Activation of Protein Kinase B and Induction of Adipogenesis by Insulin in 3T3-L1 Preadipocytes

Contribution of Phosphoinositide-3,4,5-Trisphosphate Versus Phosphoinositide-3,4-Bisphosphate

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Ectopic expression of activated protein kinase B (PKB) induces the differentiation of confluent 3T3-L1 preadipocytes into adipocytes. PKB is regulated by the lipid products of phosphoinositide 3-kinase (PI 3-kinase), phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2], and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. However, the relative contribution of each 3-phosphorylated phosphoinositide species in activating PKB remains unclear. Treatment of intact 3T3-L1 preadipocytes with synthetic 3-phosphorylated phosphoinositides revealed that only PI(3,4)P2 stimulated PKB activity. PKB was also activated by insulin, in a dose- and time-dependent manner. This activation was associated with an isolated rise in PI(3,4,5)P3, without any detectable change in PI(3,4)P2, demonstrating that this lipid was sufficient to activate PKB. Wortmannin and LY294002, inhibitors of PI 3-kinase, reduced insulin-dependent activation of PKB, whereas rapamycin, an inhibitor of p70 S6 kinase, had no effect. Platelet-derived growth factor (PDGF), which is not adipogenic, stimulated the production of both 3-phosphorylated phosphoinositide species, and this was associated with a greater activation of PKB than that observed with insulin. A low dose of PDGF (1 ng/ml), which increased the production of only PI(3,4,5)P3 and mirrored the insulin effect, was unable to induce adipocyte differentiation. In summary, insulin and PDGF differ with respect to the accumulation of 3-phosphorylated phosphoinositides and to PKB activation in 3T3-L1 preadipocytes, but these responses do not themselves explain why insulin, but not PDGF, is adipogenic. *Diabetes* 48:691–698, 1999

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BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; GPDH, glycerophosphate dehydrogenase; IRS, insulin receptor substrate; KRH, Krebs-Ringer-HEPES buffer; PDGF, platelet-derived growth factor, PDK, 3-phosphoinositide-dependent kinase; PI, phosphoinositide; PI(3,4)P2, phosphatidylinositol-3,4-bisphosphate; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PKB, protein kinase B.

Differentiation of 3T3-L1 preadipocytes into adipocytes requires insulin or insulin-like growth factor (IGF)-1 (1). IGF-1 was thought to be a more potent inducer of adipogenesis because pharmacological doses of insulin appeared to be required for this process. However, more recent reports implicate insulin as a physiologically relevant regulator of adipogenesis. Using a defined serum-free culture protocol, nanomolar concentrations of insulin, within the physiological range, were shown to induce adipocyte differentiation (2,3). Furthermore, gene inactivation through homologous recombination and chimeric receptor studies also argue for a role of the insulin receptor in the regulation of adipogenesis (4,5).

The phosphoinositide (PI) 3-kinase/protein kinase B (PKB)/p70 S6 kinase pathway has been implicated in various insulin-dependent cellular processes, including cell survival, glucose transport, and adipocyte differentiation (6–9). Both wortmannin, an inhibitor of PI 3-kinase (10), and rapamycin, which inhibits p70 S6 kinase (11), block insulin-induced 3T3-L1 adipocyte differentiation (8,12,13). Activation of PKB appears to be sufficient to induce adipogenesis because ectopic expression of constitutively active forms of PKB triggers the differentiation of confluent 3T3-L1 preadipocytes (8,9). Insulin activation of PKB has been described in various insulin-sensitive primary cultures and cell lines (14). However, despite the importance of PKB in adipocyte differentiation, the regulation of endogenous PKB by insulin in 3T3-L1 preadipocytes has yet to be characterized.

We have previously investigated the insulin-dependent response of 3-phosphorylated phosphoinositides in 3T3-L1 preadipocytes (15,16). Insulin increases only the phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] species, with no discernible effect on the levels of phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2]. This selective PI(3,4,5)P3 response appears specific to 3T3-L1 preadipocytes because differentiated adipocytes respond to insulin with increases in both PI(3,4)P2 and PI(3,4,5)P3 production (17,18). Stimulation of 3T3-L1 preadipocytes with other growth factors, such as platelet-derived growth factor (PDGF), increases both PI(3,4)P2 and PI(3,4,5)P3 (15).

Both PI(3,4)P2 and PI(3,4,5)P3 can specifically bind the pleckstrin homology (PH) domain of PKB with high affinity

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(19). Binding of 3-phosphorylated phosphoinositides translocates the kinase to the membrane, and causes a conformational change in PKB, exposing important regulatory serine and threonine residues, which can be phosphorylated by upstream kinases (20). Four of these upstream regulatory kinases, referred to as 3-phosphoinositide–dependent kinases (PDKs), have been cloned recently (21). PI(3,4)P2 and PI(3,4,5)P3 also bind to the PH domain of PDKs, and this appears to regulate their localization and/or activity. Full activation of PKB occurs when both Thr^{308} and Ser^{473} are phosphorylated by one or more PDKs (20). Although both PI(3,4)P2 and PI(3,4,5)P3 bind to PKB and PDKs in vitro, it remains unclear which 3-phosphorylated phosphoinositide actually leads to PKB activation in the cell. Reports have suggested that only PI(3,4)P2 activates PKB and that PI(3,4,5)P3 has no effect or even inhibits PKB activity (19,22–24). PI(3,4,5)P3-dependent activation of PKB, in total cell lysates, has been described by only one group (25).

In an attempt to delineate the contribution of each phosphoinositide to the activation of PKB, we measured PKB activity in 3T3-L1 preadipocytes exposed to exogenously added synthetic PI(3,4)P2 and PI(3,4,5)P3. The PKB activity and the adipogenic response of insulin- and PDGF-stimulated 3T3-L1 cells was also compared because their 3-phosphorylated phosphoinositide pattern differs.

RESEARCH DESIGN AND METHODS

Cell culture. 3T3-L1 preadipocytes, maintained at low passage, were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Confluent 3T3-L1 preadipocytes were placed in DMEM supplemented with 2% calf serum overnight, before stimulation. Cells were washed in Krebs-Ringer-HEPES (KRH) buffer and stimulated for the indicated times with 0–10 μmol/l insulin, 0–50 ng/ml PDGF, or 0–25 μmol/l phosphoinositide preparations. The following lipids were used: dipalmitoyl-PI(3,4)P2, dipalmitoyl-PI(3,4,5)P3 (26), or PI(4,5)P2 purified from bovine brain (Sigma, St. Louis, MO). Lipids were kept in chloroform:methanol:water (1:1:0.3), dried down under nitrogen, resuspended in Tris-buffered saline (10 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.1% β-mercaptoethanol, 0.001% sodium azide, and 0.1% bovine serum albumin [BSA]) at a concentration of 125 μmol/l and dispersed by sonication for 30 min before addition. Stimulation was stopped by rapidly aspirating the KRH and lysing the cells in the appropriate buffer. In some cases, as indicated, cells were incubated for 15 min with 100 nmol/l wortmannin, 10 μmol/l LY294002, or 100 nmol/l rapamycin before stimulation with insulin. For time course and inhibitors studies caused by the longer stimulation periods, cells were kept at 37°C in a 10% CO_2 incubator. Dose-response studies, in which all cells were stimulated for 5 min, were done on the benchtop (28–30°C).

Immunoprecipitation of PKB and kinase assays. Stimulated cells were lysed in phosphate-buffered saline (pH 7.4), 1% NP-40, 200 μmol/l sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 50 mmol/l NaF, 1 mmol/l β-glycerophosphate, 5 mmol/l sodium pyrophosphate, and 1 μmol/l microcystin and were centrifuged at 15,000*g* for 10 min at 4°C. Supernatants were precleared with 40 μl of protein G-agarose slurry (10% wt/vol) for 1 h at 4°C. Cleared cell extracts were incubated for 90 min with 5 μg of goat anti-PKB antibody (C-20; Santa Cruz Biotech, Santa Cruz, CA) preadsorbed to protein G-agarose. Immunoprecipitates were washed twice in lysis buffer and three times in assay dilution buffer (20 mmol/l 3-[N-morpholino] propanesulfonic acid [pH 7], 25 mmol/l β-glycerophosphate, 5 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 1 mmol/l dithiothreitol), and the final agarose pellets were resuspended in assay dilution buffer containing 40 μmol/l crosstide, an established substrate for PKB based on the consensus phosphorylation site of glycogen synthase kinase-3 (27). Reactions were started by the addition of 10 μCi [^{32}P]ATP diluted with assay dilution buffer containing 0.5 mmol/l cold ATP and 75 mmol/l $MgCl_2$, and samples were incubated for 15 min at 30°C. Aliquots of supernatant were spotted onto P81 filters, mixed with scintillation cocktail, and counted using a Beckman LS3801 scintillation counter.

Radioisotope labeling and inositol phospholipid analysis. Confluent 3T3-L1 preadipocytes were starved overnight in DMEM supplemented with 2% calf serum, and labeled with either 625 μCi/ml [^{32}P]orthophosphate or 100 μCi/ml [^{3}H]myoinositol, as previously described (15,16,28). Cells were then stimulated as indicated above, lipids were extracted and de-acylated directly, as described (15). The resulting glycerolphosphoinositols were separated on a Whatman Partisphere 5 SAX column, (Whatman, Fairfield, NJ). The peaks corresponding to the [^{32}P]glycerolphosphoinositol phosphates were measured with a Beckman model 171 on-line radioisotope flow detector. The de-acylated products of [^{3}H]PI(3,4)P2 and [^{3}H]PI(3,4,5)P3 were identified by measuring radioactivity contained in 1-ml fractions with a Beckman LS3801 scintillation counter, as previously described (16).

Adipocyte differentiation. For differentiation experiments, confluent 3T3-L1 preadipocytes were induced to differentiate, as described (3,16), in DMEM supplemented with 10% calf serum (final concentration of PDGF in the medium is <0.1 ng/ml), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and containing either 1 μmol/l insulin or PDGF (1 and 20 ng/ml). For the first 48 h, 0.5 mmol/l isobutylmethylxanthine and 0.25 μmol/l dexamethasone were present to accelerate the differentiation process (29). After 8 days, cell morphology was assessed by phase contrast microscopy, followed by measurement of triacylglycerol accumulation and glycerophosphate dehydrogenase (GPDH) activity, a specific marker of terminal adipocyte differentiation. Cellular triacylglycerol was extracted with isopropanol:heptane (2:3) and spectrometrically measured as previously described (3,30). Cellular proteins were solubilized in 0.1 N NaOH and assayed using the Biorad assay, with BSA as standard. GPDH activity was determined in cytosolic fractions, as described (3,16,31).

RESULTS

Effect of 3-phosphorylated phosphoinositides on PKB activity in 3T3-L1 cells. The lipids produced by PI 3-kinase have been implicated as second messengers leading to the activation of PKB (32). However, it is not clear which 3-phosphorylated phosphoinositide actually transduces the signals leading to PKB activation in vivo. To examine further the relative contribution of each 3-phosphorylated phosphoinositide to the activation of PKB in 3T3-L1 preadipocytes, we stimulated cell monolayers with synthetic PI(3,4)P2ipoc and PI(3,4,5)P3. PKB activity was induced 1.43 ± 0.24 fold (mean ± range) in response to a 15-min treatment with 10 μmol/l PI(3,4)P2 and further activated (4.41 ± 0.62 fold; mean ± range) with higher concentration (25 μmol/l) of

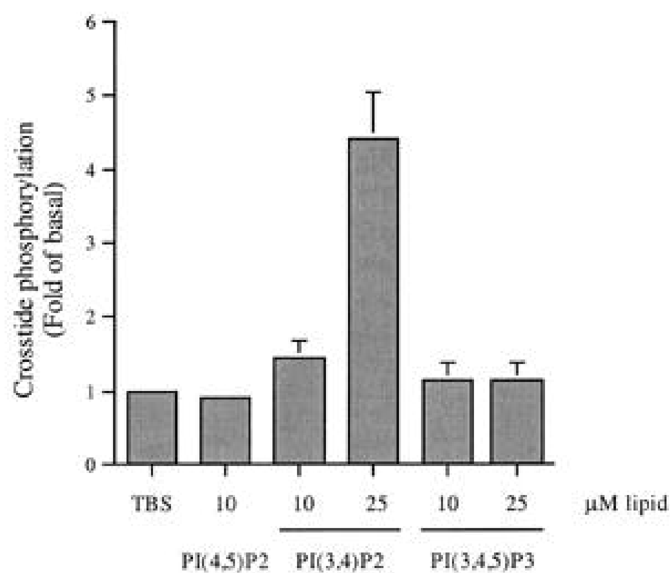

FIG. 1. PKB activation by treatment of intact 3T3-L1 preadipocytes with phosphoinositides. Confluent 3T3-L1 preadipocytes were starved overnight, then incubated with 10–25 μmol/l PI(4,5)P2, PI(3,4)P2, PI(3,4,5)P3, or TBS vehicle for 15 min at 37°C. Cells were lysed and PKB was immunoprecipitated as described in METHODS. PKB activity was measured as the phosphorylation of crosstide. Data are derived from three separate experiments, each done in duplicate, and are expressed as fold induction above basal (mean ± range).

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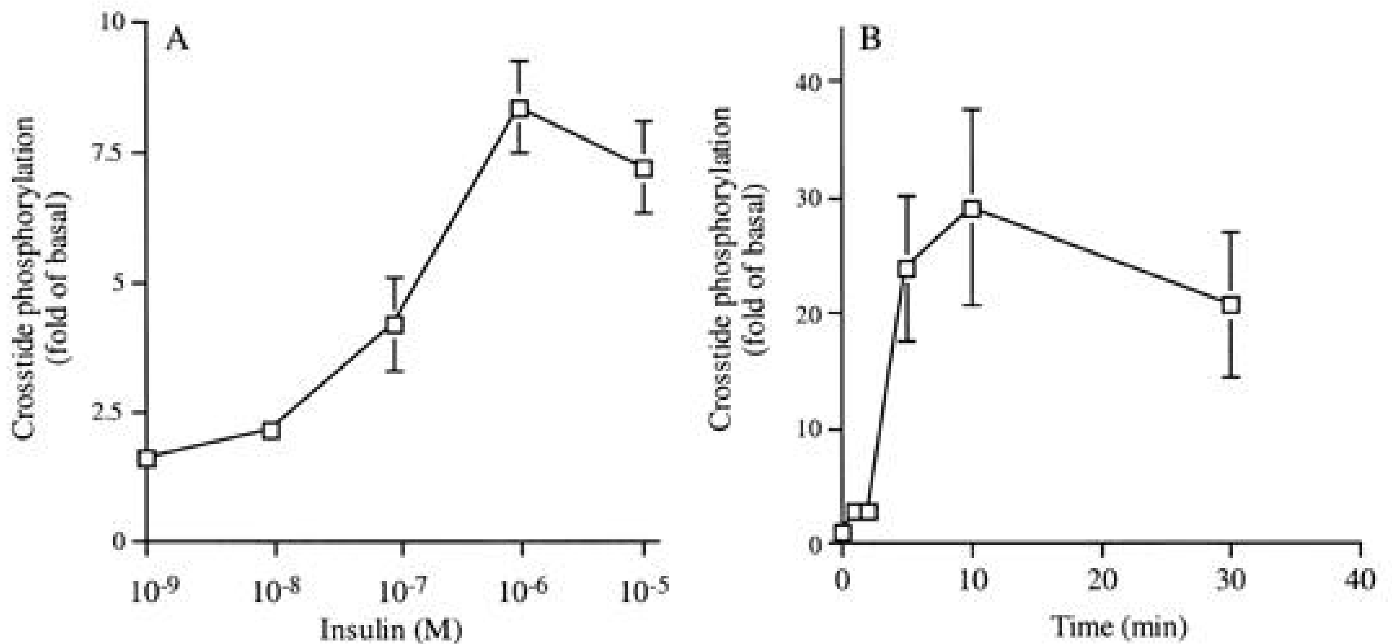

FIG. 2. Characterization of insulin-induced PKB activity in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were starved overnight, then stimulated with vehicle or insulin. Cells were lysed and PKB was immunoprecipitated as described in METHODS. PKB activity was measured as the phosphorylation of crosstide. Results are expressed as fold of basal. *A*: Stimulation for 5 min with 0–10 µmol/l insulin, on the benchtop (28–30°C). Data are derived from three separate experiments, each done in duplicate (mean ± SE). *B*: Stimulation with 1 µmol/l insulin for 0–30 min, at 37°C in a 10% CO_2 incubator. Data are derived from three separate experiments, each done in duplicate (mean ± SE) and expressed as fold of basal. Data are derived from two separate experiments, each done in duplicate (mean ± range).

the lipid (Fig. 1). However, under the same conditions, PI(3,4,5)P3, either at 10 or 25 µmol/l, had no significant effect on PKB activity. Stimulation with PI(4,5)P2, a phosphoinositide not involved in the regulation of PKB, had no effect on PKB activity.

Regulation of PKB by growth factors in 3T3-L1 preadipocytes. The PI 3-kinase/PKB pathway has been implicated in the process of 3T3-L1 adipocyte differentiation induced by insulin (6–9). Because only PI(3,4,5)P3 is induced upon insulin stimulation in 3T3-L1 preadipocytes (15,16),

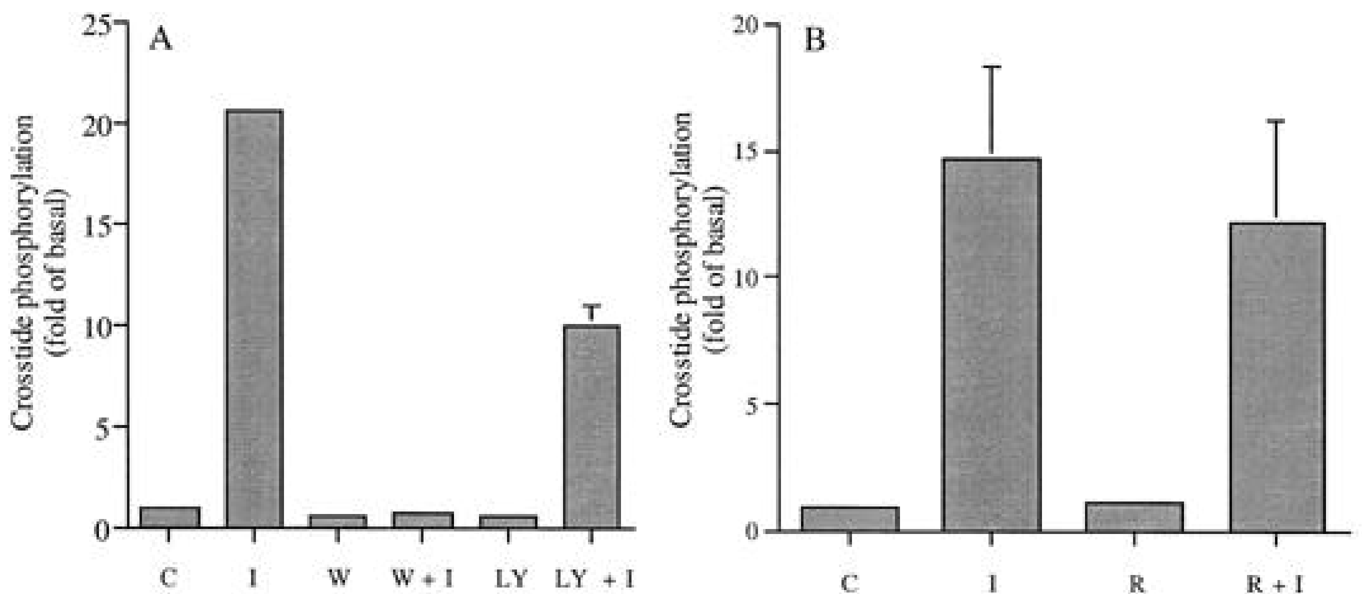

FIG. 3. Effects of wortmannin, LY294002, and rapamycin on the insulin activation of PKB in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes, starved overnight, were pre-incubated for 15 min with 100 nmol/l wortmannin (W), 10 µmol/l LY294002 (LY) (*A*) or 100 nmol/l rapamycin (R), before stimulation for 5 min with 1 µmol/l insulin (*B*). Cells were lysed and PKB was immunoprecipitated as described in METHODS. PKB activity was measured as the phosphorylation of crosstide. Data are derived from three separate experiments, each done in duplicate (mean ± SE), and expressed as fold of basal.

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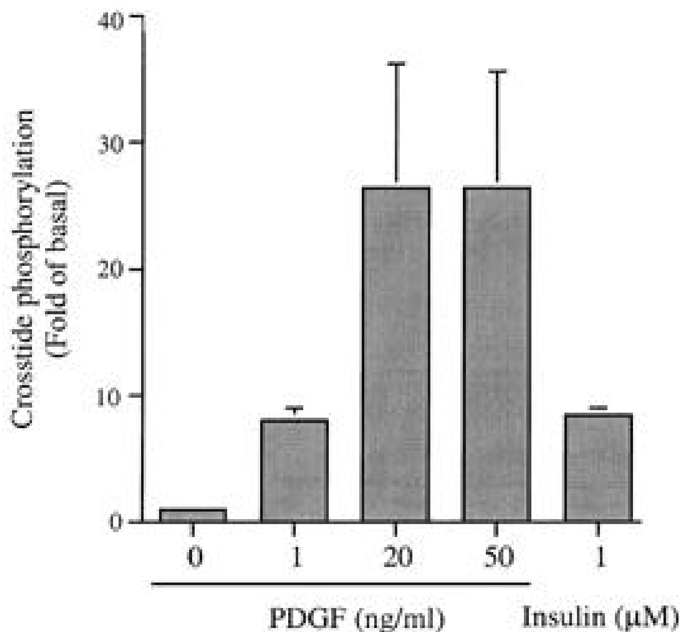

FIG. 4. Dose response of PKB activation by PDGF in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were starved overnight, before stimulation for 5 min with 0–50 ng/ml PDGF, as indicated. Cells were lysed and PKB was immunoprecipitated as described in METHODS. PKB activity was measured as the phosphorylation of crosstide. Stimulations were performed on the benchtop (28–30°C). Data are derived from two separate experiments, each done in duplicate (mean ± range) and expressed as fold of basal.

this system provides an opportunity to determine if isolated increases in PI(3,4,5)P3, generated intracellularly by insulin stimulation, are sufficient to activate PKB.

Confluent 3T3-L1 preadipocytes were stimulated for 5 min with the indicated doses of insulin. PKB was immunoprecipitated, and its activity measured as the phosphorylation of crosstide. PKB was activated 1.6 ± 0.3 fold by doses as low as 1 nmol/l insulin, and achieved maximal stimulation with 1 μmol/l insulin (8.3 ± 2.9 fold; Fig. 2*A*). We next characterized the time course of PKB activation by a maximal dose of insulin (1 μmol/l) in 3T3-L1 preadipocytes. Because of the longer incubation times, cells were maintained in a 10% CO_2 incubator at 37°C throughout the stimulation. These incubation conditions enhanced the magnitude of PKB stimulation by insulin. PKB activity began to rise within 1 min of insulin treatment (2.6 ± 0.7 fold), reached maximal levels between 5 and 15 min, and remained elevated for up to 30 min (Fig. 2*B*).

To verify that the activation of PKB by insulin in 3T3-L1 preadipocytes is PI 3-kinase–dependent, we investigated the effects of wortmannin and LY294002, two structurally unrelated inhibitors of PI 3-kinase (10), on insulin-induced PKB activity. Both wortmannin and LY294002 inhibited PKB activity induced by insulin, without significantly altering basal PKB activity (Fig. 3*A*). Insulin-induced PKB activity was completely inhibited by 100 nmol/l wortmannin and partially (42 ± 4.2%; mean ± SE) by 10 μmol/l LY294002, consistent with PI 3-kinase acting upstream of PKB. Rapamycin, which inhibits p70 S6 kinase downstream of PKB (33), had no significant effect on insulin-stimulated PKB activity in 3T3-L1 preadipocytes (Fig. 3*B*). These results demonstrate that insulin stimulates PKB activity in 3T3-L1 preadipocytes in a PI 3-kinase–dependent manner. Because insulin only induces a PI(3,4,5)P3 response in 3T3-L1 preadipocytes (15,16), our results indicate a PI(3,4)P2–independent activation of PKB by insulin.

PDGF treatment of 3T3-L1 preadipocytes also induced PKB activity in a dose-dependent manner, with large increases observed even at 1 ng/ml PDGF (8.0 ± 0.9-fold; mean ± range; Fig. 4). This response is comparable to that seen with a maximal dose (1 μmol/l) of insulin, observed during the same series of experiments. Higher doses of PDGF increased PKB activity to a greater extent than insulin, under similar experimental conditions (up to 26.3 ± 9.8-fold), reaching a plateau at 20 ng/ml (Fig. 4).

Regulation of 3-phosphorylated phosphoinositides by growth factors in 3T3-L1 preadipocytes. The greater activation of PKB observed with PDGF, compared with insulin, appeared to correlate with the ability of PDGF to

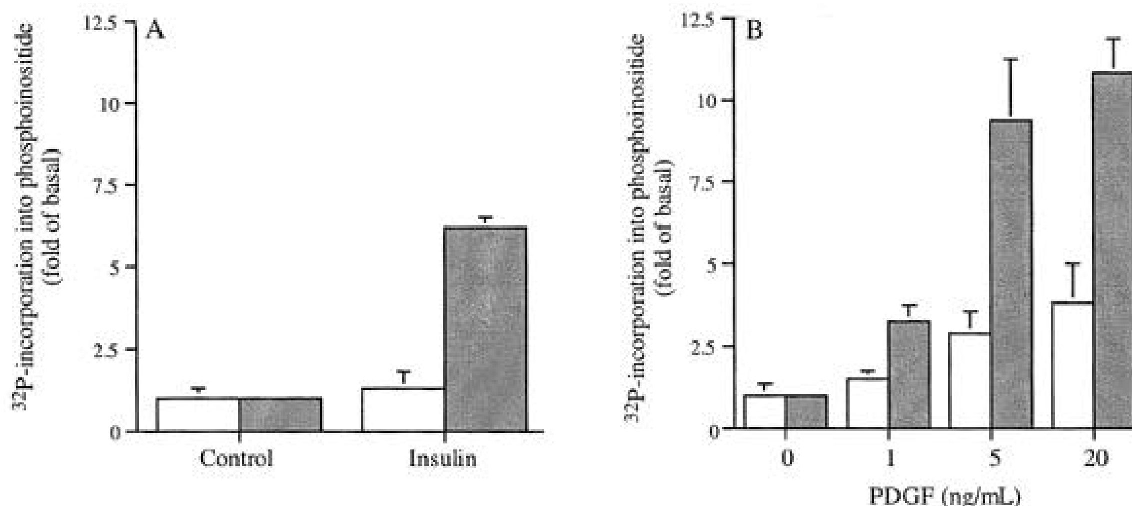

FIG. 5. The 3-phosphorylated phosphoinositide response in 3T3-L1 preadipocytes treated with PDGF or insulin. Confluent 3T3-L1 preadipocytes, starved overnight, were incubated in phosphate-free medium containing 625 μCi/ml [^{32}P]orthophosphate for 4 h, before stimulation with 100 nmol/l insulin (*A*), the indicated doses of PDGF (*B*), or vehicle. Cellular lipids were extracted, de-acylated, and analyzed by high performance liquid chromatography (HPLC), as described. The peaks corresponding to [^{32}P]PI(3,4)P2 (*A*) and [^{32}P]PI(3,4,5)P3 (*B*) were quantified using an online radioisotope detector. Data are expressed as the fold of basal. Results shown were calculated as mean ± range of a single experiment done in duplicate, and are representative of two experiments. □, PI(3,4)P2; ■, PI(3,4,5)P3.

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increase PI(3,4)P2 production. To investigate this possibility, total cellular production of 3-phosphorylated phosphoinositides was measured as the incorporation of [^{32}P]orthophosphate into PI(3,4)P2 and PI(3,4,5)P3. As we have previously described (15,16), insulin stimulation (100 nmol/l, 5 min) of 3T3-L1 preadipocytes resulted in increased production of PI(3,4,5)P3 (6.1 ± 0.3; mean ± range), without any detectable significant change in PI(3,4)P2 levels (Fig. 5*A*). No increase in PI(3,4)P2 production was observed previously using a higher dose (1 µmol/l) insulin or longer exposure to the agonist (up to 30 min) (15). Corresponding doses of insulin resulted in the activation of PKB (Fig. 1*A*). However, the maximal PI(3,4,5)P3 response was observed at 100 nmol/l insulin, without significant increases at higher doses, whereas maximal PKB activation occurred at 1 µmol/l insulin. This observation is consistent with additional PI 3-kinase–independent regulatory mechanisms, such as serine/threonine phosphorylation, being involved in the regulation of PKB activity (20).

Stimulation of 3T3-L1 cells with 5 or 20 ng/ml PDGF increased cellular production of both PI(3,4)P2 and PI(3,4,5)P3 (Fig. 5*B*). PI(3,4)P2 and PI(3,4,5)P3 rose 3.3 ± 0.1 fold and 10.8 ± 1.5 fold (mean ± range), respectively, upon a 5-min exposure to 20 ng/ml PDGF. Stimulation with a lower dose of PDGF (1 ng/ml) gave rise only to PI(3,4,5)P3 production (3.2 ± 0.6; mean ± range), without any significant increase in PI(3,4)P2, mimicking the insulin response described above. The lack of PI(3,4)P2 production in response to low-dose PDGF and insulin may reflect the lower accumulation of PI(3,4,5)P3, compared with that observed with higher doses of PDGF. We further examined this possibility by defining the threshold dose of PDGF that would elicit a PI(3,4)P2 response. PDGF, at a dose of 2 ng/ml, raised PI(3,4,5)P3 to a level similar to that observed with insulin (Fig. 6). This response was also accompanied by an increase of ~60% in PI(3,4)P2 levels. As previously noted, insulin did not induce a detectable increase in PI(3,4)P2 production. These results suggest that the insulin regulation of 3-phosphorylated phosphoinositides production in 3T3-L1 preadipocytes is distinct from that of PDGF, resulting in an isolated increase in PI(3,4,5)P3 accumulation.

The greater rise in PKB activity observed with increasing doses of PDGF, as compared with insulin (Fig. 4), correlates with the appearance of PI(3,4)P2 production, as well as further augmentation in the PI(3,4,5)P3 response. The isolated rise in PI(3,4,5)P3 observed in response to 1 ng/ml PDGF was also associated with the activation of PKB to a level similar to that observed with 1 µmol/l insulin (Fig. 4). However, the amount of PI(3,4,5)P3 generated by 1 ng/ml PDGF is ~50% of that produced in response to insulin (Fig. 5). This observation suggests that PI(3,4,5)P3 generated upon PDGF treatment may be more potent in activating PKB activity.

Differential regulation of 3T3-L1 adipocyte differentiation by insulin and PDGF. Expression of membrane-targeted, constitutively activated forms of PKB are sufficient to induce spontaneous adipocyte differentiation in confluent 3T3-L1 preadipocytes, implicating the PI 3-kinase/PKB/p70 S6 kinase pathway in the regulation of insulin-dependent adipogenesis (8,9). In 3T3-L1 preadipocytes, insulin stimulates only the production of PI(3,4,5)P3, while PDGF, at a dose (20 ng/ml) similar to that reported to have no effect on adipose cell differentiation (30 ng/ml) (1), induces both PI(3,4)P2 and PI(3,4,5)P3 (15). Growth factor–dependent differences in the mechanism of activation of PKB may play a role in their ability to induce the adipogenic process. In addition, the extent of PKB activity achieved with PDGF at 20 ng/ml is several-fold higher than that observed with a maximal dose of insulin (Fig. 4). Treatment of 3T3-L1 preadipocytes with 1 ng/ml PDGF stimulates only PI(3,4,5)P3 production and activates PKB to a level comparable to that of 1 µmol/l insulin (Figs. 4 and 5). Therefore, the potential of PDGF at 1 ng/ml to induce adipogenesis was assessed.

The 3T3-L1 preadipocytes were grown to confluence and induced to differentiate with 1 µmol/l insulin or PDGF (1 and 20 ng/ml), and differentiation was assessed visually by phase-contrast microscopy (not shown) and measurement of triacylglycerol content (Fig. 7*A*) and GPDH activity

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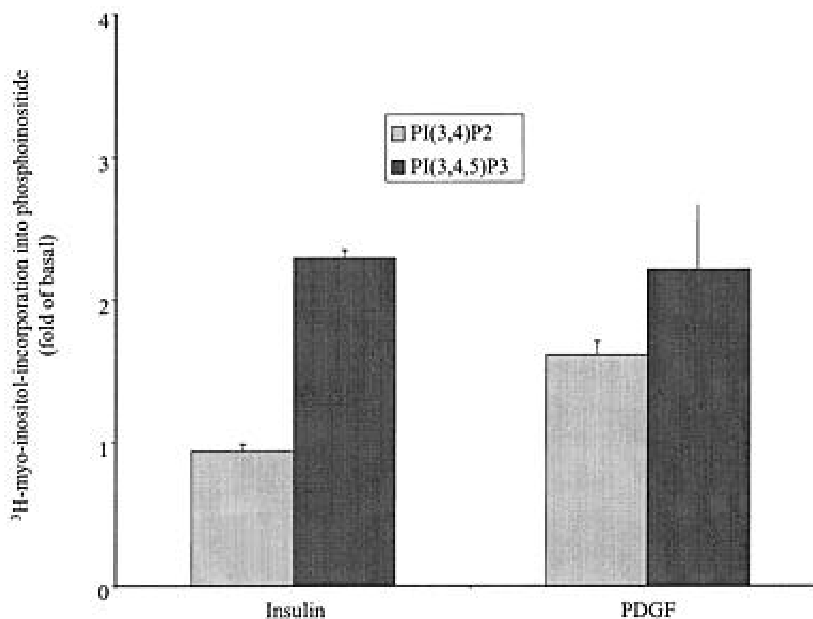

FIG. 6. PI(3,4)P2 accumulation in 3T3-L1 preadipocytes treated with doses of insulin and PDGF that generate the same PI(3,4,5)P3 response. Confluent 3T3-L1 preadipocytes were starved overnight in inositol-free medium containing 100 µCi/ml [^{3}H]myoinositol, before stimulation for 5 min with either 100 nmol/l insulin, 2 ng/ml PDGF, or vehicle. Cellular lipids were extracted, deacylated, and analyzed by HPLC, as described. The radioactivity contained in 1-ml fractions was measured and peaks representing the deacylated products of [^{3}H]PI(3,4)P2 and [^{3}H]PI(3,4,5)P3 were identified. Data are expressed as the fold of basal and were calculated as the mean ± range of two separate experiments, each done in duplicate.

(Fig. 7*B*). Dexamethasone and isobutylmethylxanthine (control) were also added for the first 48 h of the differentiation protocol to accelerate differentiation, as described (29). The presence of these en-hancers for the first 2 days, even without addition of growth factor, resulted in the differentiation of a low percentage (~10%) of 3T3-L1 preadipocytes to adipocytes, as visualized by the presence of lipid droplets, and triacylglycerol accumulation (447 ± 18.7 μg/mg protein, mean ± SE; Fig. 7*A*), and detectable GPDH activity (660 ± 158 U/mg protein, mean ± range; Fig. 7*B*). As previously described, addition of 1 μmol/l insulin for 8 days postconfluence induces differentiation of 3T3-L1 preadipocytes into adipocytes, increasing triacylglycerol content (1,176 ± 306 μg/mg protein, mean ± SE) and GPDH activity (1,410 ± 24 U/mg protein, mean ± range). PDGF (1 ng/ml) did not induce adipose cell differentiation, and triacylglycerol levels and GPDH activity were similar to those obtained with dexamethasone and isobutylmethylxanthine alone (363.7 ± 13.0 μg/mg protein, mean ± SE; and 560 ± 26 U/mg protein, mean ± range, respectively). However, high doses of PDGF (20 ng/ml) actually blocked the small percentage of differentiation observed in cells treated with dexamethasone and isobutylmethylxanthine alone, as observed by the decreased triacylglycerol value (154.8 ± 7.6 μg/mg protein, mean ± SE) and GPDH activity (95 ± 25 U/mg protein, mean ± range). Our results demonstrate that a dose of PDGF, which mimics the 3-phosphorylated phosphoinositide and PKB activation responses of insulin, does not induce adipocyte differentiation. Other subtle growth factor–dependent differences in the mechanism of PKB activation are likely operative in the control of adipogenesis.

DISCUSSION

The results described here demonstrate a novel PI(3,4,5)P3–dependent and PI(3,4)P2–independent activation of PKB in insulin-stimulated 3T3-L1 preadipocytes. PDGF-stimulated preadipocytes generate higher levels of PKB activity, likely caused by increased production of PI(3,4)P2 and further aug-

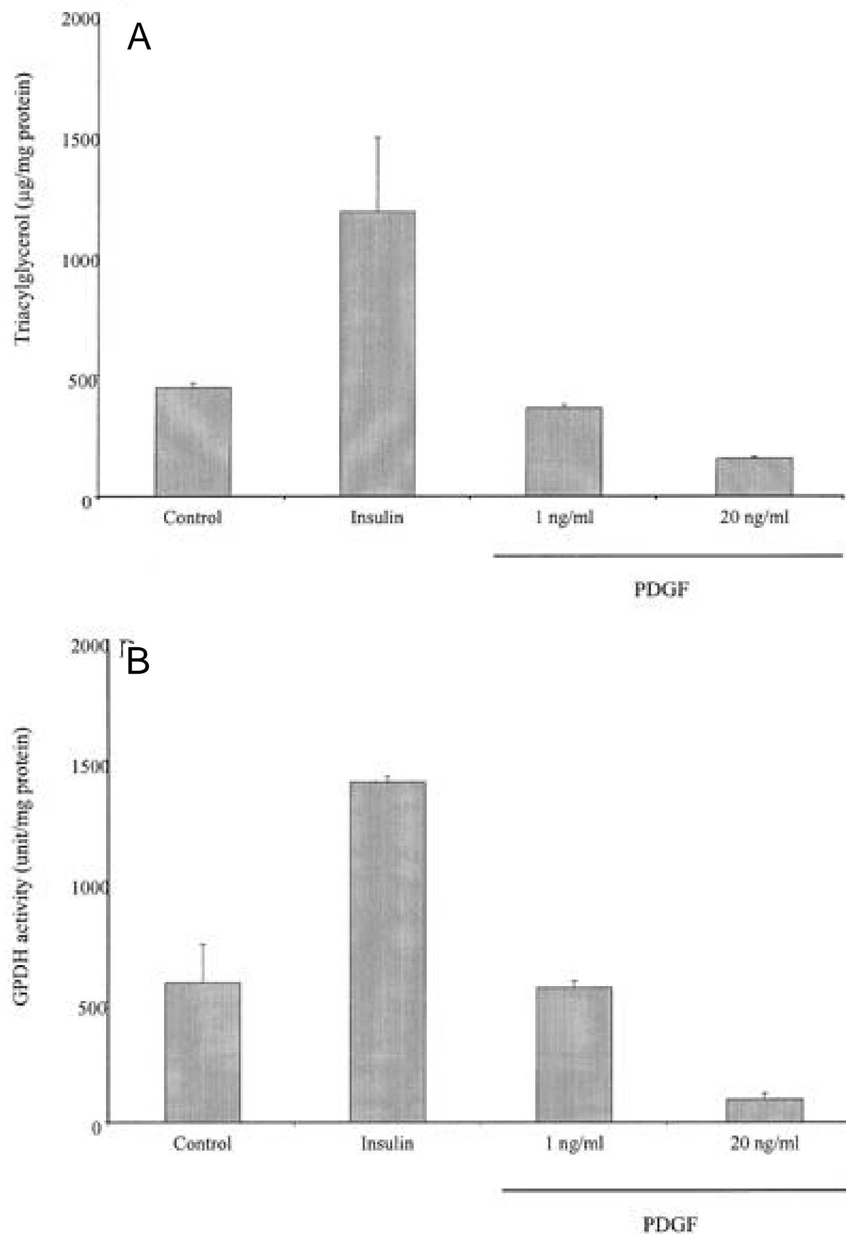

FIG. 7. Effect of insulin and PDGF on 3T3-L1 adipose cell differentiation. Two-days post-confluence, 3T3-L1 preadipocytes were induced to differentiate with either 1 μmol/l insulin, 1 ng/ml PDGF, or 20 ng/ml PDGF, in the presence of dexamethasone and isobutylmethylxanthine for the first 48 h, as described in METHODS. *A*: After 8 days, adipose cell differentiation was assessed by quantifying triacylglycerol accumulation. Results are expressed as micrograms triacylglycerol per milligram of protein and represent the mean ± SE of three separate experiments each done in duplicate. *B*: Alternatively, cytosolic GPDH activity was measured after 8 days of differentiation. Results are expressed as units per milligram protein and represent the mean ± range of two separate experiments, each done in duplicate.

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mentation in PI(3,4,5)P3. Although low-dose PDGF could mimic the PI(3,4,5)P3 and PKB response of insulin, it was unable to trigger adipogenesis.

The stimulation of PKB activity by growth factors closely follows the pattern of activation of PI 3-kinase (33). Previous studies have described a close association between PKB activity and total cellular PI(3,4)P2 levels (24). In contrast, our data demonstrate that PKB activity can be induced in 3T3-L1 preadipocytes with insulin and low concentrations of PDGF, which generate increases in PI(3,4,5)P3 only. This suggests that the PI(3,4,5)P3 response alone appears to be sufficient for PKB activation and that PI(3,4)P2 is not required. Such a PI(3,4,5)P3–dependent activation of PKB has also recently been suggested during the very early phase of platelet activation by thrombin-receptor agonists (34), although the possible contribution of a small increase in PI(3,4)P2 could not be excluded with certainty.

In 3T3-L1 preadipocytes, the greater PKB activation in response to PDGF, compared with insulin, is observed at doses of PDGF that result in significant PI(3,4)P2 increases, as well as an additional induction of PI(3,4,5)P3 production. Treatment with 1 ng/ml PDGF activates PKB to a similar level as insulin, yet PI(3,4,5)P3 production is 50% less than that observed with insulin, suggesting that PI(3,4,5)P3 produced in response to PDGF is more potent in activating PKB.

The reason for the lack of PI(3,4)P2 production in 3T3-L1 preadipocytes treated with insulin or low-dose PDGF is unknown. It is possible that the specific PI(3,4,5)P3–5-phosphatase catalyzing the production of PI(3,4)P2 from PI(3,4,5)P3 (32) is not activated or actually inhibited, by insulin treatment in these cells. Alternatively, PI(3,4)P2 production may occur only when a certain threshold of PI 3-kinase activation has been reached to phosphorylate PI(4)P. The number of insulin receptors expressed in 3T3-L1 preadipocytes is quite low, in the order of 10^4 per cell (1) compared with 10^5 PDGF binding sites per cell (35), and may not generate as complete a PI 3-kinase response compared with that obtained with high doses of PDGF. However, our results demonstrate that a dose of PDGF that increases PI(3,4,5)P3 to a similar level as that observed with insulin is also accompanied by an increase in PI(3,4)P2. These results suggest that 3-phosphorylated phosphoinositide production in 3T3-L1 preadipocytes is regulated differentially by insulin compared with PDGF.

Insulin activation of PKB was blocked by two inhibitors of PI 3-kinase, supporting its role as a downstream target of PI 3-kinase. LY294002 was less efficient than wortmannin in blocking the insulin effect on PKB activity. Wortmannin reacts with PI 3-kinase and inhibits its activity irreversibly, whereas LY294002 binds PI 3-kinase reversibly and may be removed to some extent during the immunoprecipitation protocol (10,36). Our results are consistent with previous reports demonstrating only a partial inhibition of PI 3-kinase activity using this particular dose of LY294002 (36,37). The inhibition of insulin-induced PKB activity by wortmannin described in this study can be most likely attributed to the specific inhibition of PI 3-kinase, since the concentration used was within the range specific for PI 3-kinase (10). Rapamycin, which inhibits mammalian target of rapamycin (mTOR), and ultimately p70 S6 kinase activity (11), had no effect on insulin-induced PKB activation as expected because mTOR and p70 S6 kinase are positioned downstream of PKB (20).

Ectopic expression of activated forms of PKB induces spontaneous, hormone-independent, adipocyte differentiation of confluent 3T3-L1 preadipocytes (8,9). However, PKB activation does not necessarily correlate with adipocyte differentiation because both insulin and PDGF activate PKB in 3T3-L1 preadipocytes, but only insulin induces adipogenesis. The 3-phosphorylated response of 3T3-L1 preadipocytes treated with insulin is distinctive in that only PI(3,4,5)P3 production increases. Because low-dose PDGF (1 ng/ml), which mirrored this specific PI(3,4,5)P3 response without a concomitant rise in PI(3,4)P2, did not stimulate 3T3-L1 adipogenesis, other potential growth factor-related differences in the mechanism of activation of PKB may be relevant to regulation of adipogenesis.

PI(3,4)P2 specifically binds the PH domain of isolated PKB and stimulates its kinase activity in vitro (23). PI(3,4,5)P3 also binds the PH domain of PKB with even greater affinity than PI(3,4)P2; however, the effect of PI(3,4,5)P3 binding on PKB activity is still unclear. Whereas some groups report no effect, others describe an inhibition of basal PKB activity (19,22–24). Most of these studies were all done in the context of an in vitro kinase assay in which the activity of purified PKB, bound or not to various lipid preparations, was measured. Recently, upstream regulatory kinases (PDKs), which activate PKB, have been identified and cloned (21). Therefore, determining the effect of 3-phosphorylated phosphoinositides in an in vitro kinase assay using purified PKB may not be relevant to the actual regulation of this kinase in a cellular environment.

In 3T3-L1 preadipocytes, PKB activity was stimulated in response to the addition of exogenous synthetic PI(3,4)P2 in a dose-dependent manner, confirming recent results in NIH 3T3 cells (24). The lack of PKB activation observed with PI(4,5)P2 indicates that the PI(3,4)P2 response observed is specific and does not arise from changes in membrane structure and/or integrity upon exposure to phosphoinositides. Surprisingly, we did not observe a significant increase in PKB activity in intact 3T3-L1 cell monolayers stimulated with exogenous PI(3,4,5)P3. Because insulin-stimulated PI(3,4,5)P3 was sufficient to activate PKB, this was an unexpected finding that remains under investigation.

The direct addition of synthetic 3-phosphorylated phosphoinositides to intact cells is a relatively new approach and deserves some comment. Published data in the last year indicate that these lipids fuse with the cell membrane and exert biochemical and cellular responses. As mentioned, others have observed that the addition of PI(3,4)P2 to intact cell monolayers stimulates endogenous PKB (24). Exogenous PI(3,4,5)P3 leads to increased cell motility, actin reorganization, and membrane ruffling in NIH 3T3 cells (38). Moreover, it has been shown that PI(3,4)P2 and PI(3,4,5)P3 are readily incorporated into cell membranes at similar rates (39).

In summary, in 3T3-L1 preadipocytes, both insulin and PDGF activate the PI 3-kinase/PKB pathway, which is critical to the regulation of adipocyte differentiation; however, only insulin is adipogenic. The differences in the extent of PKB activation observed with either growth factor appear to be related to distinct PI(3,4)P2 and PI(3,4,5)P3 responses, which may reflect the degree of PI 3-kinase activation. However, a dose of PDGF that mirrors the PI 3-kinase/PKB response of insulin still failed to induce adipogenesis. The reasons for this are not yet apparent. Possibilities include unique intra-

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cellular compartmentalization of PI 3-kinase and PKB, given that PI 3-kinase associates with insulin receptor substrate (IRS)-1, a cytosolic protein, and with PDGF receptor, located at the plasma membrane, upon insulin and PDGF treatment, respectively. Translocation of PKB to these IRS-1–directed compartments may bring the kinase in proximity with an alternative array of substrates potentially involved in adipogenesis.

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