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Insulin-Induced Translocation of Protein Kinase B to the Plasma Membrane in Rat Adipocytes

Olga Göransson,*[,1] Jonny Wijkander,*[,2] Vincent Manganiello,† and Eva Degerman*
**Section for Molecular Signalling, Department of Cell and Molecular Biology, Lund University, P.O. Box 94, S-22100 Lund, Sweden; and †Pulmonary/Critical Care Medicine Branch, National Heart, Lung and Blood Institutes, Bethesda, Maryland 20892*

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Protein kinase B (PKB) has previously been shown to be activated in response to insulin and growth factor stimulation. The activation mechanism has been suggested to involve translocation of PKB to membranes, where it is phosphorylated and activated. Insulin-induced translocation of PKB has not been demonstrated in a physiological target cell. Therefore we have used the primary rat adipocyte to investigate insulin-induced translocation of PKB. In the presence of 1 nM insulin translocation of PKB was detected within 30 seconds and was blocked by wortmannin, a selective phosphatidylinositol 3-kinase inhibitor. This translocation was potentiated by the tyrosine phosphatase inhibitor vanadate. Subcellular localization studies revealed that PKB translocated to the plasma membrane.

Key Words: **insulin; adipocyte; protein kinase B; plasma membrane; phosphatidylinositol 3-kinase.**

Insulin and many growth factors mediate their effects by activating phosphatidylinositol 3-kinase (PI 3-K). Recently, the serine/threonine protein kinase B (PKB), also known as RAC protein kinase or c-Akt, has been identified as an important target in PI 3-K signalling. Although PKB is activated by insulin and growth factors, its exact role remains to be determined. So far, PKB has been implicated in insulin-induced stimulation of glucose uptake (1, 2) and glycogen synthesis (3), and in promotion of cell survival (4-8).

PKB is expressed as three isoforms; α, β and γ (9-13), of which the α-form has been the most extensively studied with regard to activation mechanisms. Activation of PKBα has been proposed to involve at least two steps, namely membrane translocation and phosphorylation. Phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, products of PI 3-K, have been shown to bind to the N-terminally located pleckstrin homology (PH) domain (14) of PKBα (15, 16). This binding is thought to mediate recruitment of PKB to membranes, and to induce a conformational change of the protein. Upstream kinases, most likely including 3-phosphoinositide-dependent protein kinase-1 (17, 18), then phosphorylate and activate PKB. In support of such a hypothesis is the finding that targeting of PKB to membranes, e.g. by introduction of a myristoylation signal, results in a constitutively phosphorylated and activated kinase (19-21). Two activity controlling phosphorylation sites have been identified in PKBα (22) (Thr-308 and Ser-473) and PKBβ (23) (Thr-309 and Ser-474) of which Thr-308 in PKBα has been shown to be a substrate for 3-phosphoinositide-dependent protein kinase-1 (17, 18).

Transfected PKBα has been shown to translocate to the plasma membrane and the nucleus in HEK-293 cells in response to insulin-like growth factor-1 (IGF-1) stimulation (19). It has also been demonstrated that both endogenous and transfected PKBα (19) and transfected PKBβ (23) translocate to the nucleus in REF-52 cells in response to a combination of okadaic acid and fetal calf serum. In EL4·IL-2 fibroblasts, it has been shown that PKB translocates to the plasma membrane in response to interleukin-2 (5).

In order to elucidate the role of membrane translocation of PKB in insulin signalling, there is an obvious need to demonstrate translocation in a physiological target cell for insulin. We recently showed that the insulin mimetic agent peroxovanadate induced translo-

[1] To whom correspondence should be addressed at the Section for Molecular Signalling, Department of Cell and Molecular Biology, Lund University, P.O. Box 94, S-22100 Lund, Sweden. Fax: +46-46-222 40 22. E-mail: Olga.Goransson@medkem.lu.se.

[2] Present address: Section for Molecular Pathogenesis, Department of Cell and Molecular Biology, Lund University, P.O. Box 94, S-22100 Lund, Sweden.

Abbreviations used: PI 3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PH, pleckstrin homology; IGF-1, insulin-like growth factor-1; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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cation of PKB from the cytosol to the membrane fraction in primary rat adipocytes (24). Now we have proceeded to study translocation of PKB in response to insulin in these cells. In this paper we demonstrate a rapid, wortmannin-sensitive translocation of PKB to the plasma membrane of rat adipocytes in response to physiological concentrations of insulin.

MATERIALS AND METHODS

Preparation and stimulation of rat adipocytes. Adipocytes prepared from epididymal adipose tissue of 36-38 day-old male Sprague-Dawley rats (B&K Universal, Stockholm) (25, 26) were suspended (2 ml of 10% cell suspension) in Krebs-Ringer medium pH 7.4, 25 mM Hepes pH 7.4, 200 nM adenosine, 2 mM glucose and 1% bovine serum albumin, and were incubated at 37°C with additions as indicated. Vanadate was dissolved in water at a concentration of 300 mM and boiled prior to use. Peroxovanadate was prepared fresh by incubating vanadate and H_2O_2 (12 mM each) at 20°C, in the dark, in 40 mM Hepes pH 7.4, for 15 min prior to use. Wortmannin was dissolved in dimethyl sulfoxide (final dimethyl sulfoxide concentration in cell incubations was $\leq$0.01%). At times indicated, 5 ml of homogenisation buffer consisting of 40 mM Hepes pH 7.4, 10 mM NaF, 1 mM dithioerythriol, 1 mM phenylmethylsulfonylfluoride, 0.25 mM sodium orthovanadate, 10 μg/ml antipain, 10 μg/ml leupeptin and 1 μg/ml pepstatin A, were added to the incubations. Cells were centrifuged, resuspended in 1 ml of homogenisation buffer, homogenised (10 strokes) at room temperature and then immediately put on ice. In most experiments a crude subcellular fraction was prepared by centrifugation of the homogenates at 33 000 $\times$ g for 60 min at 4°C. The fat cake was removed, the infranatants (referred to as cytosol fractions) were withdrawn, and pellets (mainly consisting of nuclei, mitochondria, plasma membranes and cell debris, referred to as membrane fractions) were resuspended in 500 μl of homogenisation buffer.

Subcellular fractionation of adipocytes. Subcellular fractionation was performed, with modifications, as described by Simpson et al. (27). Adipocytes (700 μl packed cell volume in each incubation), stimulated as indicated, were homogenised in 3 ml of homogenisation buffer (see above), and centrifuged at 16 600 $\times$ g for 15 min at 4°C. The solidified fat was removed and the infranatant was used to prepare the microsomal fraction. The pellet, mainly containing nuclei, mitochondria, plasma membranes and broken cell debris, was resuspended in 1.5 ml of homogenisation buffer, layered on top of a 1.5 ml sucrose cushion consisting of 1.12 M sucrose, 20 mM Tris pH 7.4 and 1 mM EDTA and centrifuged at 104 000 $\times$ g for 70 min at 4°C. The pellet, comprising the nuclear- and mitochondrial fraction was resuspended in 200 μl of homogenisation buffer. Plasma membranes, collected at the sucrose-sample interface were pelleted at 48 100 $\times$ g for 45 min at 4°C, and resuspended in 200 μl of homogenisation buffer. The initial supernatant was centrifuged at 48 100 $\times$ g for 20 min at 4°C, yielding a high density microsomal pellet which was resuspended in 200 μl of homogenisation buffer. By centrifugation of the supernatant at 216 000 $\times$ g for 70 min at 4°C, the low density microsomes and the cytosol were prepared. The low density microsomal pellet was resuspended in 200 μl of homogenisation buffer.

Immunoblot analysis. Adipocyte cytosol- and membrane fractions were mixed with Laemmli sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (8% acrylamide) followed by electrotransfer of proteins onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 1 h with 0.5% gelatin in 20 mM Tris pH 7.6, 137 mM NaCl and 0.1 % (w/v) Tween-20, and were incubated for 16 h with a 1:2000 dilution of an anti-NT-PKBα antibody (Upstate Biotechnology Inc., Lake Placid, USA). Stripping of immunoblots was performed by incubation at 55°C for 30 min in a buffer containing 62.5 mM Tris pH 6.7, 100 mM β-mercaptoethanol and 2 % (w/v) SDS. The anti-adenylyl cyclase V antibody (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and the anti-nucleoporin p62 antibody (Transduction Laboratories, Lexington, USA) (both at a 1:500 dilution) were incubated with the membranes for 1.5 h. The anti-CT-PKBα antibody (1:2000 dilution, Upstate Biotechnology Inc., Lake Placid, USA) and the anti-PH-PKBα antibody (1:500 dilution, Kinetek Pharmaceuticals Inc., Vancouver, Canada) were incubated with the membranes over night. Immunoblot analysis was performed using the SuperSignal Substrate (Pierce).

Assay of PKB. PKB activity was measured as previously described (24). In summary, cytosol- and membrane fractions (10 μl) were incubated for 20 min at 30°C with 5 μl of a mixture containing 17 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) pH 7.5, 42 mM $MgSO_4$, 4.2 mM dithioerythriol, 207 mM sucrose, 170 μM [γ-^{32}P]ATP (5-15 μCi), 6.6 μM cAMP-dependent protein kinase inhibitor and 13 μg of the peptide substrate KKRNRTLTK (K9). Incubations were terminated by the addition of 10 μl of 1% bovine serum albumin, 1mM ATP, pH 3.0 and 5 μl of 30% trichloroacetic acid. After 15 min on ice, samples were centrifuged and 15 μl of the supernatants were applied onto phosphocellulose paper (Whatman P81) that was washed three times with 75 mM phosphoric acid and once with acetone. The amount of ^{32}P incorporated into the peptide substrate was determined by scintillation counting.

RESULTS AND DISCUSSION

Translocation of PKB from the cytosol to the membrane fraction in adipocytes in response to insulin. Activation of PKB by insulin and growth factors has proposedly been explained by a two-step model, which involves translocation of PKB to membranes, where phosphorylation and activation of the kinase is believed to occur. To examine translocation in a physiologically important insulin-responsive cell, we have studied insulin-induced translocation of PKB in isolated rat adipocytes. It has previously been shown (24) that vanadate and peroxovanadate, tyrosine phosphatase inhibitors with well known insulin mimetic effects (28), can induce activation of PKB, and that peroxovanadate stimulation results in a translocation of PKB to the membrane fraction of adipocytes. Insulin-induced activation (2, 24, 29, 30) but not translocation in primary adipocytes has been demonstrated in previous studies. In our investigation, insulin, vanadate and peroxovanadate were used, alone or in combination, to study the localization and activation of PKB in adipocytes. As shown in Fig. 1A, stimulation of adipocytes with peroxovanadate induced a shift in electrophoretic mobility of PKB, and caused virtually all PKB to translocate from the cytosol to the membrane fraction, in agreement with our previous findings (24). Importantly, insulin alone also induced a gel shift, as well as caused a portion of the total PKB to translocate to the membrane fraction. The insulin-induced translocation was potentiated by treatment of the cells with vanadate prior to insulin stimulation. As shown in Fig. 1B, there was an increase in PKB activity in adipocyte membranes after insulin and peroxovanadate stimulation, as compared to membranes from unstimulated cells.

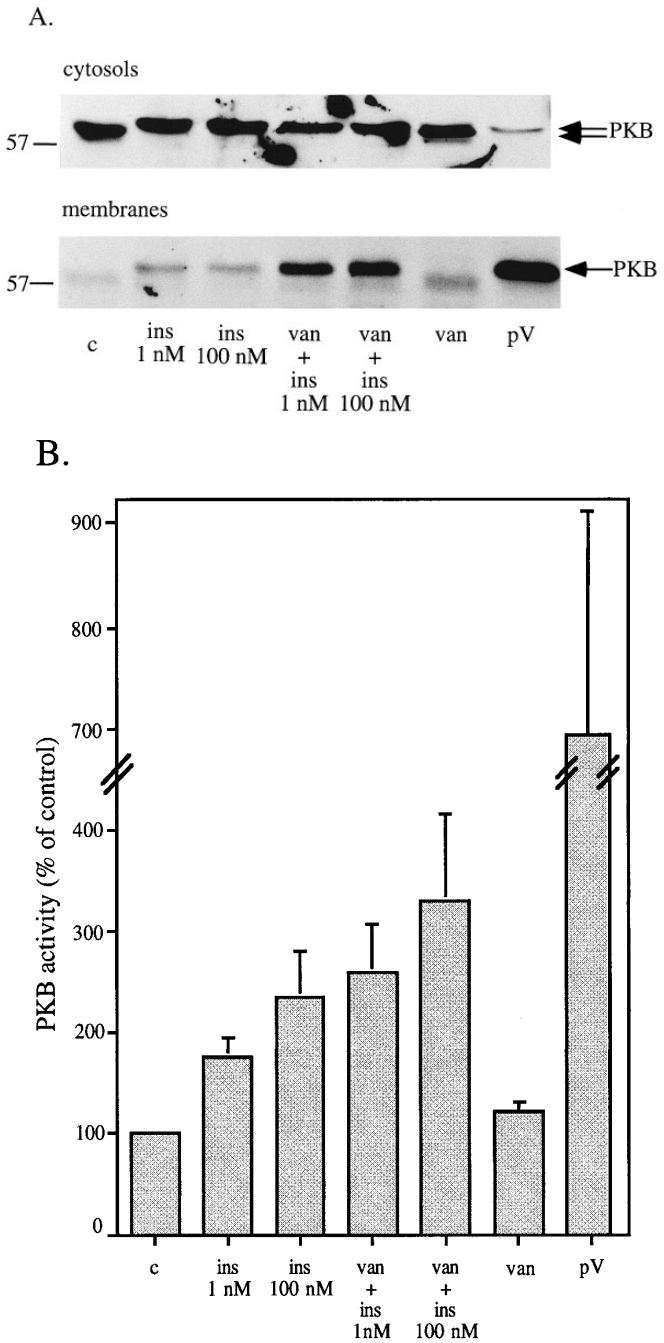

FIG. 1. Translocation of PKB to the membrane fraction of adipocytes in response to insulin, insulin in combination with vanadate, or peroxovanadate. Immunoblot analysis (A) and kinase activity (B). Adipocytes were incubated without (c, control) or with insulin (ins, 5 min), vanadate (van, 1 mM, 40 min prior to insulin stimulation) and peroxovanadate (pV, 250 μM, 40 min) as indicated in the figure, and cytosol- and membrane fractions were prepared as described in "Materials and methods". A. Cytosol- (corresponding to 30 μl packed cell volume) and membrane fractions (corresponding to 60 μl packed cell volume) were subjected to SDS-PAGE and immunoblot analysis using the anti-NT-PKBα antibody. B. Membrane fractions (corresponding to 4 μl packed cell volume) were assayed for PKB activity as described in "Materials and methods", and results are presented as percent of the activity in the control (taken as 100%)—mean values ± SEM of 3-5 individual experiments.

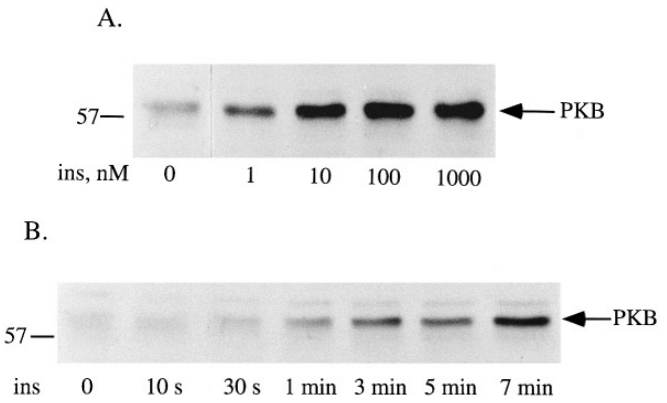

FIG. 2. Dose- (A) and time (B) dependency for the insulin-induced translocation of PKB to the membrane fraction. Immunoblot analysis. A. Adipocytes were incubated for 5 min with insulin (ins) as indicated in the figure. B. Adipocytes were incubated with 1 nM of insulin (ins) for the times indicated in the figure. Membrane fractions (corresponding to 60 μl packed cell volume) were prepared as described in "Materials and methods", and subjected to SDS-PAGE and immunoblot analysis using the anti-NT-PKBα antibody.

The insulin-induced increase in activity was potentiated by pretreatment with vanadate. Increased PKB activity could also be measured in the cytosol in response to insulin and peroxovanadate (data not shown), most likely representing PKB that had been released and /or lost to the cytosol after its activation at the membrane.

Vanadate has been shown to potentiate insulin-induced activation of PI 3-K (I. Castan, unpublished data), and to inhibit not only tyrosine phosphatases but also phosphatidylinositol 3,4,5-trisphosphate 5′-phosphatase *in vitro* (31). Either or both of these effects could enhance the accumulation of phosphatidylinositol 3,4,5-trisphosphate, one of the two proposed binding partners for PKB in the membrane, and thus provide a possible explanation to the potentiating effect of vanadate.

Dose and time dependency for the insulin-induced translocation of PKB to the membrane fraction. As shown in Fig. 2A, translocation of PKB to the membrane fraction was clearly detectable after stimulation with 1 nM insulin, and reached a maximum at 10 nM. Using 1 nM insulin, translocation could be detected within 30 s, with a maximal effect after 1-3 min (Fig. 2B). Our results show that translocation of PKB is a rapid event that takes place at low concentrations of insulin. Similar time- and dose dependencies have been reported for the activation of PKB in adipocytes (24), fortifying the suggestion that activation of PKB takes place after translocation of the protein to a membrane.

The insulin-induced translocation of PKB to the membrane fraction is inhibited by wortmannin. In order to evaluate the role of PI 3-K in the insulin-induced translocation of PKB, we used the selective PI 3-K in-

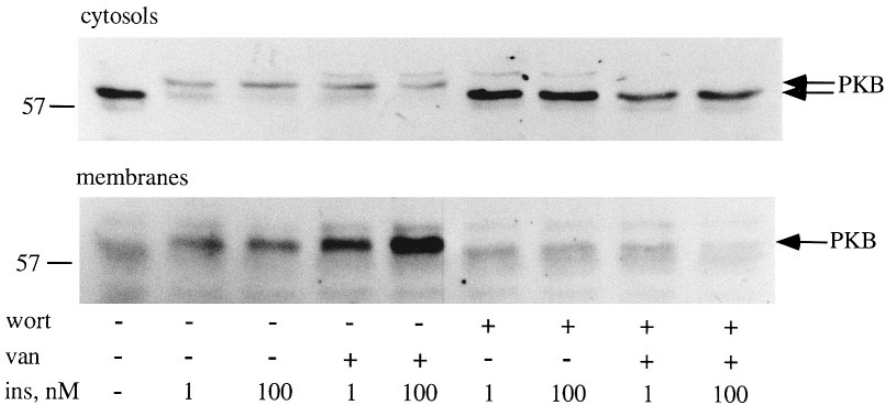

FIG. 3. Inhibition of translocation of PKB to the membrane fraction by wortmannin. Immunoblot analysis. Adipocytes were incubated with insulin (ins, 5 min), vanadate (van, 1 mM, 40 min prior to insulin stimulation) and wortmannin (wort, 100 nM, 10 min prior to vanadate and/or insulin stimulation) as indicated in the figure, and cytosol- and membrane fractions were prepared as described in "Materials and methods". Cytosol- (corresponding to 30 μl packed cell volume) and membrane fractions (corresponding to 60 μl packed cell volume) were subjected to SDS-PAGE and immunoblot analysis using the anti-NT-PKBα antibody.

hibitor wortmannin. As shown in Fig. 3, treatment of adipocytes with wortmannin prior to stimulation with insulin, or vanadate followed by insulin, blocked translocation of PKB to membranes as well as the shift in electrophoretic mobility. These results suggest that insulin-induced translocation of PKB to the membrane fraction of rat adipocytes is a PI 3-K dependent process, as was previously reported for the peroxovanadate-induced translocation of PKB (24). The recently described translocation of PKB in HEK-293 cells in response to IGF-1 stimulation was also reported to be PI 3-K dependent (19).

Translocation of PKB to the plasma membrane in adipocytes in response to insulin. To determine the more specific localization of PKB in adipocytes, subcellular fractionation of cells stimulated with insulin, vanadate and peroxovanadate was performed. The separation of nuclei/mitochondria from plasma membranes was followed by immunoblot analysis of the different fractions, using antibodies against membrane marker proteins as indicated in the legend to Fig. 4. As shown in Fig. 4, PKB from unstimulated adipocytes was localized mainly in the cytosol fraction, whereas upon stimulation with insulin a portion of the PKB translocated to the plasma membrane. In agreement with the results in Fig. 1, the insulin-induced translocation of PKB to the plasma membrane was potentiated by pretreatment with vanadate. Upon stimulation with peroxovanadate virtually all of the cytosolic PKB moved to the plasma membrane. In addition, in control- and stimulated cells there is also a band in the nuclear- and mitochondrial fraction, representing a protein of roughly the same molecular weight as PKB. Although, in most experiments, this protein had a slightly higher electrophoretic mobility than PKB, it immunoreacted with two additional PKB antibodies (anti-CT-PKBα and anti-PH-PKBα, data not shown), suggesting a presence of PKB in the nuclear- and mitochondrial fraction. However, this presence was not induced by stimulation with insulin or peroxovanadate. Translocation of PKB to the plasma membrane in response to IGF-1 (19) and interleukin-2 (5) has been demonstrated in previous studies. The presence of PKB in the nucleus has also been reported, but only as the result of stimulation of cells with IGF-1 (19) or a combination of okadaic acid and fetal calf serum (19, 23). It will now be of considerable interest to evaluate the role of different localizations of PKB regarding the mechanism of activation of PKB by insulin as compared to other growth factors, as well as PKB's action on its cellular substrates.

Our data demonstrate for the first time that in rat adipocytes, an excellent model for studies of insulin's metabolic pathways, PKB translocates to the plasma membrane in a PI 3-K dependent manner in response to insulin. This is consistent with the current hypothesis regarding the mechanism of activation for PKB (see "Introduction").

The binding of PKB to the membrane is thought to involve association of phosphatidylinositol 3,4,5-trisphosphate and/or phosphatidylinositol 3,4-bisphosphate to the PH domain of PKB. Little, however is known in detail about the nature of this interaction. We have found that in order to maintain the membrane association of PKB throughout the homogenisation and centrifugation procedures, conditions during homogenisation are crucial. After addition of sodium chloride in the range of 100 mM to the homogenisation buffer, almost no PKB could be detected in the membrane fraction (data not shown), indicating that the binding of PKB to the membrane might involve ionic interactions.

Although the PH domain is believed to be important

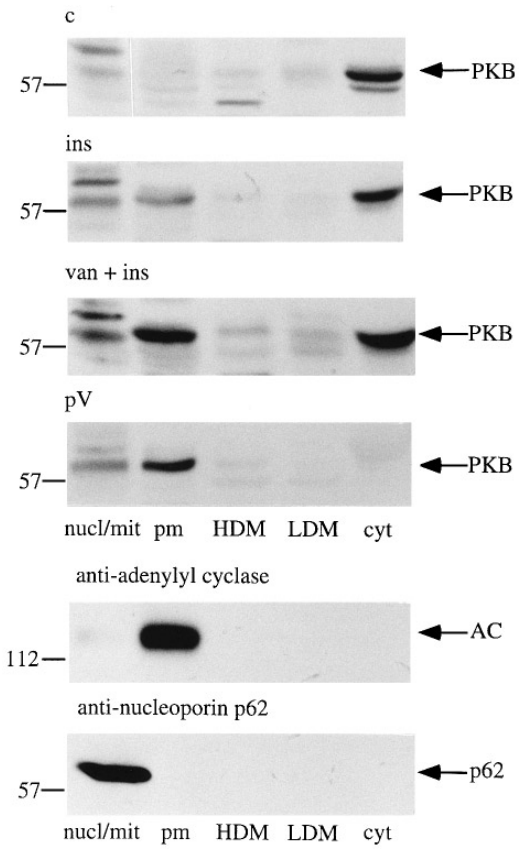

FIG. 4. Subcellular localization of PKB in adipocytes following stimulation with insulin, insulin in combination with vanadate, or peroxovanadate. Immunoblot analysis. Adipocytes were incubated without (c, control) or with insulin (ins, 10 nM, 5 min), vanadate (van, 1 mM, 40 min prior to insulin stimulation) and peroxovanadate (pV, 250 μM, 40 min) as indicated in the figure. Subcellular fractions (nucl/mit, nuclear- and mitochondrial fraction; pm, plasma membrane fraction; HDM, high density microsomal fraction; LDM, low density microsomal fraction; cyt, cytosol), prepared as described in "Materials and methods", were subjected to SDS-PAGE and immunoblot analysis using the anti-NT-PKBα antibody. Portions corresponding to, in the case of all membrane fractions, 600 μl packed cell volume, and in the case of cytosols, 70 μl packed cell volume, were loaded onto the gel. Blots were stripped and reprobed with antibodies against membrane markers: adenylyl cyclase V (AC) for the plasma membrane and nucleoporin p62 (p62), a nuclear pore protein, for the nucleus.

for the activation of PKB, it has been shown that under certain conditions activation of PKB can also occur in its absence (18, 20, 32). It has in fact been hypothesised that, in the inactive state of PKB, the PH domain constitutes an inhibitory component preventing phosphorylation, and that this constraint is removed upon membrane translocation followed by a conformational change of the protein (18).

Membrane translocation seems to be an important event in the activation of PKB, and perhaps also in directing PKB to subcellular compartments housing its substrates. Little is known about the mechanism whereby PKB mediates cellular responses to insulin, and it is therefore critical to identify and study physiologically relevant downstream targets for PKB. Glycogen synthase kinase-3 has been shown to be a substrate for PKB (3), indicating an important role for PKB in glycogen metabolism. PKB has also been reported to be involved in glucose uptake (1, 2). Another important effect of insulin is to counteract catecholamine-induced lipolysis in adipocytes, an effect mainly due to the cAMP-lowering activity of phosphodiesterase 3B. We have recently shown that phosphodiesterase 3B can be phosphorylated by PKB *in vitro* (33). The role of PKB in phosphorylation and activation of phosphodiesterase 3B in the intact cell is currently under investigation.

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