA and hepatic gluconeogenesis (36). However, multiple targets of this metal ion have been identified. In addition to GSK-3, these include adenylyl cyclase (37,38), inositol phosphatases (39), and one or two other protein kinases (34). Therefore, a GSK-3–independent mechanism could not previously be ruled out for the repressive action of lithium ions on gene transcription and gluconeogenesis. In this article, we demonstrate that mechanistically distinct inhibitors of GSK-3 (SB-216763 or SB-415286) also reduce PEPCK and G6Pase expression in hepatoma cells. These maleimides are much more

![Figure 4](image4.png)

**FIG. 4.** Each GSK-3 inhibitor blocks the activity of the PEPCK promoter. HL1C cells (H4IIE cells stably transfected with the PEPCK promoter linked to CAT reporter) were serum-starved for 20 h immediately before an incubation with dexamethasone (500 nmol/l) plus 8CPT-cAMP (0.1 mmol/l) and lithium chloride, potassium chloride, SB-216763, or SB-415286 at the concentrations shown. Cells were lysed and CAT activity determined as previously described (see RESEARCH DESIGN AND METHODS). Results are expressed as percent of glucocorticoid-induced promoter activity and are the average ± SE of three separate experiments carried out in duplicate.

![Figure 5](image5.png)

**FIG. 5.** Inhibition of GSK-3 does not affect the activity of PKB or the phosphorylation of the transcription factor FKHL1. H4IIE cells were serum-starved for 20 h before incubation with insulin (10 nmol/l), lithium chloride (20 mmol/l), SB-216763 (30 μmol/l), or SB-415286 (30 μmol/l). Cells were lysed at 0.5 or 3 h, and PKBα was immunoprecipitated and assayed (A) as previously described (see RESEARCH DESIGN AND METHODS). Results are the average ± SE of two experiments carried out in duplicate. Alternatively, 30 μg total cell lysate prepared after 0.5 h (B) or 3 h (C) treatments was subjected to SDS-PAGE, transferred to nitrocellulose, and visualized by Western blot using specific primary antibodies as labeled (phospho, phospho-specific antibody to Ser-9/21 of GSK-3, or Thr-32 of FKHL1). Similar results were obtained from three experiments, and a representative experiment is shown.
specific inhibitors of GSK-3. They have IC-50s in the low nanomolar range in vitro and do not reduce the activity of the other 24 protein kinases that were examined (12). The concentration of each compound required to reduce gene expression in H4IIE cells is similar to that required to stimulate glycogen synthesis or β-catenin–mediated gene expression in human liver cells (12). In addition, the overexpression of recombinant GSK-3 in the H4IIE cells prevents the effect of SB-216763 on PEPCK or G6Pase gene expression (Fig. 6). Because the ability of lithium to reduce hepatic gluconeogenesis is linked to the repression of PEPCK (36), these novel GSK-3 inhibitors should also reduce hepatic gluconeogenesis in vivo.

**The mechanism by which GSK-3 regulates PEPCK and G6Pase gene transcription is unclear.** Inhibition of GSK-3 reduces PEPCK promoter activity in stably transfected H4IIE cells (Fig. 4) and also in transiently transfected liver cells (36). This demonstrates that the effect of these agents is at the level of gene transcription, rather than that of mRNA stability. The GSK-3 inhibitors block basal and induced expression of G6Pase as well as PEPCK promoter activity. Therefore, the molecular target(s) of GSK-3 must be required for the PEPCK and G6Pase promoters to function. The glucocorticoid receptor (GR) is reported to be a substrate for GSK-3 in vitro and in rat cells (40), with ~40% reduction in GR transcriptional activity caused by the overexpression of GSK-3. Therefore, it is possible that GSK-3 inhibitors have effects on GR activity independent of the promoter studied. However, Rogatsky et al. (40) also found that the inhibition of GSK-3 by overexpression of PKB actually enhances gene activation by rat GR, consistent with the phosphorylation of GR by GSK-3 causing inhibition of GR activity. However, the overexpression of GSK-3α in H4IIE cells does not affect basal or dexamethasone-induced levels of G6Pase or PEPCK gene expression (Fig. 6C). This suggests that the previously reported repressive effect of GSK-3 on GR activity (40) may be promoter selective. In any case, the inhibition of GSK-3 using specific inhibitors reduces basal as well as glucocorticoid-induced G6Pase gene expression (Figs. 1–3), suggesting a GR-independent mechanism for the effects of these compounds on G6Pase and PEPCK expression. In addition, PKB has multiple cellular targets; thus, the effect of PKB overexpression on GR activity may not be mediated through inhibition of GSK-3.

Several other potential nuclear substrates of GSK-3 have been identified on the basis of in vitro phosphorylation and overexpression studies. They include c-Jun (41), cAMP-responsive element binding (CREB) protein (42), CAAT–enhancer binding protein α(C/EBPα) (43), nuclear factor of activated T-cells (NFAT) (44), and β-catenin (45). Phosphorylation of c-Jun by GSK-3 is reported to prevent DNA
binding and activity of c-Jun (46,47) and could potentially be involved in insulin activation of gene transcription. GSK-3 phosphorylation of CREB (at Ser-129) is reported to have a stimulatory effect on CREB activity (42), and CREB is involved in the cAMP-mediated activation of PEPCK expression (48). If Ser-129 is phosphorylated in vivo, the inhibition of GSK-3 might therefore inhibit CREB activity and help to reduce PEPCK expression. However, CREB has not been linked to the glucocorticoid induction of either gene or to the basal expression of G6Pase. Similarly, there is no clear evidence to date that C/EBPα, NFAT, or β-catenin are regulated by insulin in liver cells. Indeed, the available evidence suggests that C/EBPα activity is not affected by insulin in HepG2 cells, although this has only been investigated in transient transfection studies and not in the presence of glucocorticoid (49). Therefore, the mediator of the permissive activity of GSK-3 toward the PEPCK and G6Pase promoters remains uncertain.

**Inhibition of GSK-3 may underlie the effects of other agents on the PEPCK and G6Pase gene promoters.** The PEPCK and G6Pase gene promoters share a DNA sequence (TG/ATTTTG) that is required for their complete repression by insulin. The G6Pase promoter contains three such sequences, whereas the PEPCK promoter contains one (50,51). In addition, distinct DNA elements not common to both promoters are required for full repression of each gene by insulin (23,52). The signaling mechanism used by insulin to regulate PEPCK gene transcription requires PI 3-kinase (26) but not the Ras–MAP-kinase pathway (27,28), the activation of p70 S6 kinase (26,53), the small G-protein Rac, or atypical PKC (54) (all potentially downstream of PI 3-kinase). Similar conclusions have been reached concerning the insulin-signaling pathway that regulates G6Pase (29). Thus, despite the differences in promoter structure, the two genes may be repressed by a common pathway. We now find that the inhibition of GSK-3 has similar effects on both gene promoters.

The overexpression of PKB in cells results in the phosphorylation and inhibition of GSK-3 (9). When the insulin response element (IRE) from the PEPCK or G6Pase gene promoter is inserted into a normally insulin-insensitive promoter, it confers sensitivity to insulin as well as to PKB coexpression in hepatoma cells (50,55–57). This effect of PKB on the isolated element may be mediated through inhibition of GSK-3. Interestingly, coexpression of active PKB has no effect on the activity of a larger G6Pase promoter construct linked to luciferase (29); this suggests that inhibition of GSK-3 does not affect this G6Pase promoter construct. It is not certain why the promoter constructs behave differently, although perhaps the overexpressed active PKB has multiple other effects on the larger promoter that override the effect of GSK-3 inhibition.

Phorbol ester treatment of H4IIE cells causes activation of PKC and repression of PEPCK (58) and G6Pase (P.A.L., unpublished observations) gene expression. GSK-3 is phosphorylated and inhibited by PKC in vitro (50) and in phorbol ester–treated H4IIE cells (F.A.L., unpublished observations). This may underlie the effect of phorbol esters on PEPCK and G6Pase gene expression in these cells. Equally, the protein kinase p90RSK inhibits GSK-3 activity in vitro (10) and in vivo (60). Overexpression of active Ras or Raf represses PEPCK gene transcription (28). An inhibitor of a protein kinase that lies upstream in the activation of p90RSK blocks these effects of Ras and Raf. Interestingly, the regulation of GSK-3 by phorbol esters in 3T3-cells is mediated by activation of p90RSK (61). Therefore, inhibition of GSK-3 by p90RSK may underlie the effects of Ras, Raf, and phorbol esters on PEPCK gene transcription.

The overexpression of GSK-3 does not affect the regulation of PEPCK or G6Pase gene expression by insulin (data not shown). This observation coupled with the greater effect of insulin (compared with GSK-3 inhibitors) on PEPCK and G6Pase gene expression indicates that the insulin-mediated regulation of these promoters involves at least one other mechanism.

**Inhibition of PEPCK and G6Pase expression occurs in the absence of regulation of PKB or FKHRL1.** Many recent reports have demonstrated that the activity/cellular localization of members of the FKHR is regulated by PKB in intact cells (57,62–66). Phosphorylation of FKHR results in its nuclear export and reduces its transcriptional activity. Because the consensus sequence for forkhead binding of DNA is similar to the IRE found in both the PEPCK (13) and the G6Pase (50,51) gene promoters, it was possible that insulin, and potentially GSK-3 inhibitors, repressed these genes by promoting nuclear export of a forkhead protein. Indeed, in some cell lines lithium ions can partially activate PKB (67), possibly through inhibition of a GSK-3–mediated regulation of IRS-1 (68). However, our data demonstrate that neither PKB activation nor FKHRL1 inactivation underlies the action of the GSK-3 inhibitors (Fig. 5). Therefore, these promoters can be repressed in a PKB- and FKHRL1-independent manner. This is consistent with the finding that activation of PKB is not required for insulin repression of PEPCK expression (54).

In summary, we have investigated the regulatory role of GSK-3 in the expression of PEPCK and G6Pase. Inhibition of GSK-3 in H4IIE cells mimics the action of insulin on the expression of both genes. Thus, the maleimides (SB-216763, SB-415286, or similar agents) have potential therapeutic value for alleviating hyperglycemia via reduced hepatic output as well as increased glycogen synthesis, even in insulin-resistant cells.

**ACKNOWLEDGMENTS**

This work was supported by Wellcome Trust Career Development Award 051792.

The authors are grateful to Dr. Daryl K. Granner for the gift of HLIC cells.

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