

## A Role for Protein Kinase B $\beta$ /Akt2 in Insulin-Stimulated GLUT4 Translocation in Adipocytes

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**Insulin stimulates glucose uptake into muscle and fat cells by promoting the translocation of glucose transporter 4 (GLUT4) to the cell surface. Phosphatidylinositol 3-kinase (PI3K) has been implicated in this process. However, the involvement of protein kinase B (PKB)/Akt, a downstream target of PI3K in regulation of GLUT4 translocation, has been controversial. Here we report that microinjection of a PKB substrate peptide or an antibody to PKB inhibited insulin-stimulated GLUT4 translocation to the plasma membrane by 66 or 56%, respectively. We further examined the activation of PKB isoforms following treatment of cells with insulin or platelet-derived growth factor (PDGF) and found that PKB $\beta$  is preferentially expressed in both rat and 3T3-L1 adipocytes, whereas PKB $\alpha$  expression is down-regulated in 3T3-L1 adipocytes. A switch in growth factor response was also observed when 3T3-L1 fibroblasts were differentiated into adipocytes. While PDGF was more efficacious than insulin in stimulating PKB phosphorylation in fibroblasts, PDGF did not stimulate PKB $\beta$  phosphorylation to any significant extent in adipocytes, as assessed by several methods. Moreover, insulin, but not PDGF, stimulated the translocation of PKB $\beta$  to the plasma membrane and high-density microsome fractions of 3T3-L1 adipocytes. These results support a role for PKB $\beta$  in insulin-stimulated glucose transport in adipocytes.**

The ability of insulin to promote glucose storage in muscle and adipose tissue is crucial to the maintenance of glucose homeostasis. An impairment in the ability of insulin to stimulate glucose uptake in these tissues, a condition termed insulin resistance, contributes to the development of type 2 (non-insulin-dependent) diabetes, hypertension, and cardiovascular disease (25). The primary mechanism of insulin-stimulated glucose uptake is through the translocation of glucose transporter 4 (GLUT4) from an intracellular site to the cell surface (26). Defects in the insulin signal transduction pathways that regulate glucose transport have been considered likely causes of insulin resistance (28).

While the insulin signaling pathways responsible for triggering GLUT4 translocation are yet to be defined, rapid progress has been made. Activation of the insulin receptor results in the tyrosyl phosphorylation of insulin receptor substrate (IRS) proteins, docking proteins that recruit src homology 2-containing signaling proteins via phosphotyrosine moieties. Several lines of evidence suggest the involvement of IRS proteins in insulin-stimulated GLUT4 translocation. Disruption of IRS-1 and IRS-2 in mice causes mild insulin resistance and type 2 diabetes, respectively (6, 60). Overexpression of IRS-1 in rat adipocytes mimics the effect of insulin on GLUT4 translocation (43), while reduction of IRS-1 by an antisense ribozyme (43) or chronic insulin treatment (44) decreases insulin responsiveness. One of the molecules recruited by IRS proteins thought to be required for insulin-stimulated GLUT4 translocation is phosphatidylinositol 3-kinase (PI3K). Two inhibitors

of PI3K, wortmannin and LY294002, both inhibit insulin-stimulated GLUT4 translocation (14, 17, 42). Furthermore, introduction of a dominant negative p85 regulatory subunit into adipocytes significantly impairs insulin-stimulated GLUT4 translocation either when microinjected (31) or when overexpressed (47). Overexpression of constitutively active p110 catalytic subunit stimulates GLUT4 translocation to the plasma membrane (PM) in the absence of insulin (38, 53). Hence, these experiments collectively suggest that PI3K is necessary for insulin-stimulated GLUT4 translocation.

Several protein serine/threonine kinases have recently been identified as downstream targets of PI3K. These include protein kinase B (PKB; cellular homolog of v-AKT, also termed RAC-PK) (11, 20, 22), PKC $\zeta$  (8, 50), and PKC $\lambda$  (32). Several studies have examined the role of PKB in insulin-stimulated GLUT4 translocation; however, the results have been somewhat contradictory. Constitutively active PKB $\alpha$  has been expressed in either 3T3-L1 adipocytes (30) or rat adipocytes (18, 54) and found to promote GLUT4 translocation to the plasma membrane. Similarly, constitutively active PKB $\alpha$  increased glucose uptake in L6 myotubes (23, 57). Studies utilizing dominant negative PKB have produced conflicting results. In support of a role for PKB in insulin action, Cong et al. (18) found that a kinase-inactive (K179A) PKB $\alpha$  mutant inhibited insulin-stimulated GLUT4 translocation by 20% when transfected into rat adipocytes. However, Hajdуч et al. (23) found that this same construct had no significant effect in L6 myotubes. Similarly, two recent studies (29, 32) found that a double-phosphorylation site mutant of PKB $\alpha$  behaved as a dominant negative mutant with respect to 3T3-L1 adipocyte PKB activity measured in vitro but had no significant effect on insulin-stimulated GLUT4 translocation. In contrast, a kinase-inactive, phosphorylation-deficient mutant of PKB $\alpha$  was found to inhibit insulin-stimulated GLUT4 translocation in L6 myo-

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blasts (59). Part of the difficulty in reconciling these results lies in the complexity of PKB regulation, which is only beginning to be deciphered. The use of different cell types, some of which may not be bona fide insulin-responsive cells, has also complicated the interpretation. In addition, chronic overexpression of mutant forms of PKB may enable the cell to adapt via alternate pathways. Another potential problem is isoform specificity. Three isoforms of PKB have been identified; however, these were not distinguished in most studies examining the role of PKB in insulin-stimulated glucose uptake. While all of the PKB mutants studied were based on PKB $\alpha$ , PKB $\beta$  was recently identified as the isoform that associates with GLUT4 vesicles in an insulin-dependent manner in rat adipocytes (12). Interestingly, PKB $\beta$  appears to be functionally distinct from PKB $\alpha$  and PKB $\gamma$ , as stress activates PKB $\alpha$  and PKB $\gamma$ , but not PKB $\beta$  (48).

To further assess the role of PKB in insulin stimulation of GLUT4 translocation, we have utilized a different approach from previous studies. Microinjection of specific substrate peptides and antibodies was used to acutely inhibit endogenous PKB action. Microinjection of a PKB substrate peptide (KRPRAAATF) caused significant inhibition of insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. An antibody directed against the N terminus of PKB $\alpha$ , which also recognizes PKB $\beta$ , produced similar effects. We further show that PKB $\beta$  expression is switched on during adipocyte differentiation whereas PKB $\alpha$  is down-regulated. Concomitant with this change, we also observed a switch in growth factor responsiveness during differentiation in that the ability of platelet-derived growth factor (PDGF) to stimulate PKB phosphorylation is switched off in adipocytes at the expense of a robust activation by insulin. Taken together, our results strongly support PKB $\beta$  as the isoform involved in mediating the metabolic effects of insulin in adipocytes.

#### MATERIALS AND METHODS

**Antibodies.** Rabbit antibodies directed against the N terminus of PKB $\alpha$  (residues 3 to 22) were purchased from Santa Cruz Biotechnology (N19; Santa Cruz, Calif.). Sheep antibodies against PKB $\alpha$  (residues 466 to 480), PKB $\beta$  (residues 455 to 469, as described in reference 58), and PKB $\gamma$  (residues 116 to 128) were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Rabbit PKB $\beta$  antibodies were raised against a C-terminal peptide of PKB $\beta$  (CDQTHFPQF-SYASIRE). Antibodies specific for PKB phosphorylated at Ser473 or Thr308 were purchased from New England Biolabs (Beverly, Mass.). Monoclonal anti-hemagglutinin (anti-HA) antibodies (HA11, clone 16B12) were purchased from BabCo (Berkeley, Calif.). Polyclonal anti-GLUT4 antibodies (R017) were raised in rabbits against a 17-amino-acid peptide comprising the C terminus of GLUT4.

**Peptide synthesis.** Peptides were synthesized with an Applied Biosystems 430A Peptide synthesizer coupled with the FastMoc strategy. Purity was checked by reverse-phase high-pressure liquid chromatography, and their integrity was confirmed by amino acid analysis and matrix-assisted laser desorption/ionization mass spectrometry. Two peptides were examined. KRPRAAATF was a peptide closely related to that reported by Alessi et al. (1) as a specific substrate for PKB, except that we included a lysine to increase its solubility in aqueous solutions. KRPRAAAF was the same peptide, except that alanine was substituted for threonine. The first peptide was found to be a good substrate for PKB in *in vitro* assays. The alanine-substituted peptide partially inhibited PKB phosphorylation of the substrate peptide only at the highest concentration tested (1 mg/ml) (data not shown).

**Cell culture.** 3T3-L1 fibroblasts (American Type Culture Collection) were grown and differentiated into adipocytes as described elsewhere (55). Briefly, 3T3-L1 fibroblasts were grown and passaged in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. Cells were differentiated 1 to 2 days postconfluence. The differentiation medium contained 10% fetal calf serum (FCS), 250 nM dexamethasone, 500 nM isobutyl methylxanthine, and 500 nM insulin. After 3 days, the differentiation medium was replaced with postdifferentiation medium containing 10% FCS and 250 nM insulin. Cells were fed every 3 days postdifferentiation in Dulbecco's modified Eagle medium supplemented with 10% FCS. Unless otherwise stated, fibroblasts were used at confluence and adipocytes were used at 8 to 15 days after the initiation of differentiation. 3T3-L1 fibroblasts stably expressing HA-PKB $\alpha$  or HA-PKB $\beta$  have been described elsewhere (52).

**Microinjection.** Microinjection was performed as previously described (35). Cells grown to confluence and differentiated on coverslips were transferred to Krebs-Ringer bicarbonate buffer (111 mM NaCl, 4.87 mM KCl, 1.15 mM CaCl<sub>2</sub>, 1.22 mM KH<sub>2</sub>PO<sub>4</sub>, 1.21 mM MgSO<sub>4</sub>, 25.7 mM NaHCO<sub>3</sub>, 10 mM HEPES, 2.5 mM glucose, 0.5% bovine serum albumin [BSA], 1 mM Na pyruvate, pH 7.4) for 45 min. They were microinjected over a 45-min period with a Zeiss automated injection system (Carl Zeiss, Oberkochen, Germany) coupled to an Eppendorf (Hamburg, Germany) microinjector. Micropipettes were prepared with a Sutter (Novato, Calif.) P-97 micropipette puller. Reagents for microinjection were dissolved in microinjection buffer (5 mM sodium phosphate [pH 7.2] and 100 mM KCl). Sodium azide in the antibody preparation was removed by dialysis in three changes of microinjection buffer. Cells were transferred into fresh medium and allowed to recover for 60 to 90 min following injection of peptide (5 mg/ml) or antibody (0.2 mg/ml), prior to stimulation with insulin (100 nM) and analysis of GLUT4 translocation by the PM lawn assay.

**PM lawn assay.** GLUT4 translocation was determined by the PM lawn assay as described by Robinson and James (46) with modifications described by Marsh et al. (37). Briefly, 3T3-L1 cells grown on coverslips were washed in poly-L-lysine after cell treatment, hypotonically shocked with three washes in one-third intracellular buffer (70 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM EGTA, 1 mM dithiothreitol, 30 mM HEPES, pH 7.2), and sonicated with a probe sonicator (Microson, Farmington, N.Y.) at setting 0 in intracellular buffer to generate a lawn of PM fragments that remained attached to the coverslip. The fragments were then immunolabeled with rabbit anti-GLUT4 antibodies (R1159) (27) and Cy3-labeled goat anti-rabbit antibodies (Amersham, Little Chalfont, United Kingdom). Coverslips were visualized and imaged with a Bio-Rad Lasersharp MRC-500 confocal laser scanning immunofluorescence microscope. GLUT4 translocation in microinjected cells was compared to that in noninjected cells in the immediate vicinity on the same coverslip. Data was analyzed with Bio-Rad COMOS confocal imaging software. Six or more fields were analyzed for each condition within each experiment.

**Treatment of cells, metabolic labeling, and preparation of extracts.** Differentiated 3T3-L1 adipocytes were serum starved by incubating them in Krebs-Ringer phosphate buffer (KRP) containing 12.5 mM HEPES (pH 7.4), 120 mM NaCl, 6 mM KCl, 1.2 mM Mg<sub>2</sub>SO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM NaPO<sub>4</sub>, and 0.1% (wt/vol) BSA for at least 2 h at 37°C prior to stimulation. Insulin (1  $\mu$ M; Eli Lilly) and PDGF (50 ng/ml; Gibco) treatments were for 5 or 15 min. Where indicated, 100 nM wortmannin (Sigma) was added to KRP 25 min prior to addition of insulin. For metabolic labeling, cells were incubated in low (0.2 mM)-phosphate KRP containing 0.5 mCi of <sup>32</sup>P<sub>i</sub> (ICN) per ml in place of the KRP incubation and then treated as described above. After treatment, cells were washed three times with ice-cold HES buffer (20 mM HEPES [pH 7.4], 1 mM EDTA, 250 mM sucrose) and homogenized in HES buffer supplemented with protease inhibitors (10  $\mu$ g of aprotinin per ml, 10  $\mu$ g of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM ammonium molybdate, and 10 mM sodium fluoride) by 15 passes through a 22-gauge needle. 3T3-L1 fibroblasts were harvested in the same manner, except that homogenization was done through a 27-gauge needle. Subcellular fractionation of 3T3-L1 adipocytes was performed as described in the work of Clark et al. (16).

**Preparation of primary rat adipocytes.** Primary rat adipocytes were prepared from epididymal fat pads of male Wistar rats (100 to 125 g) by the collagenase digestion method (49). After incubation in KRP (containing 2% BSA) with agitation for 1 h at 37°C, cells were treated with or without 1  $\mu$ M insulin for 15 min. Adipocytes were collected by centrifugation and homogenized in HES buffer with inhibitors by 15 passes through a 27-gauge needle.

**Immunoprecipitation.** Cells were harvested in lysis buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton X-100, 25 mM  $\beta$ -glycerophosphate, protease inhibitors, and phosphatase inhibitors) and homogenized by 15 passes through a 22-gauge needle. After incubation on ice for 15 min, lysates were centrifuged at 12,800  $\times$  g for 15 min at 4°C. Immunoprecipitation was performed by adding the supernatants to Eppendorf tubes containing blocked, antibody-conjugated protein A-G beads (Pierce) and incubating them at 4°C with mixing for at least 2 h. Immunocomplexes were collected by centrifugation and washed twice with cold lysis buffer and once with low-salt buffer (10 mM NaCl, 10 mM Tris, pH 7.5). Proteins bound were eluted by addition of gel sample buffer.

To characterize PKB antibodies and for PKB $\beta$  immunodepletion experiments, immunoprecipitation was performed by adding 100  $\mu$ g of cell lysate (in HES buffer) to radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris [pH 8], 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease inhibitors and phosphatase inhibitors and incubating the lysate with antibody-conjugated protein A-G beads at 4°C. For PKB $\beta$  depletion experiments, the supernatants were subjected to a second round of immunoprecipitation. To analyze the lysate after PKB $\beta$  depletion, an aliquot of the supernatant after immunoprecipitation was subjected to methanol-chloroform precipitation (7). The resulting protein pellets were resuspended in Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Electrophoresis and immunoblotting.** Protein assays were performed using the Bradford assay reagent (Bio-Rad) or the bicinchoninic acid protein assay kit (Pierce), with BSA (fraction V; Pierce) as standard. SDS-PAGE was performed according to the method of Laemmli (34), using the SE400 system (Hofer).

Two-dimensional gel electrophoresis (2-DE) with immobilized pH gradients was performed exactly as described in the work of Hill et al. (24), with nonlinear pH 3 to 10 immobilized pH gradients in the first dimension and SDS-7.5% PAGE gels in the second dimension. To prepare samples for 2-DE, cell lysates in HES buffer were precipitated with 4 volumes of methanol at  $-20^{\circ}\text{C}$  for 1 h and then centrifuged for 10 min at  $10,000 \times g$  at room temperature. After the supernatants were removed, the pellets were air dried and resolubilized in 2D sample buffer (7 M urea, 2 M thiourea, 40 mM Tris, 4.4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 84 mM dithiothreitol, 1% Pharmalyte, 0.01% bromophenol blue, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors as described above). Samples were sonicated in a sonicating water bath (Branson) for 1 min at room temperature and then centrifuged as before. The resulting supernatants were underlaid into oil-filled sample cups positioned at the anode, and isoelectric point focusing was performed for  $53.5 \text{ kV} \cdot \text{h}$  at  $20^{\circ}\text{C}$ .

After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P from Millipore) according to the method of Towbin et al. (56). Membranes were blocked in 5% skim milk powder in Tris-buffered saline-Tween (TBST; 50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then incubated with the relevant primary antibody (diluted in blocking buffer or TBST) at  $4^{\circ}\text{C}$  overnight. After washing in TBST for 30 min with three changes of buffer, immunoreactive proteins were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG), goat anti-mouse IgG (Amersham), or rabbit anti-sheep IgG (Sigma) as appropriate and SuperSignal chemiluminescence substrate (Pierce). Images were captured on film (Fuji) or a Lumi-Imager (Boehringer).

## RESULTS

**Effect of a PKB substrate peptide or a PKB antibody on insulin-stimulated GLUT4 translocation.** Previous studies investigating the role of PKB in insulin-stimulated glucose transport have utilized overexpression of either constitutively active (18, 23, 30, 54, 57) or dominant negative (18, 23, 29, 32, 59) mutants of PKB $\alpha$ . These studies have produced conflicting results, and so we undertook a different approach to assess the role of PKB in insulin-stimulated glucose transport. Firstly, we have used microinjection in 3T3-L1 adipocytes. This approach circumvents potential adaptive responses that may occur during chronic overexpression of mutants, because we routinely perform our analysis 60 to 90 min after microinjection. This technique in combination with the PM lawn assay provides a means for examining the effects of inhibitors in single cells. Using this system, we routinely observe a 2.5-fold increase in cell surface levels of GLUT4 in response to insulin in cells microinjected with control peptides or control antibodies and in neighboring noninjected cells. Microinjection was performed with 3T3-L1 adipocytes with a peptide (KRPRAAATF) closely related to the peptide previously determined to be relatively specific for PKB (1), except that a lysine was included at the N terminus to increase its solubility. As shown in Fig. 1A, the substrate peptide inhibited insulin-stimulated GLUT4 translocation by  $(66.1 \pm 7.8)\%$  ( $n = 10$ ). A control peptide in which alanine was substituted for the phosphorylation site threonine had no significant effect on GLUT4 translocation  $([7.3 \pm 3.7]\%$ ,  $n = 6$ ). This suggests that the injected substrate peptide is interfering with the propagation of the signaling pathway leading to GLUT4 translocation, probably by competing with a physiological substrate of PKB.

To further assess the role of PKB in insulin-stimulated GLUT4 translocation, studies in which antibodies against PKB were microinjected into 3T3-L1 adipocytes were performed. An antibody directed against the N terminus of PKB $\alpha$  (N19 from Santa Cruz), which cross-reacts with PKB $\beta$ , inhibited insulin-stimulated GLUT4 translocation by  $(55.6 \pm 7.8)\%$  whereas an unrelated antibody had no significant effect on this process (Fig. 1B). We also examined the effect of a variety of C-terminal PKB antibodies, including the isoform-specific sheep PKB $\beta$  antibody described below. However, none of these antibodies had a significant effect on insulin-stimulated GLUT4 translocation in this system (data not shown). This

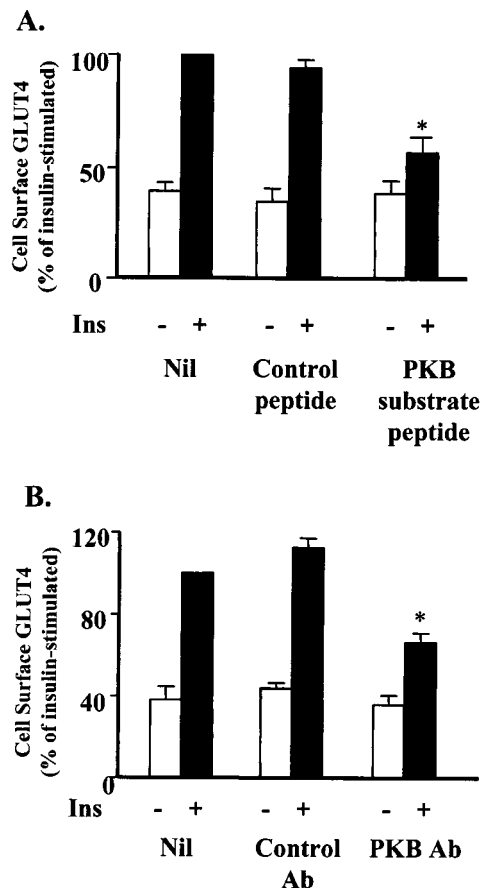


FIG. 1. Microinjection of PKB substrate peptide or an N-terminal PKB antibody inhibits insulin-stimulated GLUT4 translocation. 3T3-L1 adipocytes on coverslips were preincubated in Krebs-Ringer bicarbonate-HEPES buffer for 45 to 90 min. (A) Cells were then either not microinjected (Nil), microinjected with PKB substrate peptide (KRPRAAATF), or microinjected with control peptide (KRPRAAAF) at 5 mg/ml. (B) 3T3-L1 adipocytes were either not injected (Nil) or microinjected with a purified N-terminal PKB antibody (PKB Ab) (N19; Santa Cruz) or purified rabbit IgG fraction (Control Ab) at 0.2 mg/ml. Bathing buffer was changed, and the cells were allowed to recover for 60 min. Cells were then stimulated or not with 100 nM insulin for 20 min prior to assessment of PM GLUT4 levels by the PM lawn assay as described in Materials and Methods. Results are from four or more experiments in which GLUT4 levels in six or more fields were determined for each condition within each experiment. \*,  $P < 0.01$  compared with insulin stimulation (paired  $t$  test).

may indicate that the C terminus is inaccessible in vivo or that antibody binding to this domain is less disruptive to PKB function.

**Down-regulation of PDGF responsiveness in 3T3-L1 adipocytes.** The microinjection data indicates that activation of PKB is necessary to fully mediate insulin-stimulated GLUT4 translocation in adipocytes. However, it has been reported that PDGF also activates PKB in adipocytes without any significant effect on GLUT4 translocation (10, 51, 54), raising the possibility that PKB activation may not be sufficient for insulin action. To explore this issue further, we have compared the effects of insulin with those of PDGF on the activation of PKB in 3T3-L1 adipocytes as well as their precursor cells, 3T3-L1 fibroblasts. As phosphorylation at Ser473 and Thr308 is essential for PKB activation, the effect of insulin and PDGF on PKB phosphorylation in 3T3-L1 cells was initially examined by immunoblotting with phosphospecific PKB antibodies (Fig. 2A). Treatment of 3T3-L1 fibroblasts with either insulin or PDGF



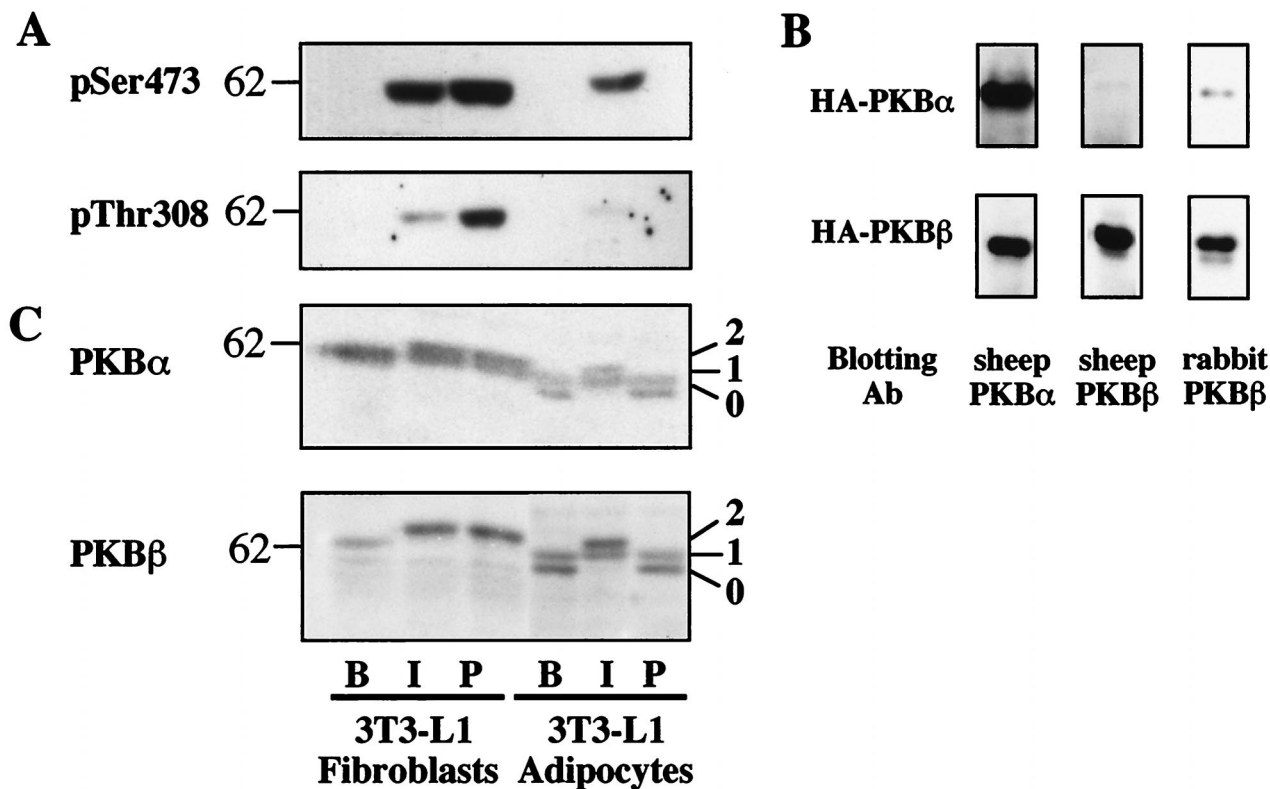


FIG. 2. Change in growth factor response accompanies differentiation of 3T3-L1 adipocytes. (A) Serum-starved 3T3-L1 fibroblasts or adipocytes were stimulated with 1  $\mu$ M insulin (I) or 50 ng of PDGF per ml (P) for 15 min or left untreated (B). Cell lysates (30  $\mu$ g) were analyzed by SDS-PAGE and immunoblotting with phospho-Ser473 (pSer473) or phospho-Thr308 (pThr308) PKB antibodies. (B) HA-PKB $\alpha$  or HA-PKB $\beta$  was immunoprecipitated from lysates (100  $\mu$ g) of 3T3-L1 fibroblasts expressing either HA-PKB $\alpha$  or HA-PKB $\beta$  and analyzed by SDS-PAGE. Immunoblotting was performed with the sheep PKB $\alpha$ , the sheep PKB $\beta$ , or the rabbit PKB $\beta$  antibodies. (C) Cell lysates (30  $\mu$ g) prepared as described for panel A were analyzed by SDS-PAGE and immunoblotting with the sheep PKB $\alpha$  or the sheep PKB $\beta$  antibody. Bands labeled as band 1 exhibited the same electrophoretic mobility.

for 15 min caused PKB phosphorylation on Ser473 and Thr308, with PDGF being more efficacious than insulin (Fig. 2A). The response to insulin compared with that of PDGF in 3T3-L1 adipocytes was quite different from that of fibroblasts. In this case, only insulin stimulated PKB phosphorylation, as detected by phosphospecific PKB antibodies (Fig. 2A).

In order to correlate these results with phosphorylation of specific PKB isoforms, the isoform specificity of PKB antibodies was examined by immunoblotting using stable cell lines of 3T3-L1 fibroblasts expressing HA-tagged forms of PKB $\alpha$  or PKB $\beta$ . HA-tagged PKB was immunoprecipitated from lysates with a monoclonal HA antibody and immunoblotted with a panel of PKB antibodies. The sheep PKB $\beta$  antibody immunoblotted HA-PKB $\beta$  but exhibited no detectable cross-reactivity with HA-PKB $\alpha$  (Fig. 2B). In contrast, the sheep PKB $\alpha$  antibody immunoblotted both HA-PKB $\alpha$  and HA-PKB $\beta$ , and the rabbit PKB $\beta$  antibody showed a slight cross-reactivity with HA-PKB $\alpha$  (Fig. 2B).

The above data suggests that the sheep PKB $\beta$  antibody is isoform specific, at least in immunoblotting; however, the sheep PKB $\alpha$  antibody cross-reacts with PKB $\beta$  to a considerable extent. Nevertheless, we proceeded to examine the relative expression and the effect of growth factors on the electrophoretic mobility of PKB $\alpha$  and PKB $\beta$  by using the sheep PKB antibodies. As shown in Fig. 2C, both sheep PKB $\alpha$  and PKB $\beta$  antibodies labeled bands in 3T3-L1 cells of an average molecular mass of 60 kDa, consistent with the calculated molecular mass of 56 kDa. While a single immunoreactive band was

detected in basal fibroblasts, an additional band with lower molecular mass (band 0) was detected in basal adipocytes, with either the PKB $\alpha$  or the PKB $\beta$  antibody (Fig. 2C). The band labeled with the PKB $\beta$  antibody was up-regulated during the differentiation process whereas the intensity of the band labeled by the PKB $\alpha$  antibody in fibroblasts was not significantly different from the combined intensity of the two bands in adipocytes (Fig. 2C). In agreement with results presented in Fig. 2A, both insulin and PDGF caused an electrophoretic mobility shift in PKB in fibroblasts, whereas only insulin caused a shift in adipocytes. Interestingly, the electrophoretic shift for both PKB $\alpha$  and PKB $\beta$  was much more pronounced in adipocytes than in fibroblasts. Moreover, in insulin-treated adipocytes, band 0 was almost quantitatively shifted to a position slightly above band 1 (labeled band 2) whereas the apparent mobility and intensity of band 1 were unchanged (Fig. 2C).

**PKB $\beta$  is highly up-regulated during adipocyte differentiation.** Key molecules mediating insulin regulation of metabolism are increased upon adipocyte differentiation. Thus, we further examined the change in PKB $\beta$  expression during the differentiation process and found that the increase in PKB $\beta$  expression during differentiation closely paralleled that observed for GLUT4 (Fig. 3A). The most dramatic increase in both PKB $\beta$  and GLUT4 occurred after day 6, when the differentiation medium was removed and cells were fed with medium supplemented only with 10% FCS (Fig. 3A). No GLUT4 expression was detected prior to initiation of differentiation (day 0, Fig. 3A). However, a low level of PKB $\beta$  was

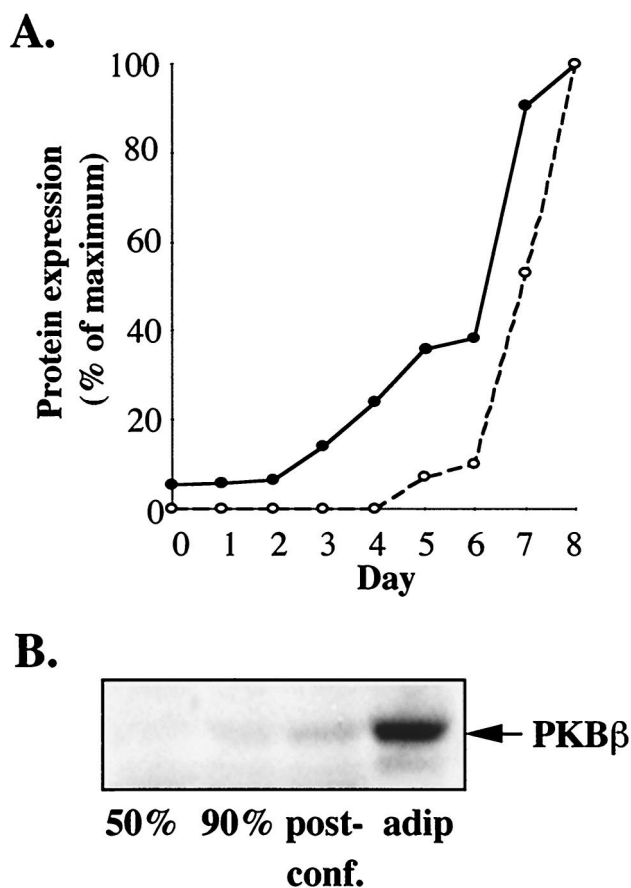


FIG. 3. PKB $\beta$  expression is induced upon adipocyte differentiation. (A) 3T3-L1 cells were harvested on each day of the differentiation procedure and analyzed for the expression of PKB $\beta$  (●) or GLUT4 (○) by immunoblotting with the sheep PKB $\beta$  antibody or a rabbit anti-GLUT4 antibody (R017), respectively. Immunoreactive signal (obtained as Lumi-Imager units) was adjusted for total protein obtained per sample and then expressed as a percentage of the maximum. Results are representative of two separate experiments. (B) 3T3-L1 fibroblasts were harvested at subconfluence (50% or 90%) or 1 day after reaching confluence (post-conf.). 3T3-L1 adipocytes were harvested after completion of differentiation, at day 8 (adip). Cell lysates (30  $\mu$ g) were analyzed for the level of PKB $\beta$  expression by SDS-PAGE and immunoblotting with the sheep PKB $\beta$  antibody.

detected (day 0, Fig. 3A). Preliminary experiments suggested that there may be changes in PKB $\beta$  expression associated with cell confluency. This possibility was examined by immunoblotting lysates harvested from 3T3-L1 fibroblasts at different confluencies. As shown in Fig. 3B, no PKB $\beta$  was detected in fibroblasts at 50% confluence, but a low level of expression was observed when 3T3-L1 fibroblasts reached confluence. Differentiation into adipocytes, however, induced a much more significant up-regulation of PKB $\beta$  expression (Fig. 3B).

**PKB $\alpha$  is down-regulated in adipocytes.** The above data clearly demonstrates an induction of PKB $\beta$  expression during adipocyte differentiation. However, while PKB $\alpha$  appears to be highly expressed in fibroblasts, its expression in adipocytes is not clear due to antibody cross-reactivity. Thus, to more clearly determine PKB $\alpha$  expression, we performed immunoblotting on cell lysates that had been depleted of PKB $\beta$  with the specific sheep PKB $\beta$  antibody. In agreement with Fig. 2C, prior to PKB $\beta$  immunodepletion, the PKB $\alpha$  antibody detected bands with similar intensities in fibroblasts and adipocytes, while a substantial induction of PKB $\beta$  was observed in adipocytes (Fig.

4A). After immunodepletion with the sheep PKB $\beta$  antibody, almost all of the immunoreactive PKB $\beta$  was removed from the adipocyte lysate (Fig. 4A). When the same samples were immunoblotted with the PKB $\alpha$  antibody, there was little difference in the fibroblasts before and after PKB $\beta$  immunodepletion, suggesting that PKB $\alpha$  is expressed at high levels in fibroblasts. However, there was a substantial reduction in immunolabeling with this antibody in adipocytes after PKB $\beta$  depletion, suggesting that most of the PKB $\alpha$  immunoreactivity is due to PKB $\beta$  in these cells and that PKB $\beta$  is the predominant isoform expressed in 3T3-L1 adipocytes (Fig. 4A). To determine if this is a general feature of adipocytes, we performed similar experiments with primary cultures of adipocytes isolated from rat fat. Prior to PKB $\beta$  immunodepletion, both the PKB $\alpha$  and the PKB $\beta$  antibodies detected a band which showed an electrophoretic mobility shift with insulin treatment (Fig. 4B). However, after PKB $\beta$  depletion, no PKB $\alpha$  was detected in rat adipocyte lysates, suggesting that these cells do not express significant levels of PKB $\alpha$  (Fig. 4B).

One trivial explanation for these observations is that PKB $\alpha$  and PKB $\beta$  hetero-oligomerize and therefore all of the PKB $\alpha$  is removed during PKB $\beta$  immunodepletion. However, this seems unlikely because interaction between PKB molecules has been shown to be isoform specific, as the Akt homology domain of PKB $\alpha$  coimmunoprecipitates PKB $\alpha$  but not PKB $\beta$  (21). Nevertheless, we addressed this possibility using 3T3-L1 adipocytes overexpressing HA-PKB $\alpha$ . As shown in Fig. 4C, no signal corresponding to HA-PKB $\alpha$  was detected in the PKB $\beta$  immunoprecipitate, demonstrating that, under these conditions, PKB $\alpha$  does not coimmunoprecipitate with PKB $\beta$ .

**Insulin but not PDGF stimulates PKB $\beta$  phosphorylation in 3T3-L1 adipocytes.** The above data suggests that PKB $\beta$  is the most pertinent isoform for the insulin-specific PKB phosphorylation in 3T3-L1 adipocytes observed in Fig. 2A. To verify this conclusion, we next examined the phosphorylation of PKB isoforms following immunoprecipitation with isoform-specific antibodies. To examine the extent of PKB $\alpha$  phosphorylation, it was necessary to first deplete lysates of PKB $\beta$  prior to immunoprecipitation with the PKB $\alpha$  antibody. In these experiments, we also examined the effect of insulin and PDGF after 5 min of stimulation, in order to assess if PDGF has a transient effect on PKB activity. Insulin, but not PDGF, stimulated the phosphorylation of immunoprecipitated PKB $\alpha$  in 3T3-L1 adipocytes as detected by phospho-PKB antibodies (Fig. 5A). However, this signal most likely represents residual PKB $\beta$  that was immunoprecipitated by the PKB $\alpha$  antibody (Fig. 5A). A marked increase in PKB $\beta$  phosphorylation was observed in response to insulin, while PDGF had little effect on PKB $\beta$  phosphorylation at 5 or 15 min of stimulation (Fig. 5A). PKB $\beta$  phosphorylation was also directly examined by immunoprecipitation from 3T3-L1 adipocytes metabolically labeled with  $^{32}$ P (Fig. 5B). A low level of constitutive phosphorylation was observed under basal conditions, and insulin stimulation caused a threefold increase in PKB $\beta$  phosphorylation (Fig. 5B). Insulin-stimulated phosphorylation was accompanied by an electrophoretic mobility shift, and the shifted band was specifically detected by phospho-Ser473 PKB antibodies (Fig. 5B). In agreement with previous results (Fig. 2 and 5A), PDGF had no significant effect on PKB $\beta$  phosphorylation in 3T3-L1 adipocytes.

2-DE was used to further examine PKB $\beta$  phosphorylation in adipocytes. PKB $\beta$  was immunoprecipitated from  $^{32}$ P-labeled 3T3-L1 adipocytes and then subjected to 2-DE and autoradiography. Two spots of similar molecular masses but different pIs were detected in basal adipocytes (spots 1 and 2, Fig. 6A). The observed pI of these phosphospots was 5.6 to 5.8, which agrees with that calculated for PKB $\beta$  (5.9). The presence of the

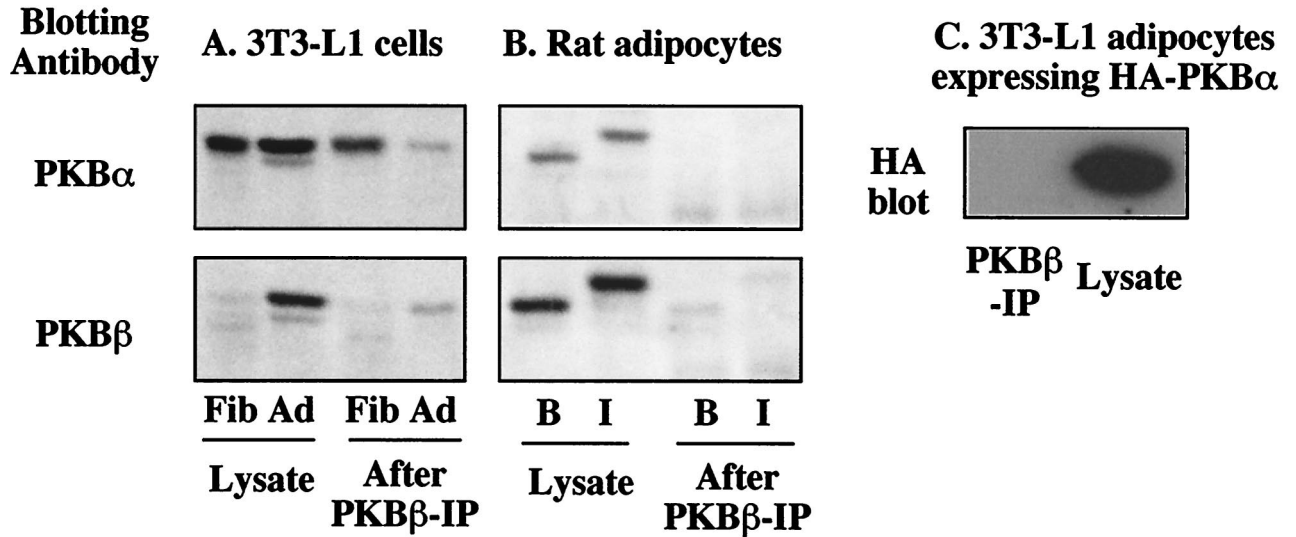


FIG. 4. PKB $\beta$  is the predominant isoform in adipocytes. (A) Cell lysates (100  $\mu$ g) from serum-starved 3T3-L1 fibroblasts (Fib) or 3T3-L1 adipocytes (Ad) were depleted of PKB $\beta$  by two consecutive rounds of immunoprecipitation with the sheep PKB $\beta$  antibody. Twenty micrograms of lysate (Lysate) and one-fifth of the immunoprecipitation supernatant (After PKB $\beta$ -IP) were analyzed by SDS-PAGE and immunoblotting with the sheep PKB $\alpha$  or the sheep PKB $\beta$  antibodies. (B) Cell lysates (100  $\mu$ g) from isolated rat adipocytes treated with 1  $\mu$ M insulin (I) for 15 min or left basal (B) were depleted of PKB $\beta$  and analyzed as described for panel A. (C) Immunoprecipitation with the sheep PKB $\beta$  antibody was performed on 100  $\mu$ g of cell lysates prepared from 3T3-L1 adipocytes overexpressing HA-PKB $\alpha$ . The immunoprecipitate (PKB $\beta$ -IP) and 10  $\mu$ g of lysate (Lysate) were analyzed by SDS-PAGE and immunoblotting for the presence of HA-PKB $\alpha$ , with a monoclonal HA antibody.

two phospho-PKB $\beta$  spots is consistent with two sites of constitutive phosphorylation, where spots 1 and 2 represent mono- and dual-phosphorylated forms, respectively. Insulin stimulation resulted in an increase in the intensity of spots 1 and 2, as well as the  $^{32}$ P labeling of an additional spot (spot 3) of more

acidic pI (Fig. 6B). To determine the relative abundance of differentially phosphorylated forms of PKB $\beta$ , we performed 2D immunoblotting. In addition to spots 1 and 2, a spot at pI 6 in 2D blots of basal adipocyte lysate which represents unphosphorylated PKB $\beta$  was detected (spot 0, Fig. 6C). A close

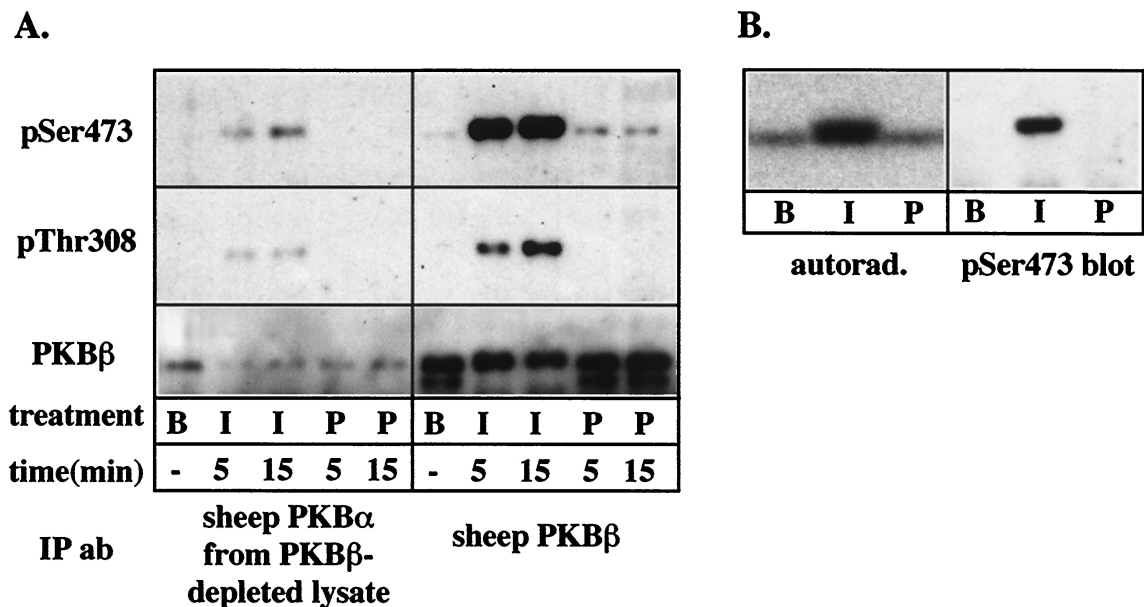


FIG. 5. Insulin but not PDGF stimulates phosphorylation of PKB $\beta$  in 3T3-L1 adipocytes. (A) PKB isoforms were immunoprecipitated from 100  $\mu$ g of 3T3-L1 lysates prepared from unstimulated cells (B) or cells treated with 1  $\mu$ M insulin (I) or 50 ng of PDGF per ml (P) for 5 or 15 min. In the case of the PKB $\alpha$  immunoprecipitation, PKB $\beta$  was first depleted from the cell lysate by two consecutive rounds of immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with phospho-Ser473 (pSer473) or phospho-Thr308 (pThr308) PKB antibodies or the sheep PKB $\beta$  antibody (PKB $\beta$ ). (B) PKB $\beta$  was immunoprecipitated from  $^{32}$ P-labeled 3T3-L1 adipocytes treated with 1  $\mu$ M insulin (I) or 50 ng of PDGF per ml (P) for 15 or left basal (B), by using the sheep PKB $\beta$  antibody, and analyzed by SDS-PAGE. The polyvinylidene difluoride membrane was subjected to autoradiography (autorad.) and then immunoblotted with the phospho-Ser473 antibody (pSer473).

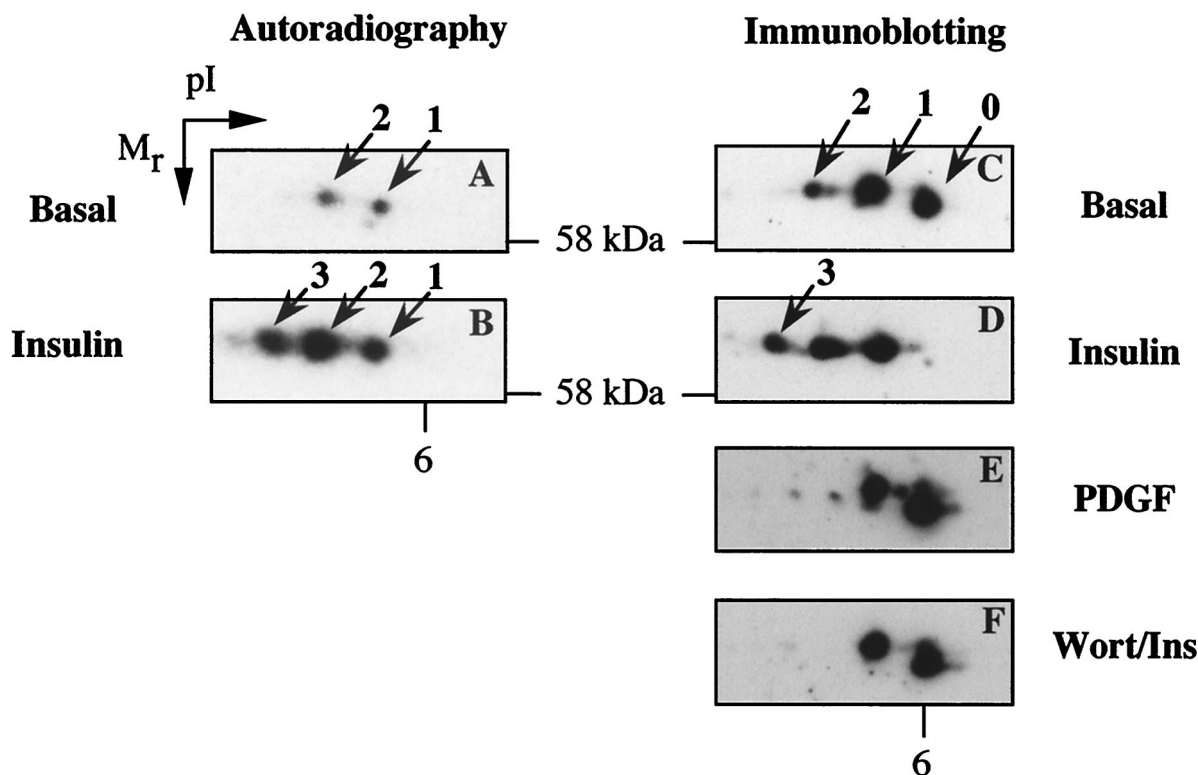


FIG. 6. Analysis of insulin-stimulated PKB $\beta$  phosphorylation in 3T3-L1 adipocytes by 2-DE. 3T3-L1 adipocytes were  $^{32}\text{P}$  labeled and then stimulated without (A) or with (B) 1  $\mu\text{M}$  insulin for 15 min. PKB $\beta$  was immunoprecipitated from cell lysates by using the rabbit anti-PKB $\beta$  antibody and analyzed by 2-DE and autoradiography. Lysates (150  $\mu\text{g}$ ) prepared from untreated 3T3-L1 adipocytes (C) or adipocytes treated with 1  $\mu\text{M}$  insulin for 15 min (D), 50 ng of PDGF per ml for 15 min (E), or 100 nM wortmannin for 40 min with 1  $\mu\text{M}$  insulin added for the last 15 min (F) were analyzed by 2-DE and immunoblotting with the rabbit anti-PKB $\beta$  antibody. Immunoblots of the polyvinylidene difluoride membranes from panels A and B yielded results similar to those for panels C and D.

examination of Fig. 6C shows that, under basal conditions, the majority of PKB $\beta$  is either unphosphorylated (spot 0) or monophosphorylated (spot 1), with only a minor proportion being dual phosphorylated (spot 2). Insulin stimulation caused the complete disappearance of spot 0 with a significant increase in the intensity of spot 2 and the appearance of spot 3 (Fig. 6D). The effect of PDGF stimulation on the 2D pattern of PKB $\beta$  spots was also examined. Consistent with previous results (Fig. 2 and 5), stimulation of 3T3-L1 adipocytes with 50 ng of PDGF per ml for 15 min did not result in the leftward shift of 2D PKB $\beta$  spots observed in response to insulin (compare Fig. 6C to E). In addition, pretreatment of cells with 100 nM wortmannin completely inhibited the insulin effect on the PKB $\beta$  2D pattern (Fig. 6F), consistent with the reported PI3K dependence of insulin-stimulated PKB activation.

**Insulin stimulates PKB $\beta$  translocation to the PM and HDM fractions.** Apart from phosphorylation, membrane translocation is thought to be required for activation of PKB. The current model of PKB activation suggests that PKB is recruited to the PM via its pleckstrin homology domain by the 3'-phosphoinositides generated by the receptor-activated PI3K, where it is phosphorylated at Ser473 and Thr308 by 3'-phosphoinositide-dependent kinase 1 (PDK1) and PDK2 (reviewed in references 2 and 40). We and others have previously reported that insulin and PDGF stimulate PI3K in adipocytes but that the subcellular distribution of the enzyme is different in each case (16, 41, 45). With PDGF, we observed a significant increase in PI3K activity in the PM fraction, whereas with insulin, it was enriched in a high-speed pellet (HSP) fraction associated with IRS-1 and/or -2 (16). Thus, we next studied the distribu-

tion of PKB $\beta$  among these fractions. Under basal conditions, almost all the immunoreactive PKB $\beta$  was found in the cytosol (Fig. 7). In agreement with previous results, basal PKB $\beta$  in the cytosol fraction was resolved into two bands, corresponding to bands 0 and 1 in Fig. 2A. A band detected in the HSP fraction in the basal state exhibited apparent electrophoretic mobility similar to that of band 0; however, in contrast to bands in the cytosol, insulin did not induce an electrophoretic mobility shift of the HSP band (Fig. 7). Insulin induced the translocation of PKB $\beta$  to the PM and high-density microsome (HDM) fractions, while PDGF was without effect. This translocation does not represent the association of PKB $\beta$  with large protein complexes because we find that it floats up through sucrose upon sedimentation, consistent with a membrane-associated protein (data not shown). Insulin-stimulated translocation of PKB $\beta$  was restricted to band 2, the activated form of PKB $\beta$ , as confirmed by immunoreactivity to the phospho-Ser473 PKB antibody (Fig. 7). Interestingly, PKB $\beta$  in the cytosol fraction underwent an almost quantitative shift from band 0 to band 2 within 5 min of insulin stimulation, coincident with increased Ser474 phosphorylation (Fig. 7). This suggests that either PKB $\beta$  was activated in the cytosol or it is activated at the PM and rapidly translocates from this site to the cytosol.

Recent studies reporting the insulin-induced association of PKB $\beta$  with GLUT4-containing vesicles (12) and phosphorylation of its components (33) suggest that PKB $\beta$  may directly mediate GLUT4 translocation. In contrast to the report by Calera et al. (12) for rat adipocytes, we did not detect PKB $\beta$  in membrane fractions in the basal state, nor did we observe significant insulin-induced translocation of PKB $\beta$  to the HSP



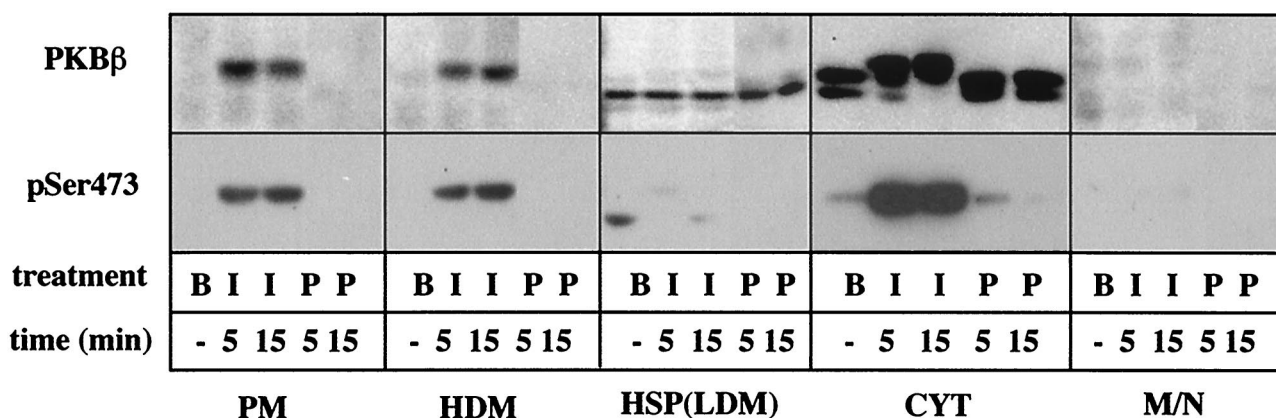


FIG. 7. Insulin, but not PDGF, stimulates the translocation of PKB $\beta$  to membrane fractions in 3T3-L1 adipocytes. 3T3-L1 adipocytes were stimulated with insulin (I) or PDGF (P) for 5 or 15 min or left basal (B). Cells were homogenized and subfractionated by differential centrifugation as described in Materials and Methods, to generate the PM, HDM, HSP (also termed LDM), cytosol (CYT), and the mitochondrial-nuclear (M/N) fractions. Twenty micrograms of each fraction was analyzed by SDS-PAGE and immunoblotting with the sheep PKB $\beta$  (PKB $\beta$ ) or the phospho-Ser473 PKB (pSer473) antibody.

fraction (also termed the low-density microsome [LDM] fraction) in 3T3-L1 adipocytes (Fig. 7). To further assess if PKB $\beta$  directly interacts with GLUT4 vesicles, we examined the ability of insulin or PDGF to stimulate GLUT4 translocation in 3T3-L1 fibroblasts overexpressing both GLUT4 and PKB $\beta$ . Activation of PKB $\beta$  by either insulin or PDGF did not alter the subcellular location of GLUT4 in these cells (data not shown), suggesting that activation of PKB $\beta$  is not sufficient to stimulate GLUT4 translocation in this system.

## DISCUSSION

In the present study, we have provided further evidence to support a role for the serine/threonine kinase PKB in insulin-stimulated glucose transport in adipocytes. We show that a PKB substrate peptide or an antibody to PKB inhibited insulin-stimulated GLUT4 translocation by 66 and 56%, respectively. Moreover, we have obtained evidence to implicate the PKB $\beta$  isoform in insulin action: (i) the expression of PKB $\beta$  is substantially increased during adipocyte differentiation whereas the expression of PKB $\alpha$  is markedly reduced during this process, (ii) primary cultures of rat adipocytes have no detectable immunoreactive PKB $\alpha$ , and (iii) insulin but not PDGF stimulates PKB $\beta$  phosphorylation and translocation to membranes in 3T3-L1 adipocytes.

Previous studies have reported contradictory findings concerning the role of PKB in insulin-stimulated glucose transport (18, 23, 29, 30, 32, 54, 57, 59). We believe the present study provides strong support for the involvement of PKB in insulin action and clarifies some potential areas of contradiction that have arisen. First, as discussed in more detail below, our study implicates a role only for PKB $\beta$  and not for PKB $\alpha$  in insulin regulation of glucose transport in adipocytes. This may be critical, as many previous studies have used reagents that either specifically target PKB $\alpha$  or, as a minimum, have questionable specificity for different PKB isoforms. Second, we have employed a strategy involving microinjection of either antibodies or substrate peptides and then examination of the consequences of these reagents for GLUT4 trafficking 60 to 90 min later. This likely overcomes potential cellular adaptive mechanisms which could occur in response to expression of cDNAs over a much longer time course.

**Role of PKB $\alpha$  versus PKB $\beta$ .** Initial comparison of the relative expression of PKB $\alpha$  and PKB $\beta$  in 3T3-L1 fibroblasts with

that in adipocytes by immunoblotting suggested that, while PKB $\beta$  was up-regulated, PKB $\alpha$  expression was not significantly altered by adipocyte differentiation (Fig. 2C). However, as the PKB $\alpha$  antibody was found to cross-react with PKB $\beta$  (Fig. 2B), part of the PKB $\alpha$  immunoreactivity in 3T3-L1 adipocytes likely represented PKB $\beta$ , which is highly expressed in these cells. We tested this hypothesis by depleting PKB $\beta$  from cell lysates prior to immunoblotting with the PKB $\alpha$  antibody and found that, indeed, most of the PKB $\alpha$  immunoreactivity in adipocytes was removed by PKB $\beta$  depletion (Fig. 4A). In contrast, substantial PKB $\alpha$  immunoreactivity remained after PKB $\beta$  depletion of fibroblast lysates (Fig. 4A). Consistent with our findings in 3T3-L1 adipocytes, we also find that there is very little PKB $\alpha$  in rat adipocytes, with PKB $\beta$  being the major isoform (Fig. 4B). Based on the present studies, we predict that previous reports of insulin-activated PKB $\alpha$  activity in rat adipocytes may be due to antibody cross-reactivity.

These results suggest that there is a switch in isoform expression during differentiation of fibroblasts into adipocytes. As PKB $\alpha$  and PKB $\beta$  are 81% identical in amino acid sequence, the specific change in isoform expression upon adipocyte differentiation is likely to be functional. Fibroblasts represent a highly proliferative cell type, and so it seems likely that PKB $\alpha$  is important in the regulation of cell growth and proliferation, and its down-regulation upon differentiation is appropriate as adipocytes are terminally differentiated and not proliferative. On the other hand, the induction of PKB $\beta$  expression during adipocyte differentiation parallels that observed for GLUT4 (Fig. 3A), implying a role for PKB $\beta$  in metabolic regulation in adipocytes. Further support comes from our observation that rat adipocytes express high levels of PKB $\beta$  (Fig. 4B) and from the reported high PKB $\beta$  mRNA expression in brown adipose tissue (3). An induction of PKB $\beta$  mRNA was also observed upon differentiation of Sol8 muscle cells (13) and C2C12 myotubes (3), supporting a role for PKB $\beta$  in insulin regulation of metabolism in muscle cells in addition to adipocytes. In our study, no PKB $\beta$  expression was observed in subconfluent, highly proliferative fibroblasts. A slight increase in PKB $\beta$  expression was observed when cells became confluent in culture, followed by a more dramatic induction when fibroblasts differentiated into adipocytes (Fig. 3). Taken together, these observations suggest that the expression of PKB $\beta$  is normally restricted to terminally differentiated, nonproliferative cells, where PKB $\beta$  plays a role in metabolic regulation. The inap-



appropriate expression of PKB $\beta$  in undifferentiated cells can lead to uncontrolled cell proliferation as demonstrated by the transforming ability of PKB $\beta$  when overexpressed in NIH 3T3 cells (15). Overexpression of constitutively active PKB $\alpha$  in 3T3-L1 fibroblasts leads to spontaneous differentiation into adipocytes (36). To further address the roles of different PKB isoforms, it may be of interest to compare the effects of overexpressing constitutively active PKB $\beta$  or PKB $\gamma$  to that observed for PKB $\alpha$  in 3T3-L1 fibroblasts.

**Activation of PKB $\beta$  is insulin specific in 3T3-L1 adipocytes.** Several recent studies have compared the signaling of insulin with that of PDGF in adipocytes, based on the hypothesis that the inability of PDGF to induce glucose uptake reflects its inability to activate a relevant downstream signaling pathway(s). Indeed, several groups have reported differences between the subcellular location of PI3K activated by insulin and that of PI3K activated by PDGF (16, 41, 45). Furthermore, PDGF was found to have little effect on the levels of phosphatidylinositol 3,4,5-trisphosphate (PIP $_3$ ) in 3T3-L1 adipocytes, whereas insulin induced a significant increase (19). These results suggest that, while PI3K is activated by recruitment to activated PDGF receptors at the PM in 3T3-L1 adipocytes, it may not have access to appropriate substrates and thus is unable to produce a rise in PIP $_3$ . In agreement with the lack of PDGF-induced PIP $_3$  production, we found no effect of PDGF on PKB $\beta$  phosphorylation in 3T3-L1 adipocytes by electrophoretic mobility shift, immunoprecipitation in conjunction with  $^{32}$ P labeling or immunoblotting with phosphospecific antibodies, or isoelectric point shifts analyzed by 2-DE. Furthermore, PDGF did not stimulate membrane translocation of PKB $\beta$ , as observed with insulin, nor did it have a transient effect on PKB $\beta$  phosphorylation (Fig. 5A). The insulin specificity further supports a role for PKB $\beta$  in the regulation of glucose metabolism in adipocytes.

Three previous studies have reported variable effects of PDGF on PKB in 3T3-L1 adipocytes (10, 51, 54). Two studies utilized phospho-Ser473 PKB antibodies and found a small PDGF-induced PKB phosphorylation (~20% of insulin effect). We were unable to detect PDGF-stimulated PKB phosphorylation in 3T3-L1 adipocytes treated with 50 ng of PDGF per ml with the same antibody in immunoblotting of cell lysates (Fig. 2A), but very weak PDGF-induced PKB $\beta$  phosphorylation was sometimes observed in immunoprecipitates (Fig. 5A). Tanti et al. observed a significant PDGF-stimulated activation of PKB (to 50% of the insulin effect) in 3T3-L1 adipocytes by immunoprecipitation-coupled kinase assays (54). The reason for the discrepancy between these studies is not clear. One possible explanation is differences in the integrity of 3T3-L1 adipocyte cultures. As PKB $\alpha$  is the predominant isoform in 3T3-L1 fibroblasts and is strongly activated by PDGF in these cells, an increased level of fibroblast contamination of adipocyte cultures may give rise to a PDGF response on PKB. This may be further amplified in the case of immunoprecipitation with an antibody with a high affinity for PKB $\alpha$ .

**Regulation of PKB $\beta$  activation in 3T3-L1 adipocytes.** A synthesis of our data concerning activation of PKB $\beta$  in 3T3-L1 adipocytes suggests that both unphosphorylated (band 0 and spots 0) and phosphorylated (band 1 and spots 1 and 2) PKB $\beta$  are present in basal adipocytes. Furthermore, separation of phospho-PKB into two spots with different pI suggests that there are two populations of differentially phosphorylated forms of PKB $\beta$  in basal 3T3-L1 adipocytes. Constitutive phosphorylation of PKB $\beta$  in 3T3-L1 adipocytes does not occur on either Ser474 or Thr309, because no significant signal was detected with phospho-PKB antibodies in basal adipocytes (Fig. 2A and 5A). Two constitutive phosphorylation sites have

been reported for PKB $\beta$  transfected into HEK-293 cells, and these are Ser125 and Thr451 (39). Thus one interpretation of our results is that ~50% of PKB $\beta$  is constitutively phosphorylated, possibly on either Ser125 or Thr451 in the basal state in 3T3-L1 adipocytes (spot 1), while a small amount is dual phosphorylated on both residues, resulting in a more acidic pI (spot 2). Insulin caused the total disappearance of nonphosphorylated PKB $\beta$  (spot 0), with an increase in the immunoreactivity of spots 2 and 3, concomitant with insulin-induced phosphorylation (Fig. 6A to D). A recent study suggests that constitutive phosphorylation at Thr450 primers PKB $\alpha$  for subsequent growth factor stimulation when expressed in NIH 3T3 cells (9). Thus, one possibility is that insulin stimulates phosphorylation of basally unphosphorylated PKB $\beta$  (spot 0) on one or both of the constitutive sites (Ser125 and Thr451) but that only basally phosphorylated PKB $\beta$  (spots 1 and 2) becomes phosphorylated on activating sites (Ser474 and Thr309). As the present data does not provide information on the sites of phosphorylation of the differentially charged PKB $\beta$  isoforms, further investigation is required to confirm this hypothesis in 3T3-L1 adipocytes. Interestingly, only a single band which exhibits an electrophoretic mobility that is intermediate between bands 0 and 1 was observed in rat adipocytes. Upon insulin stimulation, the entire band shifts to a position corresponding to band 2. The phosphorylation state of the basal band in rat adipocytes is yet to be determined and should provide valuable data on the role of constitutive phosphorylation.

Current models of growth factor-stimulated PKB activation suggest that the products of PI3K recruit both PDK1 and PKB to the PM, where PDK1 phosphorylates and activates PKB (2, 4, 40). The requirement for membrane translocation in PKB activation is supported by the finding that forced translocation to the PM by myristoylation-palmitoylation is sufficient to induce PKB phosphorylation at Thr308 and Ser473 (5). In the present study, we observed specific translocation of phosphorylated PKB $\beta$  to the PM and HDM fractions in response to insulin treatment of 3T3-L1 adipocytes. In agreement with the inability of PDGF to induce PKB $\beta$  phosphorylation, no membrane translocation was observed in response to PDGF stimulation. Interestingly, while some PKB $\beta$  remained associated with the PM and the HDM, a major portion of activated PKB $\beta$  was observed in the cytosol within 5 min of insulin stimulation (Fig. 7). These results suggest that either PKB $\beta$  can be activated in the cytosol or, more likely, following activation at the membrane, most PKB $\beta$  returns to the cytosol where it is able to phosphorylate its physiological substrates. Recent reports of insulin-induced association of PKB $\beta$  with GLUT4-containing vesicles (12) and phosphorylation of its components (33) suggest that PKB $\beta$  may directly regulate the GLUT4 translocation machinery. We did not observe any significant recruitment of PKB $\beta$  to the HSP-LDM fraction which contains the insulin-responsive pool of GLUT4 in 3T3-L1 adipocytes. However, it is possible that our assay is not sensitive enough to detect a low level of PKB $\beta$  recruitment to this fraction. Thus, we further tested the hypothesis that PKB $\beta$  directly mediates GLUT4 translocation by examining the effect of activating PKB $\beta$  by insulin or PDGF in 3T3-L1 fibroblasts expressing both PKB $\beta$  and GLUT4. Neither insulin nor PDGF stimulated GLUT4 translocation in these cells, suggesting that either another signaling pathway is required or fibroblasts lack the expression of downstream signaling molecules or machinery required for GLUT4 translocation.

In summary, our data implicates an important role for PKB in insulin action in adipocytes. We cannot exclude a role for alternate parallel pathways such as PKC $\lambda$  and/or PKC $\zeta$ . Using microinjection of either a PKB substrate peptide or a PKB

antibody, we observed a ~60% inhibition of insulin-stimulated GLUT4 translocation to the PM. Hence, it is conceivable that the residual insulin action is due to a PKB-independent pathway. Further effort is required to identify this alternate pathway and to identify downstream targets of PKB $\beta$  in adipocytes.

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The first two authors contributed equally to this paper.

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