Original Article

Characterization of a Protein Kinase B Inhibitor In Vitro and in Insulin-Treated Liver Cells

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OBJECTIVE—Abnormal expression of the hepatic gluconeogenic genes (glucose-6-phosphatase [G6Pase] and PEPCK) contributes to hyperglycemia. These genes are repressed by insulin, but this process is defective in diabetic subjects. Protein kinase B (PKB) is implicated in this action of insulin. An inhibitor of PKB, Akt inhibitor (Akti)-1/2, was recently reported; however, the specificity and efficacy against insulin-induced PKB was not reported. Our aim was to characterize the specificity and efficacy of Akti-1/2 in cells exposed to insulin and then establish whether inhibition of PKB is sufficient to prevent regulation of hepatic gene expression by insulin.

RESEARCH DESIGN AND METHODS—Akti-1/2 was assayed against 70 kinases in vitro and its ability to block PKB activation in cells exposed to insulin fully characterized.

RESULTS—Akti-1/2 exhibits high selectivity toward PKBα and PKBβ. Complete inhibition of PKB activity is achieved in liver cells incubated with 1–10 μmol/l Akti-1/2, and this blocks insulin regulation of PEPCK and G6Pase expression. Our data demonstrate that only 5–10% of maximal insulin-induced PKB is required to fully repress PEPCK and G6Pase expression. Finally, we demonstrate reduced insulin sensitivity of these gene promoters in cells exposed to submaximal concentrations of Akti-1/2; however, full repression of the genes can still be achieved by high concentrations of insulin.

CONCLUSIONS—This work establishes the requirement for PKB activity in the insulin regulation of PEPCK, G6Pase, and a third insulin-regulated gene, IGF-binding protein-1 (IGFBP1); suggests a high degree of functional reserve; and identifies Akti-1/2 as a useful tool to delineate PKB function in the liver.
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Akti, Akt inhibitor; BP1-TIRE, binding protein 1–thymine-rich insulin response element; FOXO, forkhead box class O; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDK, phosphoinositide-dependent protein kinase; PI3, phosphatidylinositol 3; PKB, protein kinase B; PRAS40, proline-rich Akt substrate of 40 kDa; smMLCK, smooth muscle myosin light-chain kinase; TSC, tuberous sclerosis complex.

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Protein kinase B (PKB) is a member of the AGC family of protein kinases (1–3). In mammals, there are three isoforms (PKBα, PKBβ, and PKBγ) (1). PKB is activated following induction of phosphatidylinositol 3 (PI3) kinase activity and the resultant generation of the lipid second messengers PI 3,4,5 trisphosphate and PI 3,4 bisphosphate (4). These lipids bind to the PH domain of PKB, altering its conformation and permitting access to upstream protein kinases (5). Phosphoinositide-dependent protein kinase-1 phosphorylates PKB at Thr^{308} (6), and a second phosphorylation (at Ser^{473}) occurs through the action of an alternative kinase, such as the rapamycin-insensitive mTOR complex 2 (TORC2) (7). Therefore, most growth factors, including platelet-derived growth factor, epidermal growth factor, and insulin, which are potent activators of PI3 kinase, also strongly induce PKB in cells.

One of the first substrates of PKB to be characterized was GSK3, as part of the insulin signaling pathway that regulates glycogen metabolism (8). Since then, multiple potential substrates of PKB have been proposed including the proapoptotic protein Bad (9,10), the tuberous sclerosis complex (TSC)2 gene product (11), the Rab-GAP AS160 (12), proline-rich Akt substrate of 40 kDa (PRAS40) (13), and the key forkhead transcription factor subfamily, forkhead box class O (FOXO). PKB phosphorylates FOXO on several residues, promoting its inactivation and exclusion from the nucleus (14–16). A growing number of insulin-inhibited genes are proposed to be targets of FOXO. These include glucose-6-phosphatase (G6Pase), PEPCK, and the insulin-like growth factor–binding protein-1 (IGFBP1) (17). All three genes are completely repressed in liver cells exposed to insulin (18) or in intact liver following feeding (19). This gene regulation requires PI3 kinase (20–22) and phosphoinositide-dependent protein kinase (PDK)1 (19) activity and can be recapitulated by overexpression of active PKB (23). Meanwhile, overexpression of FOXO will induce insulin-responsive DNA sequences within these gene promoters (24–27). These data suggest that insulin turns off these gene promoters by activating the PI3 kinase–PDK1-PKB pathway to inhibit FOXO. However the importance of PKB and/or FOXO in the regulation of these genes has been questioned (18,28). For example, overexpression of dominant-negative PKB does not block insulin action on PEPCK (29) or G6Pase (22) and inhibitors of mTOR will block insulin regulation of IGFBP1 but not PKB or FOXO (21,26), while inhibitors of GSK3 (also downstream of PKB) will inhibit these genes without regulating FOXO activity (30,31). It is also suggested that insulin can

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regulate FOXO activity independently of PKB (32). Finally, a base-by-base mutational analysis of the insulin-responsive promoter sequences from PEPCK and IGFBP1 showed that the DNA sequence required for the response to insulin is not identical to that required for the response to FOXO (33). These data suggest that although PKB and FOXO can regulate these genes, the inhibition of FOXO by PKB may not be absolutely required for their response to insulin.

Interestingly, PKBβ knockout (KO) mice exhibit reduced insulin sensitivity and can develop diabetes and lipoatrophy (34), while PKBα KO animals are smaller and have reduced lifespan and defective adipogenesis (35). PKBγ KO mice have a reduced brain size, possibly due to the relatively high expression of PKBγ in this tissue (36). These data suggest that PKBβ is the most likely link between PI3 kinase and the gluconeogenic genes PEPCK and G6Pase, although insulin regulation of these genes was not characterized in this model (34). In contrast, PKBα is the major insulin-activated PKB isoform detectable in hepatocytes (37).

Recently, selective non-ATP–competitive inhibitors of PKB were reported (38,39). Compound 16h (now termed Akt inhibitor [Akti]-1/2) potently inhibits PKBα and PKBβ in vitro and in cells, with relatively poor inhibition of PKBγ (39). Akti-1/2 also prevents phosphorylation at Ser^{308} by PDK1 in vitro and at Ser^{308} and Thr^{473} in cells without inhibiting PDK1 activity (38). Interaction of Akti-1/2 with the PH domain of PKB prevents the conformational change required for phosphorylation by upstream kinases (38). Since isolated hepatocytes contain very little, if any, PKBγ (37), we hypothesized that Akti-1/2 would produce acute and complete inhibition of PKB activity in an insulin-sensitive liver cell line (HL1c), allowing careful analysis of the requirement for PKB in processes such as insulin regulation of gene expression.

RESEARCH DESIGN AND METHODS

Actrapid (human insulin) was from Novo Nordisk (Bagsværd, Denmark); 2× Universal PCR Master Mix and No AmpErase UNG were from Applied Biosystems. Complete protease inhibitor cocktail tablets were from Roche and 8-(4-chloro-phenylthio)-cAMP from Calbiochem. All primers and probes were synthesized and purified by Sigma-Aldrich. All other chemicals were of the highest grade obtainable.

Antibodies. PKB isoform-specific antibodies have been characterized elsewhere (37). Antibodies to phospho-PKB (Thr^{308}), phospho-PKB (Ser^{473}), phospho-FOXO1 (Ser^{256}), phospho-p42/p44 mitogen-activated protein kinase (MAPK) (Thr^{202}/Tyr^{204}), and phospho-S6 ribosomal protein ($Ser^{240/244}$) were from Cell Signaling Technology (Beverly, MA); anti-PKB was from Upstate Biotechnologies (Lake Placid, NY), FOXO antibody was from Dr. Graham Rena, University of Dundee (Dundee, U.K.), and total and phospho-TSC2 and -PRAS40 antibodies were generated in the Division of Signal Transduction and Therapy, University of Dundee. Anti–β-actin was purchased from Sigma-Aldrich (St. Louis, MO).

Akti-1/2 compound synthesis. The dual PKBα/β inhibitor (compound 16h) was synthesized in house through a modification of the procedure of Lindsley et al. (39). Purity was established as >98% by H-NMR and LCMS. This compound (termed Akti-1/2 or Akt inhibitor VIII in the Calbiochem catalog) is a derivative of Akti-1/2a (a compound originally termed Akti-1/2 [38]) and exhibits increased potency.

Kinase screen. Details of the kinase selectivity screens have previously been provided (40), and complete assay conditions are provided in supplementary data (available in an online appendix at http://dx.doi.org/10.2337/db07-0343). Briefly, all assays (25.5 μl at 21°C for 30 min) were performed using a Biomek 2000 Laboratory Automation Workstation in a 96-well format (Beckman Instruments, Palo Alto, CA). Reactions contained 5–20 mU purified kinase along with substrate peptide or protein and were initiated by the addition of 10 mmol/l MgAcetate and 5, 20, or 50 μmol/l ATP ([γ-33P]-ATP, 800 cpm/pmol).

Cell culture. HL1c rat hepatoma cells were maintained in 1g/l glucose containing Dulbecco's modified Eagle's medium supplemented with 5% FCS and 100 units/ml penicillin and 100 ug/ml streptomycin.

RNA isolation and real-time quantitative RT-PCR. Following hormone treatments, total cellular RNA was extracted from HL1c cells using TRIreagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was synthesized from total cellular RNA using the Superscript II reverse transcriptase kit (Invitrogen). Real-time PCR analysis was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems). PCRs were carried out using the following cycling conditions: 50°C for 2 min ×1 and 95°C for 10 min ×1, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Transient transfections. A reporter construct encompassing a luciferase cDNA under the control of a thymidine kinase gene promoter containing the IGFBP1 thymine-rich insulin response element (BP1-TIRE) was transfected with or without an expression construct for glutathione-S-transferase-FOXO1 (pEBG-FOXO1) into HL1c cells as previously described (26,33). Cells were incubated for 16 h with hormones as indicated in the figure legends before harvesting and assaying for luciferase activity.

Luciferase assay. Luciferase activity was determined using the Luciferase Assay system (Promega) according to the manufacturer's instructions. The data are expressed as relative light units (RLU) of luciferase activity per microgram of protein.

Cell lysis and immunoblotting. Following hormone treatments, cells were harvested by scraping into ice-cold lysis buffer (50 mmol/l Tris/HCl, pH 7.5; 50 mmol/l NaF; 500 mmol/l NaCl; 1 mmol/l Na vanadate; 1 mmol/l EDTA; 1% [vol/vol] triton X-100; 5 mmol/l Na pyrophosphate; 0.27 mmol/l sucrose; and 0.1% [vol/vol] 2-mercaptoethanol) and cell debris removed by centrifugation at 4°C for 10 min at 13,000*g*. Cell lysate (10–20 μg) was separated on Novex 4–12% NuPAGE gels. Following transfer to nitrocellulose, blots were blocked with 5% (wt/vol) nonfat milk in Tris-buffered saline (containing 0.1% [vol/vol] Tween 20) for 1 h and incubated with primary antibodies at 4°C overnight before incubation for 1 h at room temperature with the appropriate secondary antibody. Protein bands were visualized using an enhanced chemi-luminescence kit (Amersham Biosciences).

Cell lysis and PKB activity. HL1c cells were harvested as above and protein concentration measured. 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 ml lysis buffer and twice with 1 ml assay buffer (50 mmol/l Tris/HCl, pH 7.5; 0.1 mmol/l EGTA; and 0.1% [vol/vol] 2-mercaptoethanol). The immunoprecipitated kinase activity was measured 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 PKI; 10 mmol/l $MgCl_2$; 0.1 mmol/l [γ^{32}P]-ATP (2×10^6 cpm/nmol); and 30 μmol/l crosstide (GRPRTSSFAEG). One unit of kinase activity is the amount that catalyzes the phosphorylation of 1 nmol/l substrate in 1 min.

RESULTS

Akti-1/2 is a specific inhibitor of PKBα and PKBβ. Akti-1/2 was synthesized as previously described (39) and dissolved in DMSO for in vitro and cell culture experiments. The compound was assayed against a panel of 70 protein kinases that includes representatives of all the major protein kinase families (Table 1). Akti-1/2 is known to inhibit PKBα (58 nmol/l half-maximal inhibitory concentration) and PKBβ (210 nmol/l) much more potently than PKBγ (2,119 nmol/l) (39). PKB lacking a PH domain is not inhibited by this class of compound, as previously reported (39); however, full-length PKBα and -β were inhibited significantly in vitro by submicromolar concentrations of Akti-1/2 (Table 1). CamKI was the only other protein kinase significantly affected by 1 μmol/l Akti-1/2 (78% inhibition). Meanwhile, smooth muscle myosin light-chain kinase (smMLCK) was potently inhibited at 10 μmol/l, and RSK1, SGK (serum- and glucocorticoid-inducible kinase)1, MAPK-activated kinase 2, Aurora B, MELK, PIM (proviral integration site)1, PIM3, EFK2, p21-activated kinase 4, and CSK all show some sensitivity to Akti-1/2 at this concentration. Previously, analysis of related compounds had suggested a strong selectivity over other members of the AGC family (e.g., cAMP-dependent protein kinase, SGK, and protein kinase C) (38,39). Our data confirm a selective advantage toward PKBα and PKBβ of 10- to 100-fold when

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TABLE 1
Akti-1/2 assayed against 70 protein kinases

	Akti-1/2 (μmol/l)			
	0.1	1.0	10.0	100.0
ΔPH-PKBα	91	84	35	3
ΔPH-PKBβ	91	84	71	3
PKBα (full length)	57	22	7.5	2
PKBβ (full length)	58	34	6	1
PKBγ (full length)	106	97	32	27
MKK1	102	103	64	24
ERK1	102	107	104	77
ERK2	98	107	104	66
JNK1	134	109	98	43
JNK2	101	93	86	23
JNK3	108	63	75	31
p38α MAPK	97	91	82	73
p38β MAPK	118	115	111	72
p38γ MAPK	104	108	97	94
p38s MAPK	94	98	96	101
ERK8	77	81	74	25
RSK1	94	90	32	10
RSK2	91	94	56	10
PDK1	73	76	63	51
SGK1	80	67	15	1
S6K1	104	96	72	27
PKA	111	109	53	12
ROCK 2	72	89	85	59
PRK2	96	106	84	92
PKCα	88	86	88	51
PKCζ	116	113	103	111
PKD1	90	89	71	32
MSK1	92	86	74	46
MNK1	95	107	109	113
MNK2	90	98	79	76
MAPKAP-K2	95	113	58	8
MAPKAP-K3	132	115	115	39
PRAK	111	105	80	15
CAMKKα	102	93	62	54
CAMKKβ	122	138	108	108
CAMK1	83	22	16	14
SmMLCK	94	86	4	5
PHK	97	97	97	50
CHK1	79	78	95	69
CHK2	93	89	83	29
GSK3β	99	98	101	44
CDK2–Cyclin A	88	90	92	89
PLK1	77	83	81	54
PLK1 (okadaic acid)	103	106	93	41
Aurora B	90	95	55	12
Aurora C	98	83	70	65
AMPK	110	107	108	83
MARK3	102	104	98	55
BRSK2	110	116	81	64
MELK	76	92	52	11
CK1	76	70	76	46
CK2	100	102	104	71
DYRK1A	100	98	73	32
DYRK2	91	96	96	47
DYRK3	110	118	80	34
NEK2α	91	91	74	48
NEK6	96	101	65	6
NEK7	92	105	74	6
IKKβ	102	105	75	9
PIM1	100	87	45	15
PIM2	107	105	80	37
PIM3	83	76	37	12
SRPK1	103	105	100	49
MST2	101	95	60	47
EFK2	101	87	49	37
HIPK2	97	102	89	63
HIPK3	108	105	108	94
PAK4	97	88	46	24
PAK5	102	111	73	58
PAK6	104	110	85	69
Src	103	92	122	52
Lck	108	107	77	43
CSK	108	99	61	9

Data are the percentage of kinase activity compared with that in control incubations (with DMSO). Protein kinases were assayed, as described in the supplementary online appendix information, in the absence or presence of the indicated amount of Akti-1/2. Assays were undertaken at approximately the Km (substrate concentration required for half the maximum velocity of the enzyme reaction) concentration of ATP for each kinase. The results are an average of a triplicate determination ± SD. Abbreviations and details of the kinase assay conditions are provided in the supplementary online appendix information.

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compared with 70 protein kinases, with the exception of CamKI and PKBγ (at least in vitro).

PKBα and -β are the major PKB activities in insulin-stimulated HL1c cells. Previous work had suggested that PKBγ was not a major contributor to total PKB activity in isolated rat hepatocytes (37). Using isoform-specific antibodies, each PKB isoform was sequentially immunoprecipitated and assayed from cells incubated with or without insulin. The main insulin-induced PKB activity was PKBα, with some PKBβ stimulation (Fig. 1*A* and Table 2); however, no PKBγ activity could be detected (although this antibody will immunoprecipitate PKBγ activity from L6 myotubes [37]). Agents that induce gluconeogenic gene expression, such as the synthetic glucocorticoid dexamethasone and/or cell-permeable cAMP, did not alter the PKB isoform activities or response to insulin in these cells (Fig. 1*A*). The activation of PKBα by insulin is rapid and sustained for at least 3 h, even in the presence of dexamethasone and cAMP (Fig. 1*B*), while significant activation can be achieved with 0.1–1 nmol/l insulin—although 10 nmol/l is required for full activation (Fig. 1*C*).

The absence of PKBγ from HL1c cells suggested that Akti-1/2 could be used to fully inhibit PKB activity at concentrations that will not affect most other protein kinases. Although the reported half-maximal inhibitory concentration of Akti-1/2 is 58, 210, and 2,119 nmol/l against PKBα, PKBβ, and PKBγ, respectively (39), 5 μmol/l Akti-1/2 was previously used to completely inhibit PKBα and PKBβ activity in intact cells (with no effect on endogenous PKBγ) (41). Therefore, we examined insulin activation of PKB in the presence of up to 10 μmol/l Akti-1/2 (Fig. 2 and Table 2). Significant reductions in both PKBα and PKBβ activities are observed in cells exposed to 0.1–1.0 μmol/l Akti-1/2 acutely or chronically, irrespective of the presence of dexamethasone/cAMP (Table 2). In addition, PKB phosphorylation in response to insulin is almost completely lost in the presence of 0.5 μmol/l Akti-1/2 (Fig. 2*A*). This is consistent with no PKBγ activity being present in this cell line, as the phospho-specific antibody recognizes all three isoforms of PKB. Cell lysates were then probed for insulin-induced phosphorylation of three known PKB substrates, namely, TSC2, PRAS40, and FOXO1 (Fig. 2*A*). A similar profile of Akti-1/2 sensitivity is observed for these substrates, with complete loss of insulin-induced phosphorylation in the presence of 0.5–1.0 μmol/l Akti-1/2. This confirms that these proteins are PKB substrates and demonstrates that 1 μmol/l Akti-1/2 almost completely prevents acute insulin activation of PKB (Table 2). Meanwhile, insulin fully activates p42/p44 MAPK even in the presence of 30 μmol/l Akti-1/2 (Fig. 2*B*).

It was noted that insulin-induced PKBα activity (Fig. 1*B*), as well as Thr^{308} and Ser^{473} phosphorylation (Fig. 3*A*), is sustained for several hours, although it returns to basal after 16 h of exposure to insulin. The presence of 1 μmol/l Akti-1/2 prevents the appearance of insulin-induced phosphorylation of PKB throughout this time period (Fig. 3*A*). However, some phosphorylation of the PKB substrates FOXO1 (Ser^{256}) and TSC2 (Thr^{1462}) is detectable after 1–3 h of exposure to insulin even in the presence of 1 μmol/l Akti-1/2 (Fig. 3*A* and *C*). This suggests that prolonged insulin exposure induces a small amount of PKB signaling even in the presence of this concentration of inhibitor, although we cannot measure this at the level of PKB phosphorylation. Meanwhile, higher concentrations of Akti-1/2 completely block downstream PKB signaling in response to

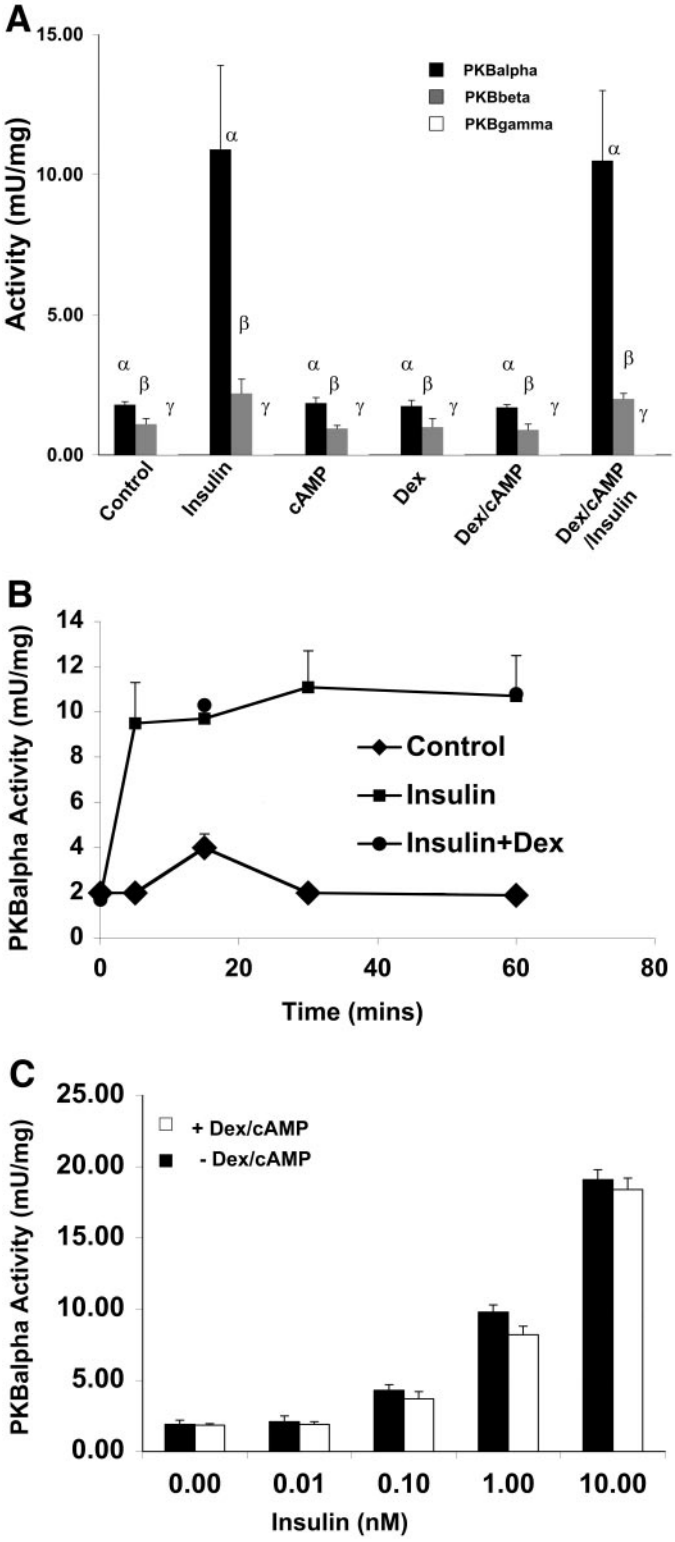

FIG. 1. HL1c cells were serum starved overnight and then incubated with 10 nmol/l insulin, 0.1 mmol/l 8CPT-cAMP, and 500 nmol/l dexamethasone (Dex) or a combination, as indicated, for 30 min. *A*: Cells were lysed, each isoform of PKB (-α, -β, and -γ) sequentially immunoprecipitated from the cell lysate, and assayed as described in RESEARCH DESIGN AND METHODS. *B*: HL1c cells were incubated with insulin (10 nmol/l) with or without 8CPT-cAMP ([0.1 mmol/l]/dexamethasone [500 nmol/l]) for the times indicated before cell lysis. PKBα was immunoprecipitated and assayed. *C*: HL1c cells were incubated with insulin at the concentrations indicated (nmol/l) for 30 min, before cell lysis. PKBα was immunoprecipitated and assayed.

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TABLE 2
PKBα and PKBβ are equally sensitive to Akti-1/2 in intact cells

Treatment	PKBα (mU/mg)			PKBβ (mU/mg)		
	0.5 h	3 h	3 h + Dex/cAMP	0.5 h	3 h	3 h + Dex/cAMP
Control	2.2 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	1.2 ± 0.2	1.4 ± 0.1	1.5 ± 0.2
Insulin + DMSO	11.5 ± 1.5	12.5 ± 1.5	12.8 ± 2.1	2.2 ± 0.4	2.4 ± 0.3	2.6 ± 0.3
Insulin + 0.1 mmol/l Akti-1/2	5.8 ± 1.2	6.6 ± 1.2	6.8 ± 1.0	1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.3
Insulin + 1.0 mmol/l Akti-1/2	2.8 ± 0.2	3.2 ± 0.4	3.5 ± 0.1	1.4 ± 0.3	1.5 ± 0.2	1.6 ± 0.2
Insulin + 2.0 mmol/l Akti-1/2	1.8 ± 0.3	2.2 ± 0.3	2.0 ± 0.2	1.1 ± 0.1	1.4 ± 0.2	1.5 ± 0.1
Insulin + 10 mmol/l Akti-1/2	0.4 ± 0.3	1.8 ± 0.3	1.6 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	1.2 ± 0.2

Data are means ± SEM of three separate experiments. HL1c cells were serum starved overnight and then incubated with 10 nmol/l insulin, 0.1 mmol/l 8CPT-cAMP, and 500 nmol/l dexamethasone (Dex) or a combination following a preincubation with Akti-1/2 or carrier for 15 min, as indicated. PKBα and PKBβ were separately immunoprecipitated from cell lysates and assayed.

insulin (Fig. 3*B*). Indeed, the presence of 10 μmol/l Akti-1/2 not only blocks insulin-induced PKB signaling but also reduces basal phosphorylation of some substrates (e.g., TSC2 and FOXO1) (Fig. 3*B* and *D*). Therefore, we conclude that 1 μmol/l Akti-1/2 prevents PKB activation by insulin over short incubation periods but that higher concentrations of this compound are required to maintain a complete block of PKB signaling for 3 h and longer.

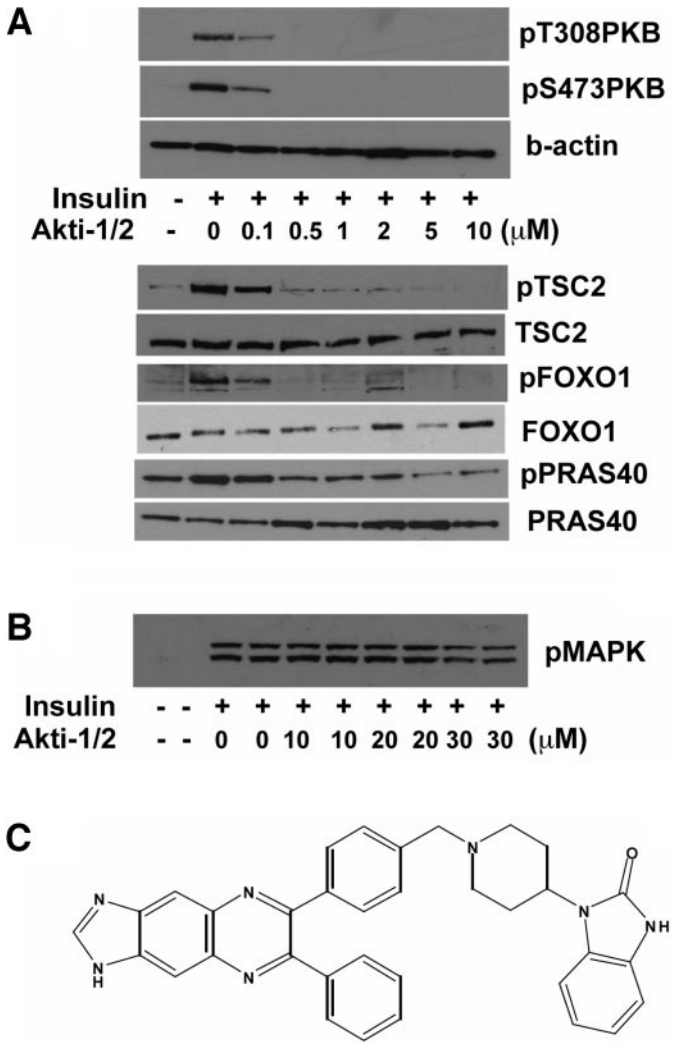

FIG. 2. The allosteric inhibitor Akti-1/2 prevents the conformational change that permits phosphorylation and activation of PKB. HL1c cells were serum starved overnight and then preincubated with or without Akti-1/2 (as indicated) for 15 min, followed by incubation with the same Akti-1/2 concentrations plus insulin (10 nmol/l), as indicated, for 30 min. Cells were lysed and analyzed by immunoblot for PKB phosphorylation (*A*, *upper*), PKB substrate phosphorylation (*A*, *lower*), or p42/p44 MAPK phosphorylation (*B*). Immunoblots representative of three experiments. The structure of Akti-1/2 (1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one) is also shown (*C*).

Insulin regulation of PEPCK, G6Pase, and IGFBP1 gene expression is PKB dependent. Insulin inhibits the transcription of the PEPCK, G6Pase, and IGFBP1 genes (Fig. 4). These gene promoters are normally active in the fasted state (glucagon and glucocorticoids) but are then turned off after feeding (insulin) (17). In the HL1c cell line, treatment with dexamethasone (synthetic glucocorticoid) and 8CPT-cAMP (to mimic glucagon signaling) strongly induces the transcription of each of the genes. Simultaneous exposure to insulin completely blocks the effects of the inducing hormones (Fig. 4). Interestingly, 1 μmol/l Akti-1/2 has almost no effect on the regulation of the genes by any hormone (Fig. 4*A*). However, 10 μmol/l Akti-1/2 significantly enhances the induction of all three genes by glucocorticoids and cAMP (Fig. 4*B*) and also prevents insulin repression of IGFBP1 and PEPCK. This concentration of Akti-1/2 only partially reduces the ability of insulin to repress G6Pase (Fig. 4*B*). The data argue that PKB activity is required for full insulin repression of these genes; however, it also suggests that only a relatively small amount of PKB activation (<5%) is actually required to permit insulin action, since 1 μmol/l Akti-1/2 has little apparent effect on the action of insulin, despite strong antagonism of insulin signaling (Fig. 3*A* and *C*). The enhanced dexamethasone/cAMP induction of all three genes by complete PKB inhibition is similar to the effect of PI3 kinase inhibitors (20). Basal PI3 kinase activity is relatively high in this cell line, and there is measurable PKB activity (Fig. 1) in the absence of insulin. Therefore, it is likely that this inductive effect is due to removal of this basal PKB activity.

Partial PKB inhibition reduces downstream insulin sensitivity. The data above demonstrate that ~90% inhibition of PKB (1 μmol/l Akti-1/2) has little affect on insulin repression of PEPCK, G6Pase, and IGFBP1 (Fig. 4). However, these experiments were conducted with a supraphysiological concentration of insulin (10 nmol/l). Insulin repression of each gene is almost maximal in cells exposed to 1 nmol/l insulin. Activation of PKB at these insulin concentrations is relatively poor (Fig. 1*C*), consistent with only a small amount of PKB activation being required for gene repression. Incubation of cells with 1 μmol/l Akti-1/2 greatly reduces repression of the genes by 0.01–1.00 nmol/l insulin, but a strong repression remains at 10 nmol/l (Fig. 5). Therefore, insulin resistance due to

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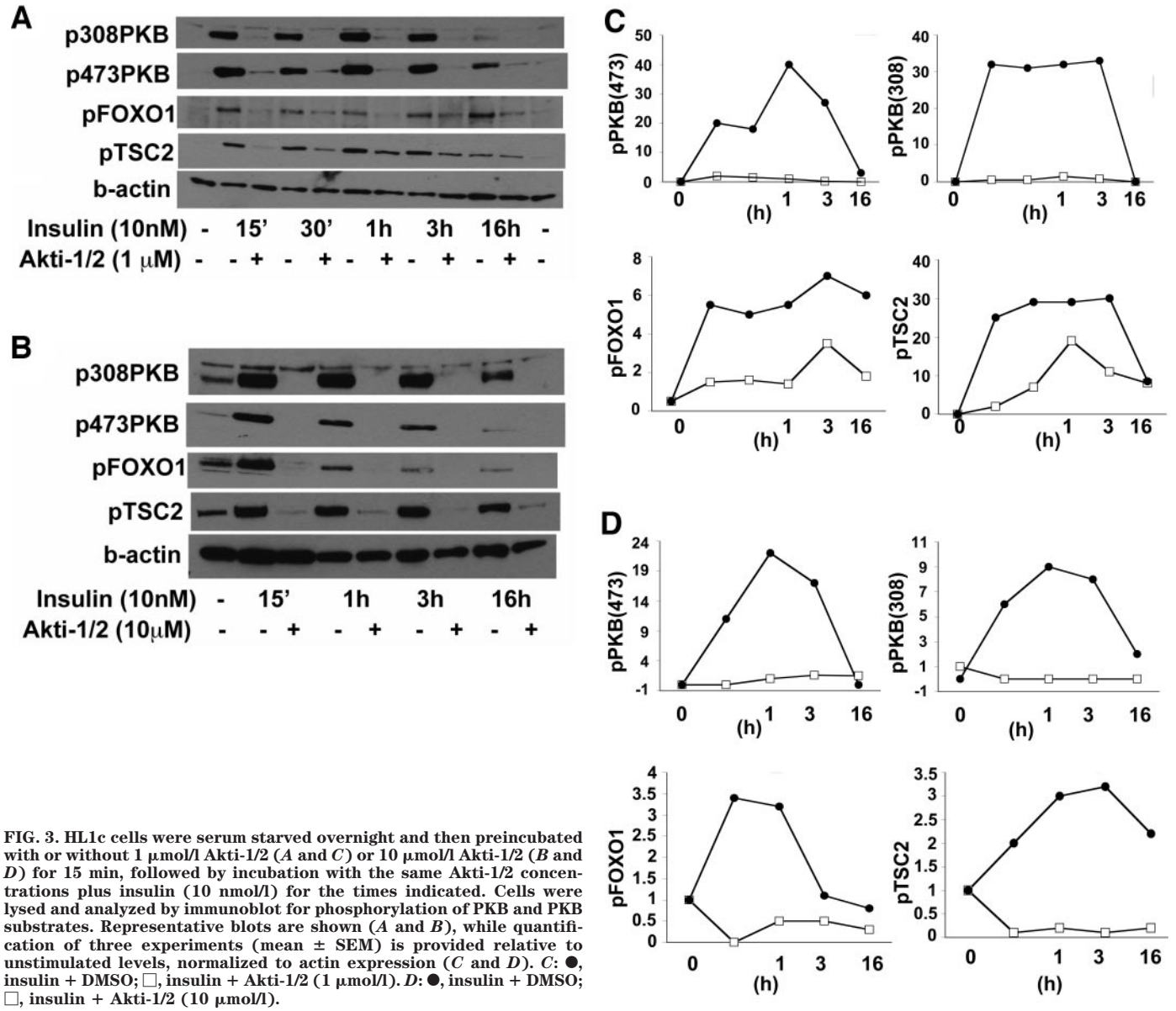

FIG. 3. HL1c cells were serum starved overnight and then preincubated with or without 1 μmol/l Akti-1/2 (*A* and *C*) or 10 μmol/l Akti-1/2 (*B* and *D*) for 15 min, followed by incubation with the same Akti-1/2 concentrations plus insulin (10 nmol/l) for the times indicated. Cells were lysed and analyzed by immunoblot for phosphorylation of PKB and PKB substrates. Representative blots are shown (*A* and *B*), while quantification of three experiments (mean ± SEM) is provided relative to unstimulated levels, normalized to actin expression (*C* and *D*). *C*: ●, insulin + DMSO; □, insulin + Akti-1/2 (1 μmol/l). *D*: ●, insulin + DMSO; □, insulin + Akti-1/2 (10 μmol/l).

reduced PKB activity can be overcome by hyperinsulinemia in this system.

Akti-1/2 completely blocks insulin regulation of FOXO1 activity. PKB regulation of these genes potentially requires the phosphorylation and inhibition of the FOXO family of transcription factors. Insulin-induced PKB phosphorylation of FOXO1 at Ser^{256} is blocked by Akti-1/2, with phosphorylation reduced to below basal in cells exposed to 10 μmol/l Akti-1/2 (Fig. 3*D*). However, phosphorylation is not completely blocked over a 16 h incubation period in the presence of 1 μmol/l Akti-1/2 (Fig. 3*C*). Therefore, we compared the effects of 1 and 10 μmol/l Akti-1/2 on insulin inhibition of FOXO1 in the HL1c cells (Fig. 6). Cells tranfected with BP1-TIRE (a FOXO reporter construct) express luciferase activity that is reduced ~50% by incubation with insulin (26). The expression of a glutathione S-transferase–FOXO1 fusion protein induces luciferase expression, but this remains sensitive to repression by insulin (Fig. 6*B*) (26). The ability of insulin to reduce luciferase expression is blocked by the presence of 1 or 10 μmol/l Akti-1/2, regardless of whether FOXO1 is overexpressed (Fig. 6). Meanwhile, the induction of luciferase expression by FOXO1 was enhanced in the presence of 10 μmol/l Akti-1/2 (Fig. 6*B*), similar to the effect on glucocorticoid induction of endogenous PEPCK, G6Pase, and IGFBP1 mRNA (Fig. 4*B*).

DISCUSSION

This work establishes Akti-1/2 as a selective tool for the characterization of PKB function in liver. The lack of hepatic PKBγ combined with a 10- to 100-fold selective preference for PKB over 70 protein kinases means that PKB can be completely inhibited in liver with little effect on other phosphorylation pathways. Of course, we cannot be certain that other enzyme classes are not affected by Akti-1/2, but our data provide details of the minimal effective concentrations required to block PKB signaling in cells, thereby reducing nonspecific effects.

Insulin action in the liver and muscle is vital for glucose homeostasis and, when disrupted, produces prolonged hyperglycemia (diabetes). In type 2 diabetes (the major form of the disease), insulin and the insulin receptor appear to be present and functional. However, much

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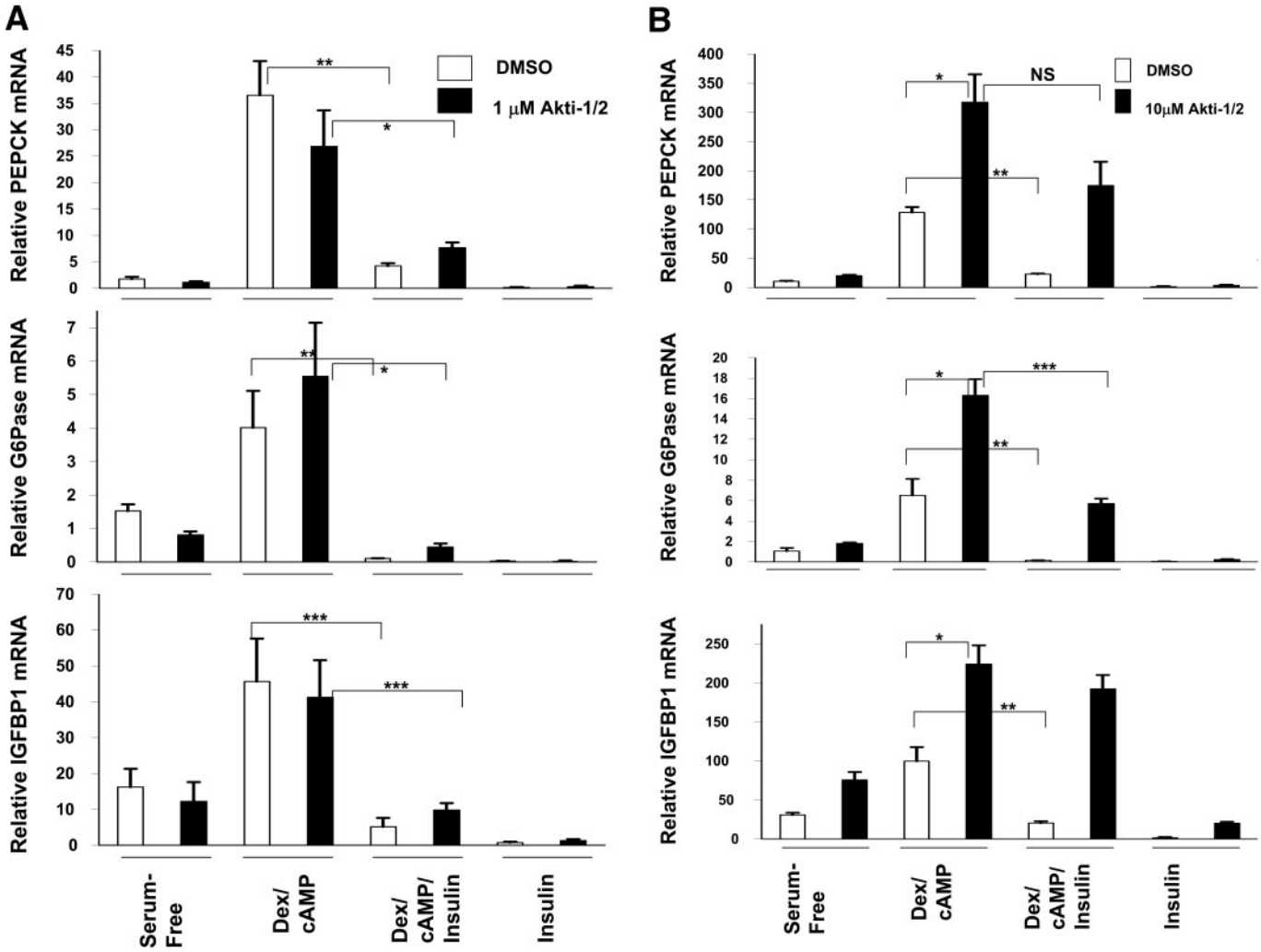

FIG. 4. HL1c cells were serum starved overnight and then incubated with or without dexamethasone (Dex)/cAMP ([500 nmol/l dexamethasone and 0.1 mmol/l 8CPT-cAMP] ± 10 nmol/l insulin) in the presence (■) or absence (□) of 1 μmol/l (*A*) or 10 μmol/l (*B*) Akti-1/2 for 3 h. RNA was extracted and PEPCK, G6Pase, or IGFBP1 gene expression measured as detailed in RESEARCH DESIGN AND METHODS. Data are presented as gene expression relative to cyclophilin expression in the same cells and are means ± SEM from at least three experiments performed in duplicate. *P < 0.05; **P < 0.05; ***P < 0.001. NS, not significant (Student's unpaired t test).

evidence is accumulating that the signaling processes that link the receptor to the regulation of glucose metabolism are defective (34,42–45). Reduced PKB signaling is a proposed mechanism in the development of insulin resistance and diabetes in humans (34). Complete ablation of PKB is embryonic lethal; however, isoform-specific genetic analysis suggests that PKBα is required for normal hepatic function and growth, while PKBβ is required for regulation of hepatic glucose output (34). The precise action of PKB in the regulation of this process is not established, although the data were consistent with a reduced regulation of gluconeogenesis following feeding. One likely explanation for this defect in the PKBβ-deficient animals is loss of insulin repression of the gluconeogenic genes PEPCK and G6Pase. However, there is some evidence that PKB activity is not required for insulin repression of these genes (22,29,32). In this work, we have used a biochemical and pharmaceutical approach to fully establish a key role for PKB in the insulin regulation of specific gene promoters in the liver. We show that complete pharmacological inhibition of PKB in a liver cell line prevents insulin repression of PEPCK and reduces regulation of G6Pase. This argues that PKB is required for proper insulin repression of these genes and suggests that the defects in the PKBβ-deficient animals are due to loss of their repression following feeding. It will be of interest to establish whether a PKBβ-specific inhibitor has a similar effect on this action of insulin; however, an isoform-specific inhibitor is not currently available. It could be argued that the inhibitory effect of 10 μmol/l Akti-1/2 on gene regulation is due to inhibition of CAMK1, smMLCK, or potentially one of the other kinases partly affected at this concentration of compound in vitro (Table 1). However, CAMK1 is poorly expressed in liver and is a calcium- but not insulin-induced enzyme. Similarly, smMLCK is a calcium-responsive rather than insulin-regulated kinase and is not really affected by the inhibitor at 1 μmol/l, a concentration that alters gene regulation (Fig. 5). In addition, neither of these kinases is regulated by PI3 kinase or PDK1 signaling, both of which are required for insulin regulation of the genes (19,20,22).

Importantly, we find that PKB inhibition (1 μmol/l, where smMLCK is not affected) reduces the sensitivity of the genes to insulin. Therefore, reduced PKB signaling capacity results in reduced insulin sensitivity of the gene promoters but does not prevent complete repression of

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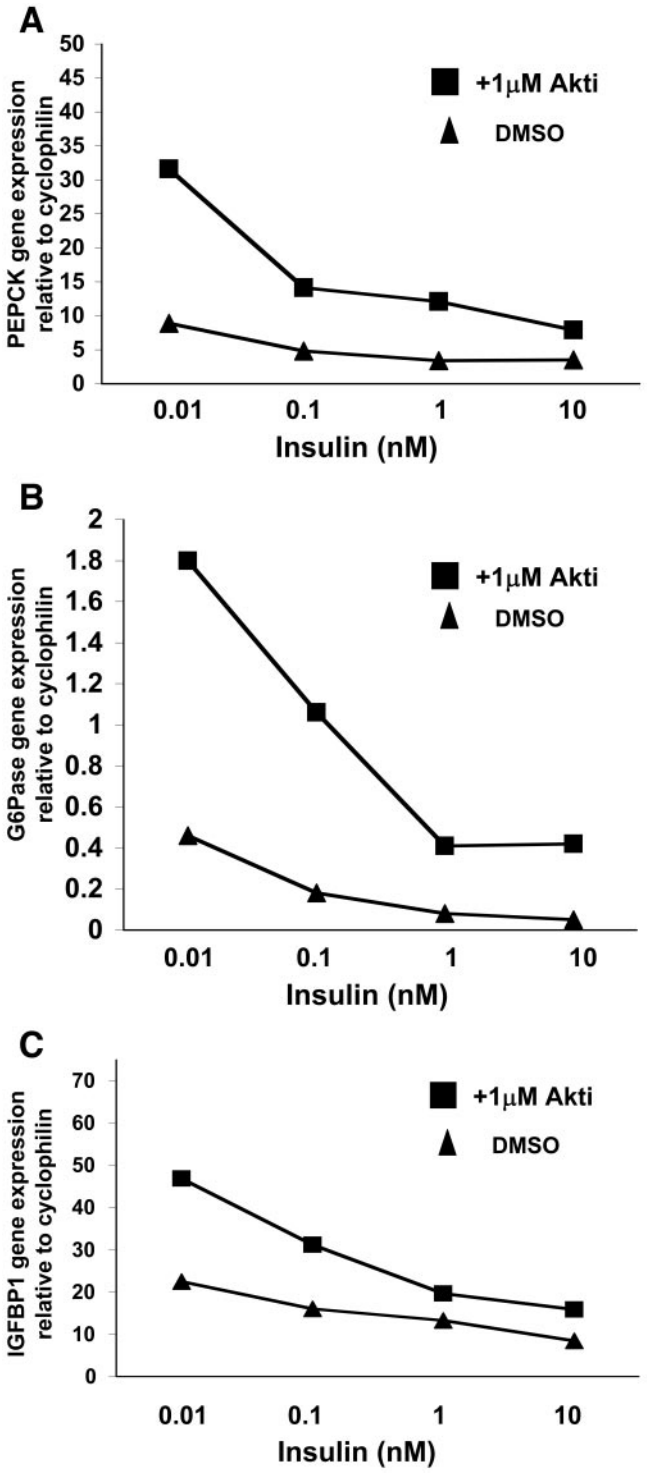

FIG. 5. HL1c cells were serum starved overnight and then incubated with or without dexamethasone/cAMP (500 nmol/l dexamethasone and 0.1 mmol/l 8CPT-cAMP) ± insulin (at the concentration stated) in the presence or absence of 1 μmol/l Akti-1/2 for 3 h. Cells were lysed and PEPCK (*A*), G6Pase (*B*), and IGFBP1 (*C*) gene expression measured by real-time PCR (TaqMan). Gene expression is presented relative to cyclophilin control gene expression in the same cells.

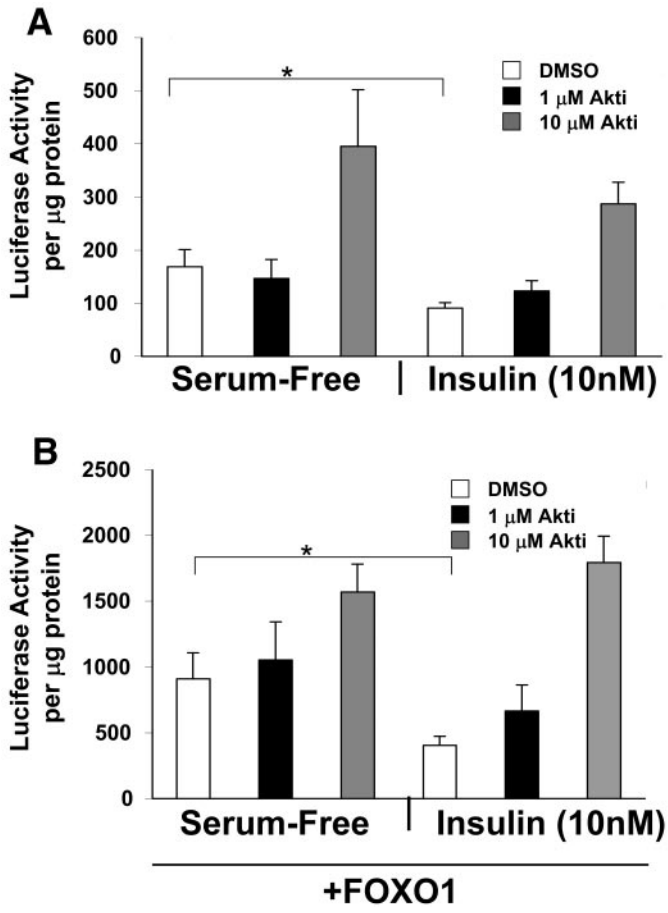

FIG. 6. HL1c cells were transfected with the insulin-sensitive reporter BP1-TIRE plus control expression plasmid pEGB-empty (*A*) or GST-FOXO1 expression plasmid pEBG-FOXO1 (*B*). Cells were then incubated with or without insulin in the presence or absence of 1 or 10 μmol/l Akti-1/2, as indicated, for 20 h. Cells were lysed and luciferase activity measured. *$P < 0.05$ (Student's t test).

the genes if the cells are exposed to high insulin concentrations. This is very similar to the presumed phenotype in an insulin-resistant (pre-diabetic) human. In these individuals, hyperglycemia does not ensue because hyperinsulinemia overcomes the effect of insulin resistance on glucose metabolism.

We find that only a small fraction of maximal PKB activation is required to repress PEPCK, G6Pase, and IGFBP1 transcription. First, 0.1 nmol/l insulin weakly activates PKB (<10% of maximum) yet strongly inhibits gene expression. Second, >90% inhibition of maximal PKB induction (in cells exposed to 1 μmol/l Akti-1/2) has no effect on the degree of repression of the genes by 10 nmol/l insulin. This is consistent with PKB expression studies where only ~5% activation of an MER-PKB (a tamoxifen-inducible form of PKB) gives maximal effects on a downstream gene reporter (23). The concept that "excess" signaling capacity allows increased sensitivity to insulin was first proposed by Kono and Barham (46), and this concept of functional reserve may now be extended to PDK1 and PKB, as well as insulin-binding capacity. Alternatively, it is possible that PKB is part of an amplification cascade, and thus only small amounts of activity are sufficient to maximally regulate downstream components. However there was little evidence that the downstream components that we measured (FOXO, TSC2, and PRAS40) were becoming fully phosphorylated at reduced PKB activation; indeed, their phosphorylation profile mirrored that of PKB quite closely. Hence, either these are not the PKB targets that link the pathway to the PEPCK, G6Pase, or IGFBP1 gene promoters or they are not part of an amplification cascade.

Our data also suggest that the contribution of PKB

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signaling to each of these gene promoters is different. For example, complete loss of PKB activation does not completely prevent insulin action on G6Pase transcription. Similarly, genetic deletion of PDK1 from liver prevents regulation of PEPCK and IGFBP1 by feeding but only partially reduces the response of the G6Pase gene promoter (19). Therefore, there is a PKB-independent regulation of this gene promoter that does not regulate the PEPCK or IGFBP1 gene promoters. This is consistent with several other studies that suggest that insulin regulates these three gene promoters by different mechanisms despite the existence of a related insulin response sequence in each (18,21,26,28,47). Nakae et al. (32) have previously demonstrated a PKB-independent regulation of FOXO, a transcription factor closely associated with the insulin repression of these gene promoters. However, we find that the isolated IGFBP1 insulin-response element (BP1-TIRE) is not responsive to insulin in cells exposed to only 1 μmol/l Akti-1/2. Overexpression of FOXO1 does not alter this sensitivity to Akti-1/2, suggesting that insulin repression of FOXO1 activity is completely PKB dependent. It is perhaps dangerous to directly compare the regulation of endogenous gene promoter with this overexpression system; however, the data would suggest that FOXO1 is not the PKB-independent target of this insulin cascade. Similarly, the distinct Akti-1/2 sensitivity of the endogenous gene promoters and the FOXO reporter construct would argue that FOXO is not the only PKB target that regulates the endogenous promoters. IGFBP1 (but not PEPCK or G6Pase) repression by insulin is sensitive to the mTOR inhibitor rapamycin (26). Clearly, PKB and mTOR activation are therefore required for full repression of IGFBP1, but only PKB activation is required for repression of PEPCK, while PKB activation and an additional PKB-independent pathway contribute to full repression of G6Pase expression.

In summary, we have established the importance of PKB activation in the insulin regulation of PEPCK, G6Pase, and a third insulin-regulated gene, IGFBP1; found further evidence of a difference in the mechanism by which insulin regulates each gene; and identified Akti-1/2 as a useful tool to delineate PKB function in liver.

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