A New Strategy for Studying Protein Kinase B and Its Three Isoforms. Role of Protein Kinase B in Phosphorylating Glycogen Synthase Kinase-3, Tuberin, WNK1, and ATP Citrate Lyase†

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ABSTRACT: Protein kinase B appears to play a key role in insulin signaling and in the control of apoptosis, although the precise targets of PKB are incompletely understood. PKB exists as three isoforms (α, *β*, and *γ*) that may have unique as well as common functions within the cell. To facilitate understanding the precise roles of PKB and its isoforms, novel tools of widespread applicability are described. These tools are antisense oligonucleotide probes that enable the specific and potent knock down of endogenous PKB α, *β*, or *γ* isoforms, individually or in various combinations, including concurrent removal of all three isoforms. The probes were applied to dissect the role of PKB in phosphorylating glycogen synthase kinase-3 (GSK-3), a critical mediator in multiple responses, and other potentially key targets. Triple antisense knock down of PKB α, *β*, and *γ* so that total PKB was <6% blocked insulin-stimulated phosphorylation of endogenous GSK-3α and GSK-3*β* isoforms by 67% and 45%, respectively, showing that GSK-3α and GSK-3*β* are controlled by endogenous PKB. Each PKB isoform contributed to GSK-3α and GSK-3*β* phosphorylation, with PKB*β* having the predominant role. Knock down of total PKB incompletely blocked insulin-stimulated phosphorylation of GSK-3α and GSK-3*β*, and a pathway involving atypical PKCs, *ζ*/*λ*, was shown to contribute to the signal. Triple antisense knock down of PKB α, *β*, and *γ* abrogated the insulin-stimulated phosphorylation of WNK1, ATP citrate lyase, and tuberin. However, antisense-mediated knock down of PKB α, *β*, and *γ* had no effect on insulin-stimulated DNA synthesis in 3T3-L1 adipocytes, indicating that pathways other than PKB mediate this response in these cells. Finally, our PKB antisense strategy provides a method of general usefulness for further dissecting the precise targets and roles of PKB and its isoforms.

Insulin promotes a variety of key biological responses, including increased glucose uptake into cells and the stimulation of glycogen synthesis, DNA synthesis, and protein synthesis (*1*). A complex system of intracellular signaling pathways mediates these insulin effects. One important target for activation by insulin is protein kinase B (PKB), a serine/threonine kinase, which exists in three isoforms, α, *β*, and *γ* (*2*, *3*). PKB also appears to be a crucial prosurvival kinase (*3*, *4*). PKB isoforms are activated by phosphorylation at two regulatory sites, one in the kinase domain (Thr^{308} for PKBα) and the other in the C-terminal domain (Ser^{473} for PKBα). The kinase that phosphorylates Thr^{308} has been identified as 3-phosphoinositide-dependent protein kinase-1 (PDK1). A distinct Ser^{473} kinase (PDK2) exists but has not been identified (*5*).

The precise targets of PKB are incompletely understood. One potential target is glycogen synthase kinase-3 (GSK-3), which is capable of phosphorylating and inactivating glycogen synthase (*6*). However, many studies now demonstrate that GSK-3 plays a more diverse role and acts as a critical downstream regulatory switch for a divergent array of responses from multiple stimuli (*7*). These pathways, when dysregulated, have been implicated in diseases such as diabetes, cancer, Alzheimer's, and bipolar disorder (*8*−*10*), thereby underscoring the importance of a clear understanding of the mechanism by which GSK-3 is regulated in vivo.

The two isoforms of GSK-3, namely, GSK-3α and GSK-3*β*, are phosphorylated in vitro by PKBα on Ser^{21} and Ser^{9}, respectively, and phosphorylation of these sites correlates with GSK-3 activity (*11*). Overexpression of constitutively active or dominant-negative mutants of PKB supports a role for PKB in regulating GSK-3 (*12*, *13*). However, the precise contribution of PKB versus other insulin-activated kinases such as the atypical PKCs, *ζ*/*λ*, or SGK in regulating GSK-3 requires further clarification (*14*−*16*).

There is a key need to develop new tools for studying PKB because current methods are inadequate in one way or another. They either lack isoform specificity or result in incomplete inhibition or do not successfully target all three isoforms (*12*, *13*, *18*−*21*). In addition, in the case of knockout mice studies the phenotypes can be influenced by unrelated changes in gene expression and developmental regulation. Moreover, there have been no studies testing the effect of removing total endogenous PKB from the cell (i.e., all isoforms together) on signaling responses. In the present study we describe antisense tools of general applicability for

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Table 1: Sequences of Oligonucleotides

antisense probes		control mismatch oligonucleotides	
name	sequence	name	sequence
single AS(α)	GCCTCCGCTCACTGTCCA	single MM(α)	GACTCCTCTCAATGTTCA
single AS(*β*)	CTCTCGGATGCTGGCTGA	single MM(*β*)	CTATTGGATGATGGATGA
single AS(*γ*)[b]	AGCCCCACCAGTCCACGG	single MM(*γ*)[b]	AGCACCAACAATCAACGG
double AS(α*β*)[a,b]	TGACCACGCCCAGCCCCC	double MM(α*β*)[a,b]	TGAACAAGCACAGCACCC
	TGACCACACCCAGCCCCC		TGAACAAACACAGCACCC

[a] The double (α*β*) probes were mixed in equal concentrations. [b] For triple oligonucleotide treatment, double (α*β*) probes were mixed with a single (*γ*) probe.

the depletion of the three known isoforms of PKB (namely, α, *β*, and *γ*) to a high level and which ablate PKB isoforms either individually or in combination. These tools have been used to test the role of PKB isoforms versus other kinases in phosphorylating GSK-3 and to test whether several other putative PKB substrates are true in vivo targets of the kinase. Development of the antisense probes is timely, given current efforts to identify new PKB substrates and the array of potential candidates recently proposed (*22*−*26*).

MATERIALS AND METHODS

Materials and Cells. 3T3-L1 fibroblasts (American Type Culture Collection) were maintained and induced to differentiate into adipocytes as described previously (*27*). Polyclonal antibodies to PKBα, PKB*β*, p70S6kinase, and PDK1 were from Upstate Biotechnology. Immobilized PKBα G1 monoclonal antibody beads and phospho-specific antibodies, including anti-phospho-WNK1(Thr^{60}), anti-phospho-tuberin (Thr^{1462}), and anti-$PKB_{substrate}$ antibodies, were from New England Biolabs. Anti-PKB*γ* antibody was from Santa Cruz. Antiphospho-(Thr) PDK1 substrate antibody and myristoylated protein kinase C ζ pseudosubstrate were from Sigma. The PKB inhibitor II was from Calbiochem. Antisense phosphorothioate oligonucleotides were synthesized using an ABI synthesizer 391 and OPC purified prior to use. Computer-aided selection procedures for antisense probes were provided by Dr. I. G. Giles of the Division.

Transfection of Cells with Antisense Oligonucleotides Single AS(α), Single AS(β), or Single AS(α) + Single AS(β) Using Lipofectin. 3T3-L1 adipocytes (day 8−12 post differentiation) or 3T3-L1 fibroblasts (typically 80% confluent) in 22 mm dishes were transfected using lipofectin transfection agent (Gibco) as described previously (*27*). For single AS(α) + single AS(*β*) transfections the phosphorothioate oligonucleotides (as indicated in Table 1) were mixed in equal concentrations in DMEM[1] (no additions) and then incubated with an equal volume of lipofectin (diluted to 40 *μ*g/mL DOTMA) for 45 min. The antisense mixture (200 *μ*L) was layered onto the cells together with a further 200 *μ*L of DMEM (no additions), and the cells were incubated at 37 °C in the presence of 5% CO_2. At 48 h the medium was removed and replaced with fresh DMEM (no addition) containing appropriate oligonucleotides and 0.25% bovine serum albumin, but in the absence of lipofectin. This was repeated every subsequent 48 h until the end of the experiment.

[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal regulated kinases 1 and 2; PHAS-I, phosphorylated heat- and acid-stable protein regulated by insulin; PI 3-kinase, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PKC, protein kinase C; SGK, serum- and glucocorticoid-regulated protein kinase; PTPs, protein tyrosine phosphatases.

Transfection of Cells with Antisense Oligonucleotides Single AS(γ), Double AS(αβ), or Triple AS(αβγ). Lipofectamine 2000 transfection agent (Gibco) was used in these studies. 3T3-L1 adipocytes (day 8−12, 22 mm dishes) were washed extensively with DMEM (no additions). Lipofectamine 2000 (diluted to 80 *μ*g/mL with DMEM containing no additions) was incubated for 5 min at room temperature. Oligonucleotides (as indicated in Table 1) were diluted to four times final concentration with DMEM (no additions), mixed with an equal volume of the lipofectamine 2000/DMEM solution, and then incubated at room temperature for 20 min. The mixture (200 *μ*L) was layered onto the cells together with a further 200 *μ*L of DMEM (no additions), and the cells were incubated at 37 °C in the presence of 5% CO_2. At 48 h the medium was removed and replaced with fresh oligonucleotide in DMEM (no addition) containing 0.25% bovine serum albumin, but in the absence of lipofectamine 2000. This was repeated every 48 h until the end of the experiment. Cells were treated with or without hormone and then extracted for Western blotting or kinase assay, as appropriate.

Incubation of Cells with Pharmacological Inhibitors. 3T3-L1 adipocytes were incubated in DMEM in the absence of serum for 4 days in the presence of 0.25% bovine serum albumin. Cells were then preincubated with 100 *μ*M PKB inhibitor II, 50 *μ*M PKCζ pseudosubstrate, or 25 *μ*M LY294002 for 2, 1.5, or 1 h, respectively, at 37 °C in the presence of 5% CO_2. Cells were then stimulated with or without insulin in the presence of inhibitors as appropriate, washed (four times) with ice-cold PBS, and solubilized into 140 *μ*L of 62.5 mM Tris-HCl (pH 7.4) containing 1% SDS. Cell extracts were analyzed by SDS gel electrophoresis and Western blotting.

DNA Synthesis. At 104 h after initiation of transfection, 3T3-L1 adipocytes were treated with or without insulin (80 nM) for 16 h. [^{3}H]Methylthymidine (3 *μ*Ci/well) was then added and the incubation continued for 1.5 h. Cells were washed (four times) with ice-cold PBS and solubilized into 140 *μ*L of 62.5 mM Tris-HCl (pH 7.4) containing 1% SDS, and triplicate samples of 25 *μ*L each were added to 750 *μ*L of 10% TCA. Precipitates were collected onto GF/C filters following incubation at 4 °C for 1 h. The filters were washed with further TCA and dried, and radioactivity was measured using a scintillation counter.

Western Blotting and Presentation of Quantitative Scanning Data. Samples were separated by SDS gel electrophoresis and transferred to nitrocellulose. The membranes were blocked for 1 h at room temperature in TBS−1% Tween 20 (TBS-T) containing 5% milk fat protein (MFP) and then incubated overnight at 4 °C in primary antibody (1:1000 to 1:10000 dilution) in TBS-T containing 5% BSA

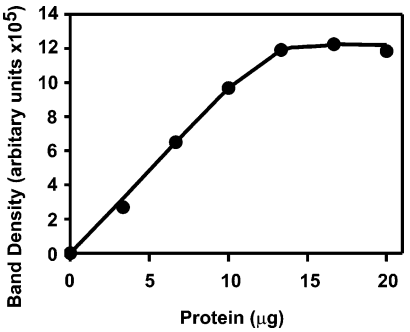

FIGURE 1: Standard curve showing total PKB band density vs amount of cell lysate protein analyzed by Western blot. The curve is representative of four independent experiments.

(phospho-specific antibodies, ERK1/2 antibody) or 5% MFP (isoform-specific PKB antibodies, p70S6kinase). For determination of phosphorylation of WNK1 on Thr^{60} and tuberin on Thr^{1462}, 30 μg of total protein was loaded per gel track, and the membranes were incubated overnight with appropriate antibody at a dilution of 1:1000. Levels of phosphorylation of ATP citrate lyase on Ser^{454} were determined in accordance with Berwick et al. (*23*) using anti-$PKB_{substrate}$ antibody. Following incubation, membranes were washed (four times) with TBS-T and incubated with appropriate secondary antibody for 1 h at room temperature in the presence of 5% MFP, and the bands were visualized by ECL and autoradiography. Bands were quantified by densitometric scanning and analyzed using Phoretix 1D software. Figure 1 shows a typical standard curve for total PKB protein from 3T3-L1 adipocytes obtained by this method. All quantitative Western blot data for antisense knock downs and other effects were obtained from band densities that fell within the subsaturating linear range of the standard curve, as confirmed by developing blots for at least four different times. Data are given as the mean ± SEM (*n*), where *n* is the number of independent experiments. All error bars are SEMs for the number of independent experiments indicated.

In Vitro Kinase Assay. Cells were serum starved for 5 days and where appropriate were stimulated with 100 nM insulin for 5 min prior to extraction. Lysates from 3T3-L1 adipocytes were prepared by extraction into lysis buffer [20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate, 2 μg/mL leupeptin, 1 mM PMSF, and 1% (v/v) Triton X-100]. Immunoprecipitation of PKB (400 μL total volume, 0.15 mg of protein) was carried out in the presence of immobilized PKBα G1 monoclonal antibody beads and lysis buffer for 2 h at 4 °C. The beads were washed three times in lysis buffer followed by a further two washes in kinase buffer (20 mM Tris-HCl, pH 7.5, containing 5 mM β-glycerol phosphate, 0.1 mM sodium vanadate, 10 mM $MgCl_2$, and 2 mM DTT). The kinase assay was carried out at 30 °C for 30 min (assay conditions were linear) in the presence of kinase buffer, crosstide substrate (2 μg per reaction), and ATP (10 μCi of [γ-^{32}P]ATP and 200 pmol of cold ATP per reaction). The reaction was stopped by spotting the kinase mixture onto P81 filter paper. The P81 paper was washed four times in 1% phosphoric acid and once in acetone. Incorporation of phosphate into crosstide was determined by scintillation counting.

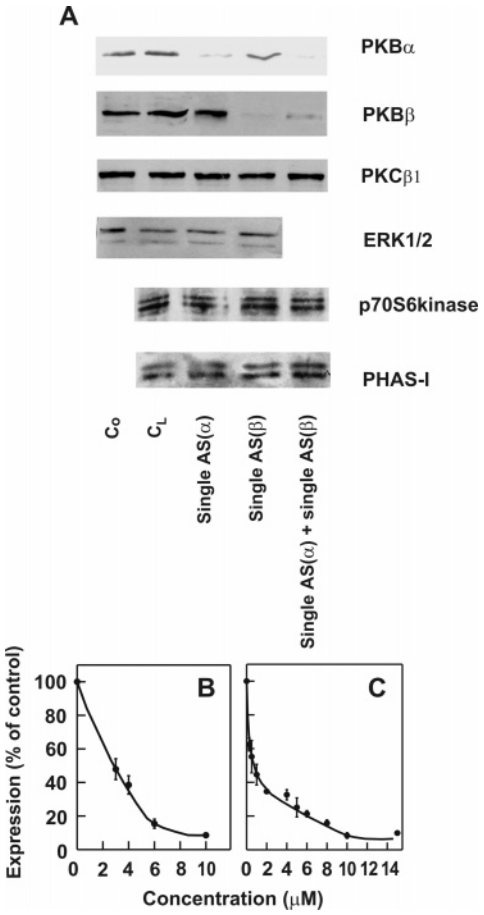

FIGURE 2: Single AS(α) and single AS(β) specifically deplete PKBα and PKBβ, respectively. Panel A: 3T3-L1 adipocytes were incubated with the indicated oligonucleotides (10 μM each) according to Materials and Methods. Cells were extracted after 120 h, and Western blotting was performed to determine the expression of PKBα, PKBβ, PKCβ1, ERK1/2, p70S6kinase, and PHAS-I. Key: C_o, control untreated cells; C_L, lipofectin-only treated cells. The upper two blots are representative of in excess of 20 independent experiments, and other blots are representative of three independent experiments. Panels B and C: 3T3-L1 adipocytes were exposed to single AS(α) (B) or single AS(β) (C) at the concentrations indicated. Cells were extracted after 120 h, and PKBα (B) or PKBβ (C) expression was determined by Western blotting and densitometric scanning. Results are expressed as a percent of lipofectin-only controls and are the mean ± SEM for three to six independent experiments.

RESULTS

Development of PKB Antisense Probes. Antisense phosphorothioate probes were designed using specific criteria as discussed in ref *27*. 18mer probe sequences were selected on the basis of sequence specificity, high T_m, no self-complementarity, and no duplex formation, which would otherwise impede uptake. Antisense probes were selected which targeted individual isoform-specific C-terminal regions of PKB. These probes, referred to as single AS(α) and single AS(β), depleted PKBα and PKBβ isoforms, respectively (Figure 2). In 3T3-L1 adipocytes, depletion of PKBα by single AS(α) did not affect levels of PKBβ or the expression of other cellular proteins including PKCβ1, ERK1/2, p70S6kinase, or PHAS-I (Figure 2A). Similarly, single AS(β) had no effect on levels of PKBα, PKCβ1, ERK1/2, p70S6kinase, or PHAS-I (Figure 2). Depletion of PKBα and PKBβ by single AS(α) and single AS(β), respectively, was

concentration dependent in 3T3-L1 adipocytes, with maximal depletion of 91% ± 1% (n = 5) and 92% ± 2% (n = 6), respectively, achieved using 10 μM oligonucleotide for 5 days for both single AS(α) and single AS(β) (Figure 2B,C). The concentrations of single AS(α) and single AS(β) required to deplete 50% of their target isoforms ($K_{0.5}$) from 3T3-L1 adipocytes were approximately 3 and 0.75 μM, respectively. Concerted depletion of both PKBα and PKBβ within the same cells was achieved using a mixture of single AS(α) and single AS(β) in 3T3-L1 adipocytes, as shown in Figure 2. Both probes were also effective in depleting their respective PKB isoforms in 3T3-L1 fibroblasts in a concentration-dependent manner (data not shown).

A double probe, referred to as double AS(αβ), was also developed that potently depleted PKBα and PKBβ in combination. The double probe target site is highly homologous between PKBα and PKBβ, is close to the kinase domain, and is at a region distinct from that targeted by single AS(α) and single AS(β). This double probe is a mixture of two slightly different sequences complemetary to PKBα and PKBβ and was used in 3T3-L1 adipocytes as described in Materials and Methods. As shown in Figure 3A−C, this double probe depleted PKBα and PKBβ in a concentration-dependent manner with 94% ± 1% (n = 12) and 97% ± 1% (n = 12) depletion, respectively, occurring after 5 days with probe concentrations of 5 μM. Double AS(αβ) was specific for PKBα and PKBβ since levels of PKBγ, PDK1, PKC, and p70S6kinase were unaffected by the antisense treatment (Figure 3A). A final probe, called single AS(γ), was also developed that specifically targeted PKBγ. Single AS(γ) treatment achieved 96% ± 1% (n = 3) depletion of PKBγ from 3T3-L1 adipocytes (Figure 3A,D). The single AS(γ) probe could be used alone or in combination with the double AS(αβ) probe. Use of this triple antisense probe mixture, referred to as triple AS(α$\beta\gamma$), resulted in depletion of >94% of all three isoforms of PKB, namely, α, β, and γ, from 3T3-L1 adipocytes without affecting the levels of other proteins, including PDK1, PKC, and p70S6kinase (Figure 3). Loss of PKB protein was associated with a corresponding loss of PKB activity as determined by kinase assay and confirmed by inhibition of insulin-stimulated phosphorylation of PKB on Ser^{473} (Figure 4). Treatment of cells with the triple AS(α$\beta\gamma$) antisense probe mixture reduced insulin-stimulated PKB activity to a level equivalent to that in unstimulated control cells, thereby attesting to the efficacy of our antisense strategy.

For all studies undertaken, parallel experiments were performed in which the antisense probe(s) were replaced with appropriate control mismatch phosphorothioate oligonucleotides. The mismatch controls were identical in sequence to the antisense probes but with base changes along the length of the probe (Table 1). Additional control oligonucleotides, namely, sense or random (same base composition as the corresponding antisense probe but in scrambled order), were also employed in some experiments, and these gave results similar to those with mismatch oligonucleotides (data not shown). In each experiment treatment of the cells with control oligonucleotides at concentrations identical to that for the corresponding antisense probes did not affect levels of endogenous PKB protein or activity (for example, see Figures 3−5 and 9).

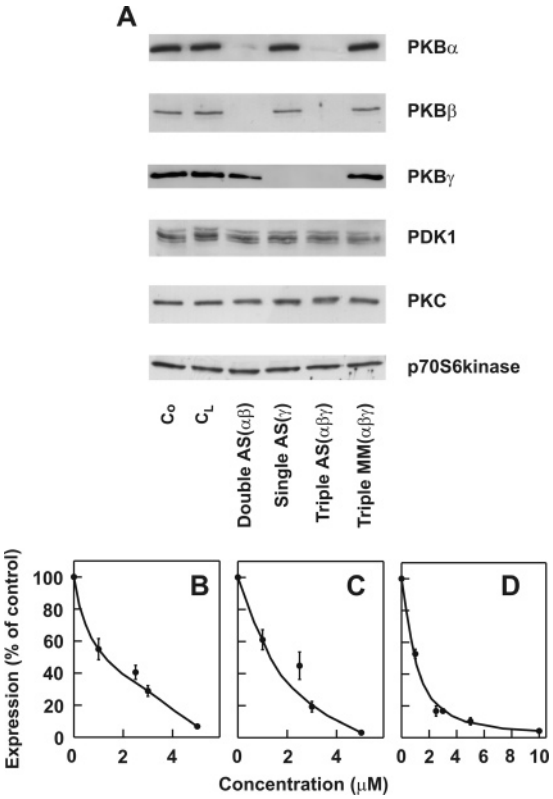

FIGURE 3: Specific depletion of both PKBα and PKBβ by double AS(αβ) and PKBγ by single AS(γ). Panel A: 3T3-L1 adipocytes were treated with the indicated oligonucleotides, as detailed in Table 1. After 120 h cells were extracted by scraping and Western blots performed to determine the expression of PKBα, PKBβ, PKBγ, PDK1, PKC, and p70S6kinase. PKC was blotted with phospho-(Thr) PDK1 substrate antibody. The 80 kDa PKC band is shown. Oligonucleotide concentrations were 5 μM except for single AS-(γ) and single MM(γ), which were 10 μM. Key: C_o, control untreated cells; C_L, lipofectamine 2000-only treated cells. Panels B and C: 3T3-L1 adipocytes were treated with double AS(αβ) at the indicated concentrations. Cells were extracted after 120 h by scraping, and PKBα (B) and PKBβ (C) expression was determined by Western blotting and densitometric scanning. Results are expressed as a percent of lipofectamine 2000-only controls and are the mean ± SEM for six independent experiments. Panel D: As above but using single AS(γ) as oligonucleotide at the concentrations shown. PKBγ expression was determined by Western blotting and densitometric scanning. Results are the mean ± SEM for three independent experiments.

In summary, we have developed a library of antisense probes (see Table 1) that deplete PKB isoforms, either individually or in combination, including removal of all three isoforms together. These tools allowed us to obtain a series of different cells highly deficient in one, two, or all three PKB isoforms.

Role of PKB in Insulin Signaling of GSK-3 Inactivation. Having established specific antisense probes that depleted PKB to a high level, it was important to test and validate them. For this GSK-3 was chosen. Triple antisense knock down of total PKB was first examined. The effects of concurrent removal of the three endogenous PKB isoforms on insulin-stimulated GSK-3 phosphorylation have not been previously determined. 3T3-L1 adipocytes were stimulated with or without insulin in the presence or absence of oligonucleotides, and the phosphoryation of GSK-3α and GSK-3β isoforms was determined by Western blotting and quantified by densitometric scanning. Results are summarized

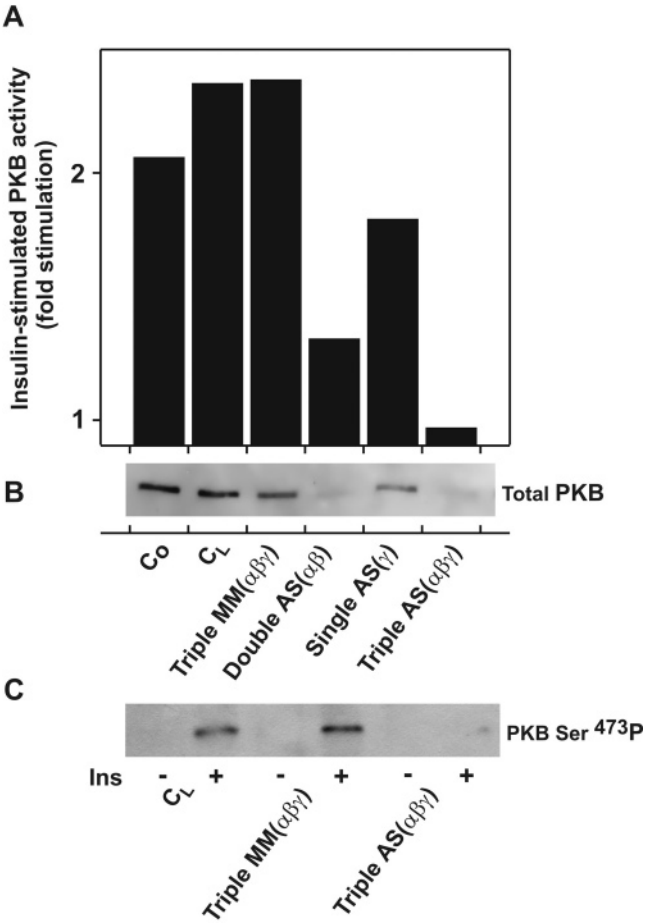

FIGURE 4: PKB knock down is associated with loss of insulin-stimulated PKB activity. 3T3-L1 adipocytes were treated with or without oligonucleotides for 120 h. Cells were then stimulated with (+) or without (−) 100 nM insulin for 5 min and whole cell extracts used for (A) determination of insulin-stimulated PKB activity, (B) Western blot of total cell PKB, or (C) determination of phosphorylation of PKB on Ser[473]. Oligonucleotide concentrations were 5 μM except for single AS(γ) and single MM(γ), which were 10 μM. Insulin-stimulated PKB activity is expressed as the fold increase in activity relative to that of unstimulated, untreated control cells. Results are representative of three independent experiments.

in Figure 5. In control cells treated with or without lipofectamine 2000, insulin caused a marked increase in the phosphorylation of both GSK-3α and GSK-3β. Comparable levels of insulin-dependent phosphorylation of GSK-3α or GSK-3β were also observed in cells treated with lipofectamine 2000 in the presence of the appropriate control mismatch oligonucleotides, showing that phosphorothioate oligonucleotides did not exert nonspecific effects on GSK-3 phosphorylation in 3T3-L1 adipocytes. The concentrations of the appropriate mismatch oligonucleotides used were identical to those of the corresponding antisense probes. Treatment of the cells with the triple AS(α$\beta\gamma$) antisense probe mixture so that total PKB protein and activity were <6% of controls, resulted in a significant attenuation of the insulin-dependent phosphorylation of both GSK-3α and GSK-3β, with the greatest effect observed for GSK-3α. This extensive removal of endogenous PKB isoforms resulted in a 67% ± 4% (n = 3) reduction in the phosphorylation of GSK-3α at Ser[21] and a 45% ± 1% (n = 3) reduction in GSK-3β phosphorylation at Ser[9] in response to insulin (Figure 5C,D). Additional Western blotting of individual PKB isoforms in the antisense-treated cells showed that, relative to lipofectamine 2000-only controls, PKBα and PKBβ were depleted by 93% ± 1% (n = 3) and 94% ± 4% (n = 3), respectively, indicating that individual PKB isoforms had been ablated extensively and by comparable amounts in these cells.

Role of PKB Isoforms in Insulin-Dependent Phosphorylation of GSK-3α and GSK-3β. We next applied our antisense strategy to test and dissect the role of the different PKB isoforms, either individually or in combination, in mediating the insulin-dependent phosphorylation of GSK-3α and GSK-3β in vivo. The combined role of PKBα and PKBβ in regulating insulin-dependent phosphorylation of GSK-3 was evaluated using the double AS(αβ) probe strategy in 3T3-L1 adipoyctes as detailed in Materials and Methods. Treatment of the cells with double AS(αβ) depleted endogenous PKBα and PKBβ by 94% ± 2% (n = 3) and 98% ± 1% (n = 3), respectively, and caused a marked reduction in insulin-stimulated phosphorylation of both GSK-3α at Ser[21] and GSK-3β at Ser[9] of 58% ± 2% (n = 3) and 42% ± 5% (n = 3), respectively, relative to controls (Figure 5). These antisense-mediated effects were specific since the double MM(αβ) mismatch oligonucleotide did not significantly alter the phosphorylation of either GSK-3α or GSK-3β relative to the controls (Figure 5B−D).

We next extended the study to examine the individual roles of PKBα, PKBβ, and PKBγ in mediating the insulin-stimulated phosphorylation of GSK-3α and GSK-3β. 3T3-L1 adipocytes were treated with the single isoform-specific antisense probes as detailed in Materials and Methods. Specific antisense-mediated removal of PKBα, PKBβ, or PKBγ by >90% (see Figures 2 and 3) reduced insulin-stimulated phosphorylation of GSK-3α at Ser[21] by 36% ± 2% (n = 3), 60% ± 4% (n = 3), and 34% ± 5% (n = 3), respectively, and reduced insulin-stimulated phosphorylation of Ser[9] of GSK-3β by 22% ± 2% (n = 3), 37% ± 2% (n = 3), and 27% ± 5% (n = 3), respectively (Figure 6). Thus, in each case, removal of a single PKB isoform in 3T3-L1 adipocytes attenuated insulin signaling of the phosphorylation of both GSK-3α and GSK-3β, with removal of PKBβ causing the greatest level of inhibition. Moreover, these results demonstrate that the removal of single PKB isoforms produced effects which were correspondingly smaller than that observed when multiple PKB isoforms were eliminated (e.g., compare Figures 5 and 6). Effects of the knock downs on insulin-stimulated phosphorylation of both GSK-3α and GSK-3β were in the ratio ~1:2:1 (PKB α:β:γ knock down).

An important conclusion from the above antisense studies is that the removal of near total PKB using the triple AS-(α$\beta\gamma$) probe mixture incompletely reduced insulin-stimulated phosphorylation of GSK-3. Thus, additional pathways appear to play an important role by which insulin signals increased phosphorylation, and hence inactivation, of GSK-3 in 3T3-L adipocytes. Possible candidates that may be involved are the atypical PKCs, ζ/λ. Since PKCζ has been reported to phosphorylate GSK-3β on Ser[9] in vitro (*17*), we next conducted pharmacological studies to evaluate the role of the atypical PKCs, ζ/λ, in mediating insulin-dependent phosphorylation GSK-3 and to compare and contrast this with the role of PKB in the process.

Effect of Pharmacological Inhibition of PKCζ/λ or PKB on GSK-3 Phosphorylation. PKCζ/λ was inhibited in 3T3-L1 adipocytes using the myristoylated pseudosubstrate

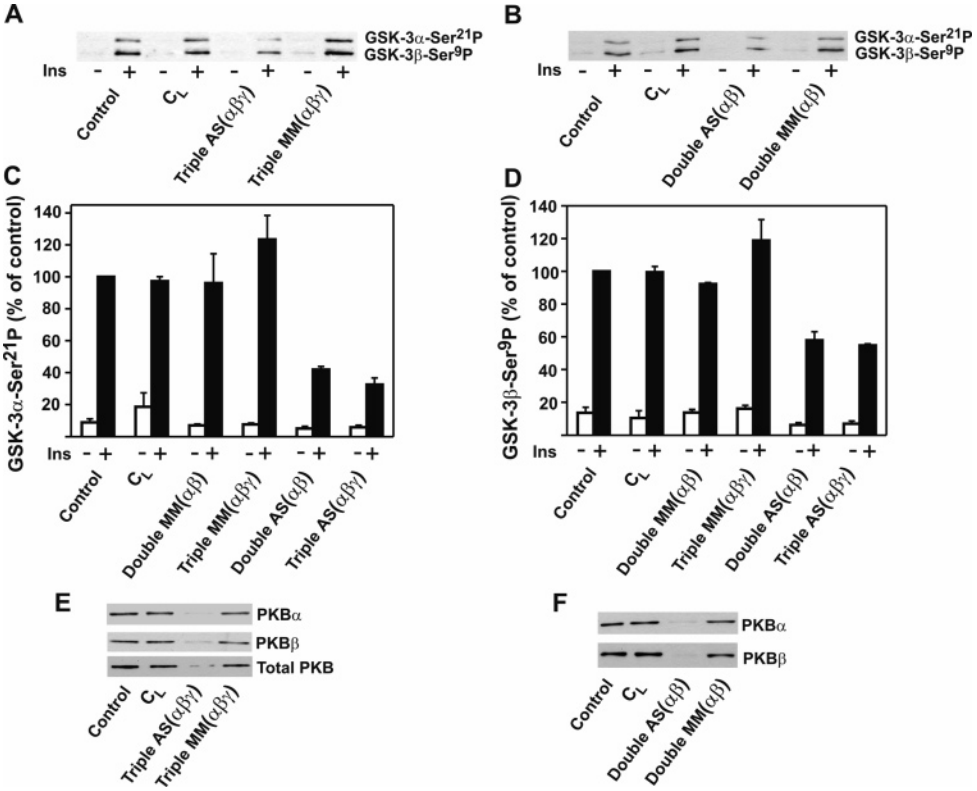

FIGURE 5: Insulin-stimulated phosphorylation of GSK-3α and GSK-3β is attenuated by antisense-mediated ablation of PKB. 3T3-L1 adipocytes were incubated with or without triple AS(α$\beta\gamma$) or double AS(αβ) or appropriate mismatch oligonucleotides as indicated. Cells were then stimulated without (−) or with (+) 20 nM insulin for 10 min and extracts prepared according to Materials and Methods. (A, B) Western blots showing phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9}. (C, D) Quantification of phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9}. Results are expressed as percent of unstimulated, untreated controls. (E, F) Western blots showing PKB protein. Densitometric analysis of blots from triple AS(α$\beta\gamma$) or double AS(αβ) treated cells showed that PKBα was depleted by 93% ± 1% or 94% ± 2%, respectively, and PKBβ was depleted by 94% ± 4% or 98% ± 1%, respectively. In the triple antisense treated cells, total PKB protein was depleted by 94% ± 5%. Values are expressed as the mean ± SEM for three independent experiments. Key: control, control untreated cells; C_L, lipofectamine-only treated cells. The concentration of each oligonucleotide was 5 μM except for single AS(γ) and single MM(γ), which were 7.5 μM.

inhibitor of PKCζ. We also tested a new PI analogue that has recently been reported to block PKB activation in some cell types (*28*). 3T3-L1 adipocytes were exposed to the inhibitors according to Materials and Methods, and insulin-dependent phosphorylation of GSK-3 isoforms was determined by Western blotting and densitometric scanning. Results are shown in Figure 7. In the absence of pharmacological inhibitors, insulin caused marked phosphorylation at Ser^{21} of GSK-3α and Ser^{9} of GSK-3β. Insulin signaling of phosphorylation of these sites was markedly reduced by treatment of the cells with either PKCζ pseudosubstrate (PKCζ-I) or the PI analogue (PKB-I). Analysis of results showed that PKCζ-I inhibited the phosphorylation of GSK-3α at Ser^{21} and GSK-3β at Ser^{9} by 56% ± 5% ($n = 3$) and 46% ± 8% ($n = 3$), respectively, while PKB-I caused a 75% ± 5% ($n = 3$) and 59% ± 4% ($n = 3$) reduction in the phosphoforms of GSK-3α and GSK-3β, respectively (Figure 7B,C). Thus, while PKCζ-I significantly attenuated insulin-stimulated phosphorylation of GSK-3 at the indicated sites in 3T3-L1 adipocytes, the inhibitory effect of PKB-I on GSK-3 phosphorylation was greater. A further difference in the action of these inhibitors was indicated by the fact that PKB-I, like the triple AS(α$\beta\gamma$) probe, exerted a differentially greater effect on GSK-3α when compared to GSK-3β, while the effect of PKCζ-I on the phosphorylation of the two GSK-3 isoforms was more comparable.

These results demonstrate a role for PKCζ/λ in the phosphorylation, and hence inactivation, of GSK-3 in response to insulin in 3T3-L1 adipocytes. Furthermore, the present PI analogue inhibitor studies are consistent with our antisense studies, which demonstrate an important role for PKB isoforms in GSK-3 phosphorylation. However, the specificity of this inhibitor has not been rigorously tested under the conditions required for extensive PKB inhibition. Further studies showed that pharmacological block of the upstream signaling component PI 3-kinase with LY294002 also attenuated, but not completely abolished, insulin-stimulated phosphorylation of GSK-3α and GSK-3β (Figure 7).

We extended our studies to investigate the effects of simultaneous inhibition of PKB and PKCζ. Combined treatment of 3T3-L1 adipocytes with the pharmacological inhibitors to PKB and PKCζ elicited a near total loss of all insulin-stimulated phosphorylation of both GSK-3α and GSK-3β (Figure 7). This result showed that PKB and PKCζ represent key signaling intermediates responsible for the insulin regulation of GSK-3 isoforms in 3T3-L1 adipocytes and that they act in concert. Thus, while each of these protein kinases is necessary for the insulin response, they are not sufficient on their own. Only when PKB and PKCζ are active simultaneously are they sufficient to elicit normal levels of insulin-stimulated phosphorylation of GSK-3.

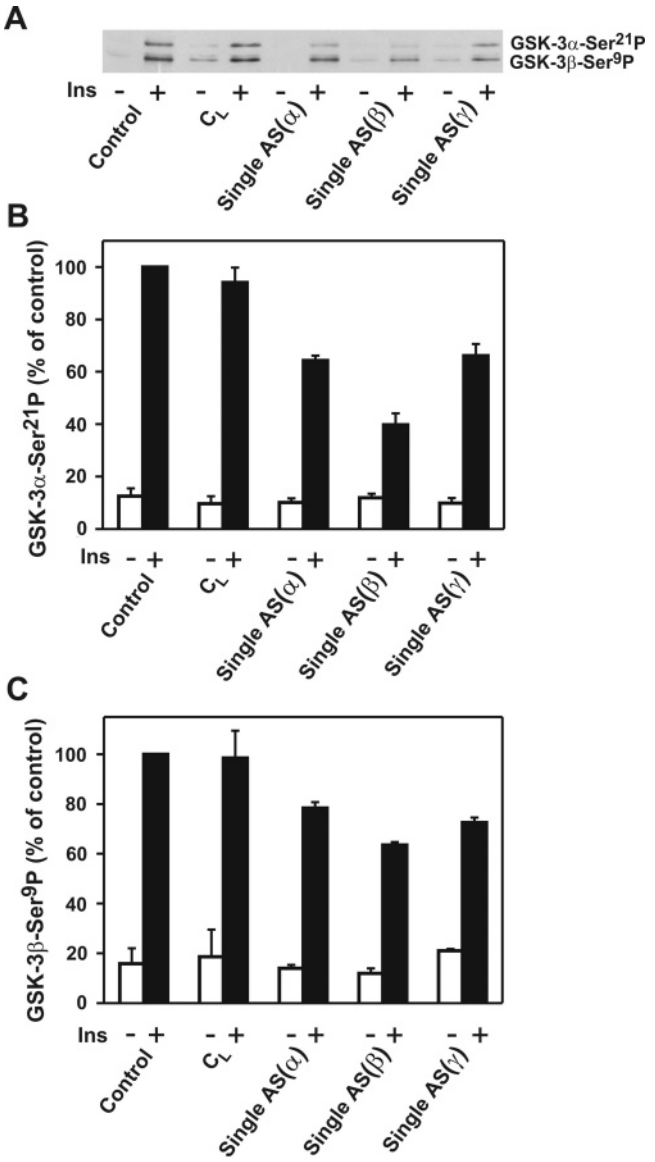

FIGURE 6: Knock down of individual PKB isoforms inhibits insulin-stimulated phosphorylation of GSK-3α and GSK-3β. 3T3-L1 adipocytes were treated with or without single AS(α), single AS(β), or single AS(γ). Cells were then stimulated without (−) or with (+) insulin and samples prepared as given in Figure 5. (A) Western blots showing the phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9}. (B, C) Graphs showing the phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9} relative to unstimulated, untreated controls. Values are the mean ± SEM for three independent experiments. Key: control, control untreated cells; C_L, transfection agent-only treated cells. The concentration of each oligonucleotide was 10 μM except for single AS(γ), which was 7.5 μM.

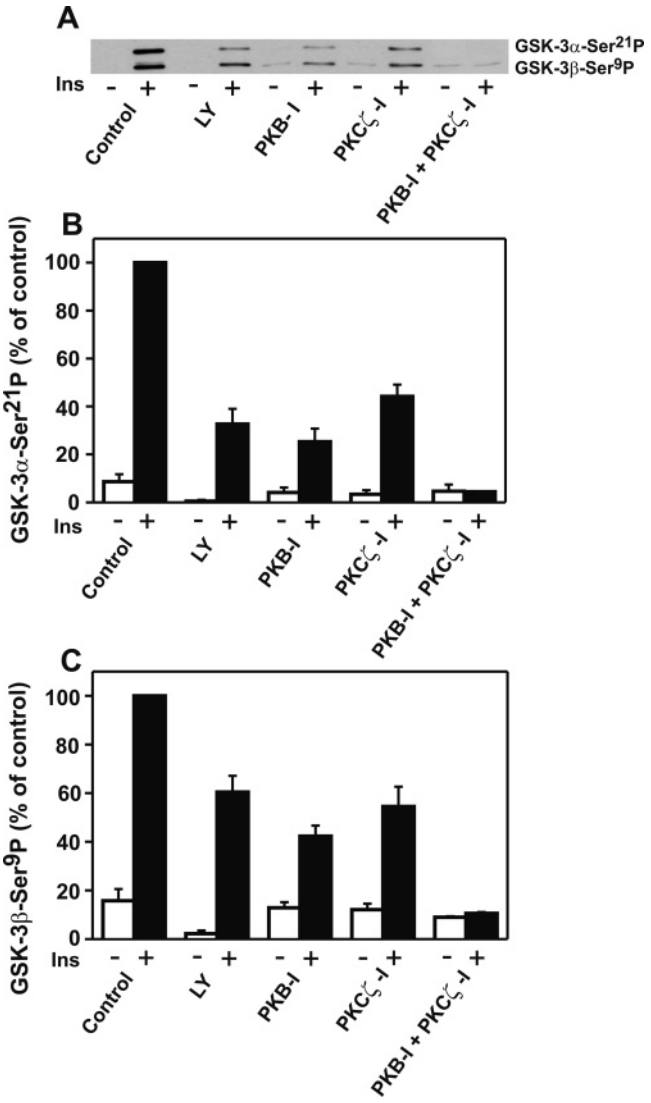

FIGURE 7: PKCζ and PKB both mediate in signaling insulin-stimulated phosphorylation of GSK-3α and GSK-3β. 3T3-L1 adipocytes were serum starved and pretreated with or without the PKB inhibitor (PKB-I), PKCζ myristoylated pseudosubstrate peptide (PKCζ-I), or LY294002 (LY) according to Materials and Methods. Cells were then incubated for 10 min in the presence or absence of 20 nM insulin. (A) Western blots showing the phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9}. (B, C) Quantification of phosphorylation of GSK-3α on Ser^{21} and GSK-3β on Ser^{9}. Results are expressed as percent relative to unstimulated, untreated control cells. Values are the mean ± SEM for three independent experiments.

PKB Knock Down Inhibits the Phosphorylation of WNK1, ATP Citrate Lyase, and Tuberin. Recent studies have suggested that PKB may play an important role in the phosphorylation of a number of other substrates, including the tumor supressor TSC2 gene product tuberin, WNK1 [with no K (lysine) protein kinase-1], and ATP citrate lyase (*23*−*26*). Antisense knock down was used to test the role of PKB in these responses. As shown in Figure 8, removal of all three PKB isoforms by the triple antisense method resulted in a marked inhibition of insulin-dependent phosphorylation of WNK1, tuberin, and ATP citrate lyase at the indicated sites, showing that PKB mediates in these effects. The result for WNK1 was particularly striking, suggesting that there is an absolute requirement for PKB in this phosphorylation event. Mismatch oligonucleotides were without effect in each case.

Role of PKB in Insulin Signaling of DNA Synthesis. We next employed our antisense probes to investigate the role of PKB in the insulin signaling of increased DNA synthesis. Previously, using antisense strategies we have demonstrated that ERK1/2 are required for the stimulation by insulin of thymidine incorporation into DNA in 3T3-L1 adipocytes (*27*). This result demonstrated a critical role of the MAP kinase pathway in mediating insulin signaling of DNA synthesis. To test whether the PKB pathway was required for insulin to signal this response, we utilized our PKB antisense strategies. 3T3-L1 adipocytes were treated with the

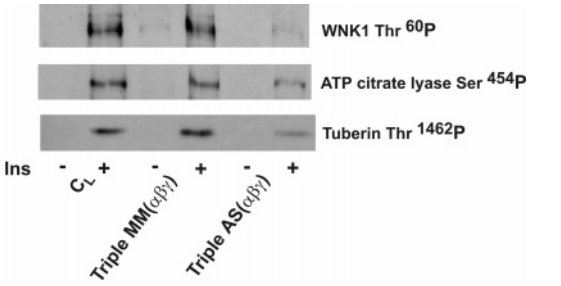

FIGURE 8: Antisense knock down of PKB inhibits insulin-stimulated phosphorylation of WNK1, ATP citrate lyase, and tuberin. 3T3-L1 adipocytes were treated with or without oligonucleotides for 120 h. Cells were then stimulated with (+) or without (−) 100 nM insulin for 5 min and cell lysates centrifuged at 10000*g* for 10 min. The supernatants were analyzed by Western blotting using phosphospecific antibodies to determine phosphorylation at the indicated sites. Oligonucleotide concentrations were 5 μM except for single AS(γ) and single MM(γ), which were 10 μM. Blots are representative of three independent experiments. Triple AS($\alpha\beta\gamma$) treatment depleted PKBα, PKBβ, and total PKB protein by 93% ± 1%, 94% ± 4%, and 94% ± 5%, respectively, and reduced insulin-stimulated phosphorylation of WNK1 to 11% ± 2%, ATP citrate lyase to 33% ± 2%, and tuberin to 27% ± 5% relative to that in lipofectamine-only controls. Results are the mean ± SEM for three independent experiments.

triple AS($\alpha\beta\gamma$) probe mixture to deplete PKBα, PKBβ, and PKBγ protein. Antisense-mediated removal of 96% of total PKB protein resulted in no significant effect on insulin-stimulated thymidine incorporation into DNA when compared to control cells, including triple MM($\alpha\beta\gamma$) mismatch oligonucleotide-treated cells (Figure 9). Next we worked further upstream in the pathway and tested the involvement of PI 3-kinase using the pharmacological inhibitor LY294002. Pretreatment of 3T3-L1 adipocytes with LY294002 to inhibit PI 3-kinase did not prevent insulin-stimulated increase in thymidine incorporation (Figure 9).

DISCUSSION

A strategy that eliminates all three isoforms, PKBα, PKBβ, and PKBγ, either individually or in combination is essential for a clear understanding of the roles of PKB isoforms in signal transduction. To achieve this, we have developed a library of novel antisense probes that specifically target the three known PKB isoforms within 3T3-L1 adipocytes, namely, PKBα, PKBβ, and PKBγ. We have shown that these antisense phosphorothioate oligonucleotide probes can be used successfully either individually or in combination. In this way we can manipulate one, two, or all three isoforms of PKB within the cell, achieving >90% knock downs, and use this to test the specific roles of the PKB isoforms in mediating key insulin responses.

The probes were designed to be specific and were selected on the basis that they did not bind to any other sequences in the database. Critically, kinases such as PDK1, SGK isoforms, p70S6kinase, PKCλ, PKCζ, and MAP kinase isoforms do not contain sequences which will bind the probes. The specificity of the phosphorothioate oligonucleotide antisense probes was rigorously verified in a number of ways. First, in all studies undertaken, control phosphorothioate oligonucleotides were used in parallel experiments at identical concentrations. These took the form of mismatch oligonucleotides (consisting of the antisense sequence with base changes along the length of the probe). In each case,

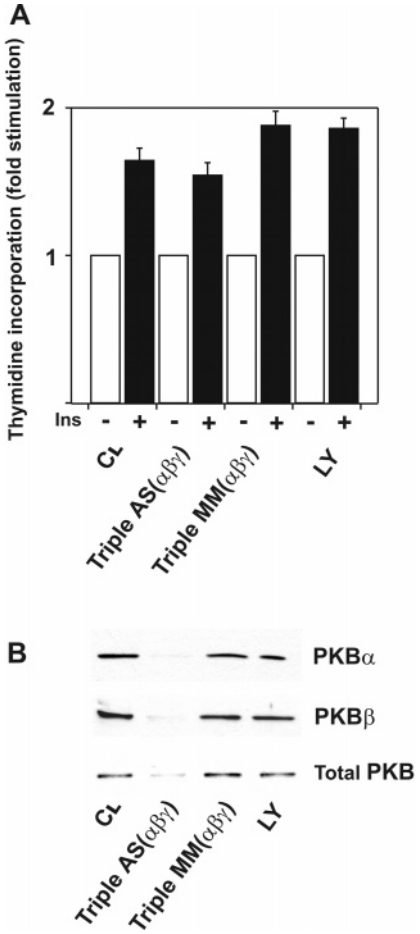

FIGURE 9: Insulin-stimulated DNA synthesis occurs independently of PKB in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with oligonucleotides for 120 h as in Figure 5 except that 80 nM insulin was absent (−) or present (+) during the final 16 h. Cells were then incubated with [^{3}H]methylthymidine and cell extracts used for (A) determination of thymidine incorporation as described under Materials and Methods or (B) Western blotting of PKB. [^{3}H]-Methylthymidine incorporation (mean ± SEM for three independent determinations) is expressed as a fold effect of that in appropriate unstimulated control cells. The concentration of each oligonucleotide was 5 μM except for γ probes, which were 7.5 μM. Key: C_L, lipofectamine 2000-only treated cells; LY, cells incubated without oligonucleotides but in the presence of 25 μM LY294002 for the final 16.5 h of the experiment. Densitometric analysis of blots showed that PKBα, PKBβ, and total PKB protein in antisense-treated cells were depleted by 97% ± 1%, 98% ± 1%, and 96% ± 1%, respectively. Results are the mean ± SEM for four independent experiments.

treatment of 3T3-L1 adipocytes with control oligonucleotides did not significantly affect the levels of PKBα, PKBβ, or PKBγ within the cell. Second, treatment of cells using the PKB antisense probes did not alter the amounts of other key components upstream and downstream of PKB (Figures 2 and 3) or levels of other major endogenous proteins as assessed by protein staining of Western blots (data not shown). Additionally, the expression of ERK1/2 (e.g., Figure 2) and signaling of events that occur through the ERK1/2 pathway were normal (e.g., Figure 9), showing that this parallel pathway was unperturbed. Third, the antisense probe treatments did not affect general cell morphology of 3T3-L1 fibroblasts or adipocytes. Fourth, the antisense knock down of either individual PKB isoforms or all three isoforms together did not induce any apoptosis in 3T3-L1 adipocytes,

as measured by various methods including DNA laddering and assays of caspase 3 activity (K. Green, E. M. Sale, C. P. Hodgkinson, and G. J. Sale, unpublished observations). Lastly, the antisense probe treatments had no effect on the ability of insulin to stimulate DNA synthesis (Figure 9), a response that does not require PKB, showing that there was no general impairment of cell function.

In previous studies we have successfully employed antisense strategies to test the role of endogenous ERK1/2 in insulin signaling (*27*, *29*−*34*). In the present studies, we have used our PKB probes to dissect the role of PKB and its three isoforms in regulating GSK-3 and its role in phosphorylating other putative substrates.

Antisense-mediated removal of all three isoforms of PKB using triple AS(α*β*γ) so that total endogenous PKB activity and protein were <6% that of controls resulted in a 67% reduction in insulin-stimulated phosphorylation of GSK-3α on Ser^{21} and a 45% reduction in insulin-stimulated phosphorylation of GSK-3*β* on Ser^{9}. These results show that both endogenous GSK-3α and GSK-3*β* are controlled by endogenous PKB and validate the antisense strategy as an effective molecular tool. The results also demonstrate that PKB plays a more significant role in mediating in the phosphorylation of GSK-3α than GSK-3*β*.

Given the central role of GSK-3 in cell signaling, it was important to determine precisely which PKB isoforms phosphorylated this key target. Using the isoform-specific antisense probes, each PKB isoform was shown to contribute to the insulin-stimulated phosphorylation of both GSK-3 with PKB*β* having the greater role. Effects of the knock downs on insulin-triggered phosphorylation of both GSK-3α and GSK-3*β* were in the ratio ∼1:2:1 (PKB α:*β*:γ knock down). Each of these effects was significantly lower when compared with removal of total PKB. Moreover, antisense-mediated removal of PKBα and PKB*γ* from PKB*β*-depleted cells caused only a modest further increase in the level of inhibition of GSK-3α or GSK-3*β* phosphorylation. Thus while all PKB isoforms contribute to GSK-3 phosphorylation in 3T3-L1 adipocytes, PKB*β* appeared to play a more predominant role in the process relative to PKBα and PKB*γ*. This conclusion is consistent with the impaired insulin-dependent GSK-3*β* inactivation reported in muscle cells from obese insulin-resistant patients where only PKBα activation is normal (*35*). Our study represents the first complete investigation into the role of PKB and its individual isoforms in phosphorylating both GSK-3α and GSK-3*β*. Other attempts using siRNA approaches have been incomplete in one way or another, e.g., not studying all PKB isoforms, only achieving partial depletion of targeted isoforms and not targeting total PKB (*20*, *21*). Furthermore, recent studies have demonstrated that siRNA may cross-react with targets of limited sequence similarity, leading to some concerns regarding this strategy (*36*).

Importantly, our antisense strategy also showed that ablation of total endogenous PKB protein did not result in the complete loss of insulin-stimulated phosphorylation of either GSK-3α or GSK-3*β*. This indicates that pathways additional to the PKB pathway also mediate in these insulin effects. The relative contribution of these other pathways to the overall insulin signal was greater for GSK-3*β* than for GSK-3α. Possible candidates that may mediate in these effects are the atypical PKCs, PKCζ/λ or SGK. PKCζ/λ are activated in response to insulin and have been implicated in signaling increased glucose uptake in adipocytes (*37*, *38*). Furthermore, several studies have raised the possibility that PKCζ/λ may regulate GSK-3 phosphorylation and activity (*9*, *14*, *17*). In the present studies pharmacological blockade of PKCζ/λ in 3T3-L1 adipocytes markedly attenuated insulin-dependent phosphorylation of both GSK-3α at Ser^{21} and GSK-3*β* at Ser^{9}, while inhibition of PKB and PKCζ/λ together caused total ablation of these insulin responses. These results demonstrate that both PKB and PKCζ/λ are required for maximal insulin-stimulated phosphorylation of GSK-3α at Ser^{21} and GSK-3*β* at Ser^{9} in 3T3-L1 adipocytes and that their combined actions are additive and sufficient. In further support of a dual mechanism for GSK-3 regulation, we have shown that PKB and PKCζ come together to form a complex containing PDK2 (*5*). Formation of such an active signaling complex may be crucial in ensuring that GSK-3 becomes fully phosphorylated. As PKCζ has been reported to phosphorylate GSK3 on Ser^{9} (*17*), a model whereby PKB and PKCζ converge and phosphorylate the same regulatory site is favored.

Antisense knock down of total PKB was used to definitively establish that the lipogenic enzyme, ATP citrate lyase, is an in vivo target for PKB in insulin signaling. We also showed by eliminating PKB from the cell that tuberin is a proper in vivo PKB target in insulin action. Triple antisense knock down also abrogated the insulin-stimulated phosphorylation of WNK1. These results support peptide mapping, mutation, and siRNA studies that have indicated that WNK1 is an endogenous substrate for PKB (*25*, *26*).

In further studies, we showed that LY294002 and antisense-mediated knock down of total PKB did not inhibit insulin-stimulated DNA synthesis in 3T3-L1 adipocytes. These cells are terminally differentiated, and the ERK pathway is known to signal increased DNA synthesis in response to insulin in these cells (*27*). In growing cells, such as fibroblasts, inhibition of the PI 3-kinase and the ERK pathways both inhibit DNA synthesis in response to growth factors, and in such cell types both of the pathways may be involved (*39*−*41*).

Our antisense probes are of potential therapeutic usefulness. The ability to target individual PKB isoforms offers higher selectivity and specificity than would be achieved by targeting all PKB isoforms. This is important because PKB may have isoform-specific roles and because specific isoforms are elevated in certain cancers. It is noteworthy that a number of antisense oligonucleotides are already in human clinical trials (*42*).

In summary, we have developed for the first time tools, namely, antisense probes, which enable the knock down of total endogenous PKB or its individual isoforms from the cell. The antisense probes developed provide powerful molecular tools of broad biological importance for investigating the role of endogenous PKB isoforms in signaling key cellular processes and for testing further putative PKB substrates that are likely to be identified in the future.

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