Role of Translocation in the Activation and Function of Protein Kinase B*

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We have investigated the role of subcellular localization in the regulation of protein kinase B (PKB) activation. The myristoylation/palmitoylation motif from the Lck tyrosine kinase was attached to the N terminus of protein kinase B to alter its subcellular localization. Myristoylated/palmitoylated (m/p)-PKB was associated with the plasma membrane of transfected cells, whereas the wild-type kinase was mostly cytosolic. The activity of m/p-PKB was 60-fold higher compared with the unstimulated wild-type enzyme, and could not be stimulated further by growth factors or phosphatase inhibitors. In vivo 32P labeling and mutagenesis demonstrated that m/p-PKB activity was due to phosphorylation on Thr308 and Ser473, that are normally induced on PKB following stimulation of the cells with insulin or insulin-like growth factor-1 (IGF-1). A dominant negative form of phosphoinositide 3-kinase (PI3-K) did not affect m/p-PKB activity. The pleckstrin homology (PH) domain of m/p-PKB was not required for its activation or phosphorylation on Thr308 and Ser473, suggesting that this domain may serve as a membrane-targeting module. Consistent with this view, PKBα was translocated to the plasma membrane within minutes after stimulation with IGF-1. This translocation required the PH domain and was sensitive to wortmannin. Our results indicate that PI3-K activity is required for translocation of PKB to the plasma membrane, where its activation occurs through phosphorylation of the same sites that are induced by insulin or IGF-1. Following activation the kinase detached from the membrane and translocated to the nucleus.

Many growth factors elicit cellular responses by activating phosphoinositide 3-kinase (PI3-K); reviewed in Ref. 1. Recently, protein kinase B (PKB), also known as RAC protein kinase or c-Akt (2–4) was recognized as a downstream target of PI3-K (5, 6). Three mammalian isoforms of PKB have been identified so far, termed PKBα, β, and γ (7–9). All three isoforms contain a pleckstrin homology (PH) domain at the N terminus (10), followed by a catalytic domain related to protein kinases A and C, and a C-terminal regulatory region. PKBα was found to mediate insulin- and insulin-like growth factor (IGF-1)-induced cellular responses, such as the inhibition of glycogen synthase kinase-3 (11), the stimulation of glucose uptake (12), and the promotion of cell survival by inhibiting apoptosis (Ref. 13; reviewed in Refs. 14 and 15). PKBα is the cellular homologue of the oncogene product v-Akt encoded by the AKT8 retrovirus, which induces thymic lymphomas in mice (16). Cloning of v-akt revealed that it was created by fusion of viral Gag sequences to the N terminus of mouse PKBα, which adds an N-terminal myristoylation signal to the oncoprotein and could account for its transforming ability (2, 17). Overexpression of PKBα or β is associated with some human ovarian, pancreatic, and breast carcinomas (8, 18–20).

PKBα is activated by a variety of growth factors and phosphatase inhibitors (5, 6, 21) through a phosphorylation mechanism (21–23). The activation of PKBα by insulin or IGF-1 is mediated by phosphorylation of Thr308 in the catalytic domain and Ser473 at the C terminus (22). The phosphorylation of both sites is blocked by pretreatment of the cells with the PI3-K inhibitor wortmannin. Substitution of both regulatory sites by aspartic acid residues to mimic phosphorylation by the introduction of a negative charge, produces a constitutively active enzyme (22). This work predicted the existence of an upstream kinase(s) that phosphorylate(s) these sites, and recently a protein kinase activity was identified and purified capable of phosphorylating Thr308 in the presence of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) or phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) (Refs. 24 and 25; reviewed in Ref. 26). The enzyme has therefore been termed 3-phosphoinositide-dependent protein kinase-1 (PDK1).

The PH domain of PKB has been reported to play a role in the activation process (6), but PKB activation can also occur in its absence, depending on the agonist and the type of deletion mutants used (21, 23, 27). The PH domain of PKB binds PtdIns(3,4,5)P3 and PtdIns(3,4)P2 at low micromolar concentrations (28, 29), but the precise role of inositol phospholipid

gen-activated protein kinase-activated protein kinase; Pipes, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

2 P. Cron and B. A. Hemmings, unpublished data.

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binding to PKB is not fully understood (28–31). Since PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are located in the plasma membrane, the interaction of PKB with one or both of these phosphoinositides may play a role in recruiting PKB to the membrane. To address this question, we have added a membrane targeting signal to the N terminus of PKBα and find that this is sufficient for maximal phosphorylation of Thr320 and Ser473, and activation of PKBα in the presence or absence of the PH domain. Furthermore, we provide evidence that the IGF-1-induced activation of PKB is accompanied by its translocation to the membrane, followed by the translocation to the nucleus.

**Materials and Methods**

**Construction of Expression Vectors**—The pECE and pCMV constructs encoding hemagglutinin (HA) epitope-tagged PKBα have been described (21, 22). Myristoylated/palmitoylated (m/p)-HA-PKBα was created by polymerase chain reaction with HA-PKBα as template, using a 5′ oligonucleotide encoding the 12-amino acid N-terminal sequence of Lck, which carries the myristoylation/palmitoylation signal, followed by two Ala residues and the HA epitope, and a 3′ oligonucleotide encoding amino acids 468–480 of PKBα. The resulting product was subcloned as a SalI fragment into pECE.HA-PKBα. The pECE.HA-PKBα constructs encoding PKBα phosphorylation site mutants at Ser473 and Thr320, or both have been described (22). To create the membrane targeted versions of these mutants, the C/EBPα/EcoRI fragment of pECE.HA-PKBα-S473A and NotI/EcoRI fragment of pECE.HA-PKBα-T308A were subcloned into the respective restriction sites of pECE.m/p-HA-PKBα. The pECE construct encoding m/p-HA-PKBα-αPHI was made by polymerase chain reaction using a 5′ oligonucleotide encoding the 12-amino acid N terminus of Lck, followed by two Ala residues, the HA epitope and amino acids 119–125 of PKBα, and a 3′ oligonucleotide encoding amino acids 468–480. The product was subcloned as a SalI/EcoRI fragment into the same restriction sites of the pECE vector (32). The Bg/II/XbaI fragments from the above described pECE constructs were transferred into the same restriction sites of the pCMV5 vector (33). m/p-HA-PKBα-T308D/S473D was created by subcloning NotI/XbaI fragments from pECE-HA-PKBα-T308D/S473D and pECE.HA-PKBα-ΔC14 into pCMV5.m/p-HA-PKBα. The constructs were confirmed by restriction analysis and sequencing. The constructs pRS-A-p85α 478–513 has been described (34).

**Cell Culture**—Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc.) at 37 °C, in an atmosphere containing 5% CO2. Cells seeded at 10^6/10-cm dish and 0.5% calf serum (FCS; Life Technologies, Inc.) at 37 °C, in an atmosphere supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.).

**Immunofluorescence**—Cells were transfected the following day by a modified calcium phosphate method (35), with 1–2 μg of DNA constructs containing 30 μl of immunoprecipitation in kinase buffer, 30 μM peptide GRPRTSFFAEQ as substrate (11), 10 mM MgCl2, 1 μM protein kinase A inhibitor peptide (Bachem), and 50 μM ATP (Amersham; 1,000–2,000 cpm/μmol) and the activity determined as described previously (22).

**Immunoblot Analysis**—Cell extracts and immunoprecipitates were resolved by 7.5% SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon P membranes (Millipore). The filters were blocked for 30 min with 5% skimmed milk in 1 × Tris-buffered saline, 1% Triton X-100, 0.5% Tween 20, followed by a 2-h incubation with 50-fold diluted rabbit polyclonal anti-PKBα antibody specific for the C terminus (Abcam, Inc. Cat. No. 46948, Ref. 4), or recombinant PH domain containing N-terminal 131 amino acids (Ab72/335) or with the anti-HA epitope 12CA5 monoclonal antibody that was 1,000-fold diluted in the same blocking solution. The secondary antibodies were 1,000-fold diluted alkaline-phosphatase conjugated anti-rabbit IgG (Sigma) and anti-mouse Ig (Southern Biotechnology Associated), or 5,000-fold diluted horseradish peroxidase-linked Ig (Amersham). Detection was performed using the AP color development reagents from Bio-Rad or by enhanced chemiluminescence (Amersham). To normalize expression levels of PKB, FITC-labeled secondary antibodies were employed at a 200-fold dilution, the signal detected by chemiluminescence using a Storm 840/860 PhosphorImager and quantified with ImageQuant Software (Molecular Dynamics).

**Immunofluorescence**—293 cells were plated and transfected on sterile cover slips in 12-well plates for immunofluorescence. Cells were serum-starved for 24 h when fixation with 0.2% Triton X-100 were performed according to Ref. 37. The 12CA5 monoclonal antibody diluted 50-fold in phosphate-buffered saline (PBS) was applied for one hour at 37 °C. The cells were subsequently washed twice with PBS and incubated with FITC-conjugated anti-mouse IgG (Sigma) at a 1:50-fold dilution, or with 100-fold diluted biotinylated anti-mouse IgG (Sigma), followed by 200-fold diluted streptavidin coupled to Texas Red (Amersham). DNA was stained with 4,6-diamidino-2-phenylindole. The coverslips were washed twice with PBS, once with H2O, mounted on glass slides using Gelvatol, and photographed with a LEITZ DMRD Leica camera. Confocal images were collected on a Leica TCS 4D microscope. REF-52 cells were subcultured on either 25-mm glass coverslips (Schutt Labortechnik, Göttingen, Germany) or acid-washed coverslips. Microinjection was performed with a normal Leitz micromanipulator, as described previously (38). Cells were injected with 0.5 mg/ml PKB construct in the presence of 1 mg/ml biotinylated rabbit IgG (Sigma). Cells were serum-starved for 36–48 h before stimulation with 1 μM okadaic acid/10% FCS. Cells were fixed with 3.7% formaldehyde and further treated as described previously (36). PKB was detected using Ab60624989 followed by a FITC-conjugated anti-rabbit antibody. HA-PKBα was detected with the 12CA5 monoclonal antibody followed by a FITC-conjugated anti-mouse antibody. Microinjected cells were identified by containing the cells with streptavidin coupled to Texas Red (Amersham). DNA was stained using Hoechst stain 33342 (1 μg/ml bisbenzimide).

**Immunoelectron Microscopy and Quantification**—The cells were fixed in 4% formaldehyde in 0.2 M Pipes, pH 7.2, for at least 20 min, washed twice with PBS, scraped from the dish using a rubber policeman, and embedded in 10% pig skin gelatin before cryoprotection in 2.3 M sucrose in Ultratwin sections. Ultratwin sections were cut at −110 °C in a Reichert Ultratc E cryomicrotome, mounted on carbon/Formvar-coated grids, and labeled using the 12CA5 monoclonal antibody followed by a rabbit anti-mouse antibody and finally protein A gold (7 or 5 nm particle size prepared as described by Luocoz. Ref. 39). Sections were embedded in methylcellulose uranyl acetate as described in Ref. 40. To quantitate immunolabeling, sections were scanned systematically and gold label identified. All visible parts of the plasma membrane and adjacent cytoplasm of labeled cells were photographed at magnification ×15,000. Cytoplasm areas and membrane profile length were estimated using a square lattice grid with 1-cm line spacing as described in Ref. 41. Gold particles over the nucleus were not included in the analysis and gold particles were only assigned to the plasma membrane if they lay within 2 particle widths of the plasma membrane profile.

**32P Labeling of 293 Cells and Immunoprecipitation of HA-PKBα**—293 cells transfected with HA-PKBα DNA constructs were serum-starved overnight, washed twice with phosphate-free DMEM, and incubated for 4 h (Fig. 5) or 12 h (Fig. 10) with carrier-free [32P]orthophosphate (1 mCi/ml) in either the presence or absence of 100 nM IGF-1. The cells were harvested for 4 h (Fig. 5) or 12 h (Fig. 10) after addition of 100 ng/ml IGF-1 and placed on ice. The medium was aspirated, the cells washed twice with ice-cold DMEM washed in PBS, scraped from the dish using a rubber policeman, and mixed with 30 μl of immunoprecipitation in kinase buffer, 30 μM peptide GRPRTSFFAEQ as substrate (11), 10 mM MgCl2, 1 μM protein kinase A inhibitor peptide (Bachem), and 50 μM [γ-32P]ATP (Amersham; 1,000–2,000 cpm/μmol) and the activity determined as described previously (22).
RESULTS

Membrane Targeting Promotes the Activation of PKBα—We previously proposed that PKB activation occurs by phosphorylation, following recruitment of the kinase to the membrane via its PH domain (21, 22). To investigate the role of membrane targeting in the activation of PKB, the N-terminal membrane localization sequence from Lck was attached to the N terminus of HA-PKBα (see Fig. 1). This signal was chosen because it contains the consensus sequence for both myristoylation and palmitoylation (42) and has been shown to be sufficient to localize a number of cytosolic proteins to the plasma membrane (43). Several mutants of m/p-HA-PKBα were also prepared in which the ATP-binding site (Lys179) or the phosphorylation sites (Thr308, Ser473) were mutated to Ala, or in which the N-terminal 118 amino acids containing the PH domain, or the C-terminal 14 amino acids encompassing the Ser473 phosphorylation site were deleted (Fig. 1).

To confirm that the Lck myristoylation/palmitoylation signal provides membrane attachment of m/p-HA-PKBα, we determined the subcellular localization of the proteins expressed in 293 cells by immunofluorescence using the anti-HA epitope antibody. HA-PKBα was found in the cytosol of serum-starved, unstimulated 293 cells (Fig. 2, A and B). However, all forms of m/p-HA-PKBα (Fig. 2, C–G) were highly concentrated at the plasma membrane. No immunostaining occurred if the 293 cells were transfected with vector alone (Fig. 2H). Overexpression of m/p-HA-PKBα, m/p-HA-PKBα-S473A, or m/p-HA-PKBα-ΔPH in 293 cells resulted in rounding of the cells, which was not observed when either wild-type HA-PKB or other m/p-HA-PKBα mutants were overexpressed.

The activity of m/p-HA-PKBα in unstimulated cells was over 60-fold higher than that of HA-PKBα. This is higher than the activity of PKBα obtained after stimulation of the wild-type kinase with insulin, IGF-1, or vanadate (Fig. 3A). Consistent with this finding, the activity of m/p-HA-PKBα could not be increased further by stimulation of the cells with insulin, IGF-1, or vanadate (Fig. 3A). m/p-HA-PKBα from unstimulated cells, like HA-PKBα from IGF-1-stimulated cells, could be in-

Fig. 1. Schematic presentation of membrane-targeted PKBα mutants expressed in 293 cells. PKBα was tagged at the N terminus with the HA epitope. To achieve membrane localization of HA-PKBα, the Lck myristoylation/palmitoylation signal (MGCGCSS-NPEDD) was added to the N terminus. The predicted Mr presented here accommodate the Lck-derived sequence and the HA epitope.

Fig. 2. Intracellular localization of wild-type and membrane-targeted PKBα in unstimulated cells. 293 cells grown and transfected on coverslips were stained with the anti-HA epitope 12CA5 monoclonal antibody, followed by a FITC-conjugated secondary antibody to reveal the presence of the epitope-tagged protein in the cytosol or membrane. A, cells transfected with HA-PKBα show cytoplasmic staining by immunofluorescence. B, the same cells stained with 4,6-diamidino-2-phenylindole, confirming that HA-PKBα is absent from the nucleus. C, m/p-HA-PKBα; D, m/p-HA-PKBα-K179A; E, m/p-HA-PKBα-S473A; F, m/p-HA-PKBα-T308A; G, m/p-HA-PKBα-ΔPH; H, mock transfected cells.
PKB Activation by Recruitment

**Fig. 3.** Effects of membrane targeting on the activity of wild-type and mutant PKBα expressed in 293 cells. A, cells transfected with HA-PKBα, m/p-HA-PKBα, K179A, S473A, T308A, ΔPH, HA-PKBα-ΔC14, and m/p-HA-PKBα-ΔC14 were serum-starved for 24 h prior stimulation with buffer, 100 nM insulin, 50 ng/ml IGF-1, or 0.1 mM 3-PT. PKB was immunoprecipitated with the anti-HA epitope 12CA5 monoclonal antibody and kinase activity determined. Kinase activity is the average (± S.D.) of two experiments each with duplicate immunoprecipitates and is presented relative to HA-PKBα activity from unstimulated cells. The activity was corrected to different expression levels of the constructs. B, immunoblot showing expression and migration of HA-PKBα and membrane-targeted mutants. Proteins were immunoprecipitated with the anti-HA epitope 12CA5 monoclonal antibody from the extracts of serum-starved cells to obtain comparable amounts of PKB, and were detected with Ab469/480 (left panel), or in the case of the ΔC14 mutants with Ab17/213 (right panel). The apparent M₆ of HA-PKBα and HA-PKBα-ΔPH are indicated.

Activated in vitro by treatment with protein phosphatase 2A in vitro (data not shown). No PKB activity was detected when kinase-inactive m/p-HA-PKBα-K179A was expressed in 293 cells (Fig. 3A).

The intracellular localization of wild-type HA-PKBα and m/p-HA-PKBα were confirmed by biochemical studies. About 80% of HA-PKBα activity and 75% of HA-PKBα protein were detected in the 100,000 × g supernatant (S100) of unstimulated 293 cells, whereas virtually all of the m/p-HA-PKBα activity and protein was recovered in the 100,000 × g pellet (P100) (Fig. 4, A and B).

**Membrane-targeted PKBα Is Constitutively Active due to Phosphorylation on Thr³⁰⁸ and Ser⁴⁷³—**The m/p-HA-PKBα-T308A and m/p-PKBα-S473A mutants possessed only ~2% and ~10–15% of the activity, respectively, of the membrane-targeted wild-type enzyme in unstimulated or stimulated 293 cells (Fig. 3A), suggesting the involvement of these sites in the activation of m/p-HA-PKBα. Furthermore, all membrane-targeted forms of the kinase expressed in 293 cells displayed reduced electrophoretic mobility indicative of phosphorylation (Fig. 3B). To establish which residues in PKB were phosphorylated in vivo, 293 cells were ³²P-labeled, and either m/p-HA-PKBα or HA-PKBα was immunoprecipitated, digested with trypsin, and the resulting phosphopeptides analyzed by C18 chromatography. As observed previously (22), two peptides phosphorylated on Ser⁴⁷³ and Thr³⁰⁸ were obtained from HA-PKBα immunoprecipitated from unstimulated cells (Fig. 5A), while stimulation with IGF-1 induced the appearance of two further phosphopeptides labeled on Thr³⁰⁸ and Ser⁴⁷³, without significantly affecting the labeling of Ser¹²⁹ and Thr³⁵⁰ (Fig. 5B). In contrast, m/p-HA-PKBα from unstimulated cells was heavily phosphorylated on residues Thr³⁰⁸ and Ser⁴⁷³, as well as at Ser¹²⁹ and Thr³⁵⁰ (Fig. 5C), and IGF-1 treatment did not lead to any further increase in the ³²P labeling of m/p-HA-PKBα at Thr³⁰⁸ or Ser⁴⁷³ (Fig. 5D). Membrane targeting led to more efficient incorporation of phosphate into Thr³⁰⁸ and Ser⁴⁷³ than did IGF-1 stimulation of HA-PKBα (Fig. 5, B–D). m/p-HA-PKBα was also phosphorylated on two peptides each containing phosphothreonine (peptides TX1 and TX2 in Fig. 5C), that were absent in HA-PKBα (Fig. 5A).

Thr³⁰⁸ and Ser⁴⁷³ were also heavily phosphorylated in the membrane-targeted mutant, which lacks the PH domain in both unstimulated and IGF-1-stimulated cells (Fig. 5, E and F). Consistent with this finding, m/p-HA-PKBα-ΔPH was as active as m/p-HA-PKB. Significantly, only the TX1 phosphopeptide was detected in m/p-HA-PKBα-ΔPH, implying that TX2 residues within the N-terminal 118 amino acids and that it is not important for the activation by membrane targeting. The TX1
peptide was found to be phosphorylated on Thr at position 4, and predicted tryptic cleavage sites suggested that it could be Thr371. However, this phosphopeptide was found not to be crucial for m/p-HA-PKBα activity (see below), and further characterization of this phosphorylation site was not pursued.

Deletion of the C-terminal 14 amino acids of HA-PKBα prevented kinase activation by insulin, IGF-1, or vanadate, and m/p-HA-PKBα-D14 possessed a similarly low activity in unstimulated or stimulated cells (Fig. 3A). This suggests that the extreme C terminus not only contains an activating phosphorylation site, but may also provide an important conformational determinant for activation.

The kinase-inactive m/p-HA-PKBα-K179A mutant (which was expressed at much lower levels than m/p-HA-PKBα) was phosphorylated on Thr450 and Thr308 in unstimulated cells, but the level of phosphorylation of Ser473 was much lower (Fig. 5G). Following IGF-1 stimulation, 32P-incorporation into Thr450 doubled and incorporation into Ser473 increased 5-fold (Fig. 5H). As reported previously for HA-PKBα-K179A (22), the 32P labeling of Ser124 was extremely low in the membrane-targeted, kinase-inactive mutant (Fig. 5G).

**PKB Activation by Recruitment**

FIG. 5. Tryptic phosphopeptides of wild-type and membrane-targeted HA-PKBα mutants. 293 cells expressing the indicated form of HA-PKBα were incubated with 32P for 3 h, and then stimulated for 10 min with either buffer or 100 ng/ml IGF-1. The cells were lysed and the HA-PKB immunoprecipitated with the anti-HA epitope 12CA5 monoclonal antibody, digested with trypsin, and analyzed by chromatography on a C-18 column as described under "Materials and Methods." The figure shows the tryptic phosphopeptide maps of HA-PKBα, m/p-HA-PKBα, m/p-HA-PKBα-ΔPH, and m/p-HA-PKBα-K179A from unstimulated (A, C, E, and G) and IGF-1-stimulated cells (B, D, F, and H). Each 32P-labeled peptide that eluted from the C18 column was subjected to phosphoamino acid analysis, and the location of each phosphorylation site was established by solid phase sequencing in which the 32P radioactivity released after each cycle of Edman degradation was measured (51). The peptides corresponding to Ser124 (S124), Thr308 (T308), Thr450 (T450), and Ser473 (S473) contained the expected phosphoamino acid and released 32P radioactivity at the 3rd, 1st, 14th, and 8th cycles of Edman degradation as expected (Ref. 22 and data not shown). Similar results were obtained in three separate experiments.
PI3-K Activity—A mutant form of the p85 regulatory subunit of PI3-K (Δp85) that fails to bind and activate the p110 catalytic subunit has been reported to act as a dominant negative mutant of this enzyme (34) and to abolish platelet-derived growth factor-induced activation of PKB in cotransfection experiments (5). Coexpression of Δp85 with HA-PKBα reduced insulin-induced PKB activation by 80% (Fig. 6A), but had no effect on the activity of m/p-HA-PKBα in unstimulated cells (Fig. 6B).

To gain further insight into the regulation of m/p-HA-PKBα cells were treated with inhibitors of PI3-K, following a 12-h serum starvation. Treatment with either 250 nM wortmannin or 100 μM LY 294002 for 3 h reduced m/p-HA-PKBα activity by ~50% (data not shown). However, longer treatment could only be performed with LY 294002, as prolonged wortmannin treatment was toxic. Following a 12-h incubation with LY 294002 in the absence of serum, m/p-HA-PKBα and m/p-HA-PKBα-S473A activities were reduced by ~80% and ~60%, respectively, and the activity of m/p-HA-PKBα was rapidly restored to the initial level within 30 min following the removal of LY 294002 (Fig. 7, A and B). During this 30-min period, no activity changes for untreated or LY 294002-treated m/p-HA-PKBα were observed (data not shown). LY 294002 withdrawal did not promote the activation of wild-type HA-PKBα (data not shown). The activation of m/p-HA-PKBα in the absence of any growth factors after the inhibitor removal could be explained by an autocrine loop mechanism in which membrane-targeted kinase might induce the production and secretion of a signal, which then stimulates the activation of PI3-K. However, this possibility is unlikely, since we found that conditioned medium obtained from cells expressing m/p-HA-PKBα failed to activate the endogenous PKB in unstimulated 293 cells (data not shown).

The 32P-phosphorylation of m/p-HA-PKBα was reduced by ~50% following a 12-h treatment with 100 μM LY 294002, due to dephosphorylation on Ser473 (>70%), Thr450 (60%), and Thr308 (40%) (Fig. 10). There was no change in the 32P labeling of the peptide containing Ser114 or of peptide ThrX1 (Fig. 8). PKB Associates with the Plasma Membrane and Subsequently Translocates to the Nucleus Following IGF-1 Stimulation—To investigate whether dynamic changes in the subcellular distribution of PKB occur during cell stimulation, we employed quantitative immunogold localization on ultrathin cryosections. In unstimulated 293 cells expressing HA-PKBα, there was substantial immunolabeling in the cytoplasm with little evidence for membrane localization (Figs. 9A and 10A). However, after stimulation for 2 min with IGF-1, there was clear evidence for accumulation of gold labeling at the plasma membrane (Fig. 9A). Labeling was more marked 5 min after stimulation with IGF-1, by which time the concentration of
epitope 12CA5 monoclonal antibody and the 32P-phosphopeptides isolated as described in the legend to Fig. 5. The amount of 32P in the HPLC fractions containing peptides corresponding to Ser 124, Ser 473, Thr308, Thr450, and peptide TX1 was determined. The results are shown as cpm/mg, which is defined as the 32P radioactivity measured in each HPLC peak divided by the amount of protein (mg) in the cell lysate used to immunoprecipitate m/p-HA-PKBa. The results are presented as the average (± S.E.) from three experiments each carried out in duplicate.

The rapid translocation of HA-PKBa to the plasma membrane correlates with the rate of activation of PKB by IGF-1 in 293 cells, which is half-maximal after about 1 min and maximal after 5 min (data not shown). IGF-1-stimulated translocation of HA-PKBa to the plasma membrane was prevented by treatment of the cells with 100 nM wortmannin, and also in cells expressing the HA-PKBa-ΔPH mutant (Figs. 9B and 10C). Changes in PKB localization during IGF-1 stimulation were also monitored by indirect immunofluorescence. HA-PKBa was detected mainly in the cytoplasm of unstimulated 293 cells (Fig. 11A; see also Fig. 3, A and B). Membrane staining could be observed 2 and 5 min after IGF-1 stimulation (Fig. 11, B and C), whereas 30-min IGF-1 treatment led to considerable nuclear labeling, excluding nucleoli (Fig. 11D). To study immunolocalization of endogenous PKB, we used REF-52 cells treated with 1 μM okadaic acid, 10% FCS. These agents exert a synergistic effect on PKB activation (21), and their concomitant application resulted in maximal stimulation of kinase activity in REF-52 fibroblasts (data not shown). Significant nuclear staining could be detected following 15-min stimulation of quiescent REF-52 cells with 1 μM okadaic acid, 10% FCS, which increased further with 50-min treatment (Fig. 12A). Similar changes in subcellular distribution were observed upon 30-min stimulation of REF-52 fibroblasts expressing HA-PKBa (Fig. 12B), as well as kinase-deficient HA-PKBa-K179A (data not shown). Taken together, the data indicate that PKB transiently associates with the plasma membrane of stimulated cells, which is followed by translocation to the nucleus.

DISCUSSION

Growth factor-induced activation of PKBα is mediated by PI3-K (5, 6, 11, 27), and inhibitors of PI3-K prevent the insulin- and IGF-1-induced phosphorylation of PKB on Thr308 and Ser473 (22). Based on the fact that the PI4 domain binds 3-phosphoinositides at low micromolar concentrations (28, 29), it was proposed that these lipids may act to recruit PKBα to the plasma membrane where the activating phosphorylations take place (15, 26). The present study has provided three pieces of evidence supporting this concept. First, HA-PKBα translocates to the plasma membrane within minutes following IGF-1 stimulation (Figs. 9 and 11), whereas an HA-PKBα mutant that lacks the PH domain that is required for membrane association of HA-PKBα, which is blocked by deletion of the PH domain or by wortmannin treatment. A, 293 cells expressing HA-PKBα were stimulated for 2, 5, and 20 min with 100 ng/ml IGF-1 before formaldehyde fixation and immunoelectron microscopy as described under “Materials and Methods.” B, cells expressing HA-PKBα or HA-PKBα-ΔPH were stimulated with buffer or 100 ng/ml IGF-1 for 5 min, and in the case of HA-PKBα treated with 100 nM wortmannin (WM) 10 min before and during IGF-1 stimulation. Quantification was carried out as described under “Materials and Methods.” Results are expressed as ratio estimates (± S.E.) from three experiments with n ≥ 10 per condition for A, or from two experiments with n ≥ 8 per condition for B.

FIG. 8. Effects of LY 294002 on 32P labeling of membrane-targeted PKBα. 293 cells expressing m/p-HA-PKBα were incubated with 32P either in the presence or absence of 100 μM LY 294002 for 12 h before lysis. m/p-HA-PKBα was immunoprecipitated with the anti-HA epitope 12CA5 monoclonal antibody and the 32P-phosphopeptides isolated as described in the legend to Fig. 5. The amount of 32P in the HPLC fractions containing peptides corresponding to Ser 124, Ser 473, Thr308, Thr450, and peptide TX1 was determined. The results are shown as cpm/mg, which is defined as the 32P radioactivity measured in each HPLC peak divided by the amount of protein (mg) in the cell lysate used to immunoprecipitate m/p-HA-PKBα. The results are presented as the average (± S.E.) from three experiments each carried out in duplicate.

FIG. 9. IGF-1 induces plasma membrane association of HA-PKBα, which is blocked by deletion of the PH domain or by wortmannin treatment. A, 293 cells expressing HA-PKBα were stimulated for 2, 5, and 20 min with 100 ng/ml IGF-1 before formaldehyde fixation and immunoelectron microscopy as described under “Materials and Methods.” B, cells expressing HA-PKBα or HA-PKBα-ΔPH were stimulated with buffer or 100 ng/ml IGF-1 for 5 min, and in the case of HA-PKBα treated with 100 nM wortmannin (WM) 10 min before and during IGF-1 stimulation. Quantification was carried out as described under “Materials and Methods.” Results are expressed as ratio estimates (± S.E.) from three experiments with n ≥ 10 per condition for A, or from two experiments with n ≥ 8 per condition for B.
The identity of the kinase which phosphorylates PKBα at Ser473 is not yet known. MAPKAP kinase-2 is able to phosphorylate PKBα in vitro on Ser473, but it does not appear to be the in vivo PKBα kinase, as it is activated by stress stimuli and proinflammatory cytokines, which do not activate PKBα in the cells that we have studied (22). Moreover, the drug SB 203580, which prevents the activation of MAPKAP kinase-2 in cells (44), has no effect on the activation of PKBα by insulin or IGF-1 (22).

The PH domain was not required for the activation of membrane-targeted PKBα (Fig. 3A), indicating that it is not essential for phosphorylation of m/p-HA-PKBα on Thr308 and Ser473 by upstream protein kinase(s). Kohn et al. (23) also showed that a membrane-targeted form of PKBα lacking the PH domain was highly active in unstimulated cells. However, it should be noted that PKBα constructs lacking the PH domain can be activated by insulin and IGF-1 (23, 27), under conditions where we detected no apparent translocation of the protein.

Basal PI3-K activity is required to maintain m/p-HA-PKBα in its phosphorylated, active state. This implies that the role of PI3-K in PKB activation is twofold: one is to provide membrane translocation in cooperation with the PH domain thus “priming” the kinase for the activation, and second is to enable phosphorylation and activation. Membrane targeting circumvents the former regulatory step, yielding the fully active, fully phosphorylated m/p-HA-PKBα. This is different from v-Akt or Gag-PKB, whose activity can be stimulated further by mitogens in a wortmannin-sensitive manner (5, 45). It appears therefore that the attachment of a short peptide carrying the Lck membrane-targeting motif provides an optimal conformation for maximal PKB activity, without the activation of PI3-K. This is in agreement with the data from Stokoe et al. (25, 53), who demonstrated that PtdIns(3,4,5)P3, through its binding to the PH domain facilitates phosphorylation and activation of PKB by Thr308 kinase. In addition, the removal of the PH domain reduces or eliminates the requirement for PtdIns(3,4,5)P3 for phosphorylation of Thr308 (25, 53), which also explains the ability of HA-PKBαΔPH to be activated without the apparent membrane translocation. It therefore seems likely that the plasma membranes of unstimulated cells contain sufficient PtdIns(3,4,5)P3 to maintain the activity of Thr308 kinase/PDK1 at a high enough level to phosphorylate membrane-targeted PKBα at Thr308. PI3-K may also maintain m/p-HA-PKBα activity through the inhibition of PP2A (46), which is likely to be the in vivo PKB phosphatase (21).

The subcellular distribution of the wild-type kinase is tightly regulated. PKBα translocates to the plasma membrane, which can be detected after 2-min stimulation by IGF-1 (Figs. 9–11) or vanadate. The PH domain, which is required for the membrane translocation, may also be responsible for the detachment from the membrane, for example by binding inositol triphosphate that is generated in the cell though the action of mitogen-stimulated phospholipase Cγ (47). The mechanism by which PKB translocates to the nucleus is still not clear. Kinase activity is not required for this process, judged by the fact that HA-PKBα-K179A can also be detected in the nuclei of stimulated REF-52 cells. It is likely that the PH domain does not play a role in this process, as HA-PKBαΔPH was also found in the nuclei of stimulated REF-52 cells.5

According to the available data, we propose the following model for the regulation of PKB activity. After cell stimulation, the kinase translocates to the membrane, which allows a correct conformation for the activating phosphorylation. Following

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3 D. R. Alessi, unpublished data.
4 M. Andjelkovic and B. A. Hemmings, unpublished data.
5 A. Fernandez and N. J. C. Lamb, unpublished data.

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**FIG. 10.** Morphological demonstration of IGF-1-induced translocation of HA-PKBα to the plasma membrane, which can be prevented by wortmannin treatment. 293 cells expressing HA-PKBα were fixed and processed as described for Fig. 9. A, significant labeling of HA-PKBα in the cytoplasm of unstimulated cells with low labeling over the plasma membrane. B, the plasma membrane of cells stimulated with 100 ng/ml IGF-1 for 5 min is intensely labeled with gold particles and, in this case, there is little cytoplasmic label. C, lack of appreciable labeling at the plasma membranes in cells pretreated with 100 nm wortmannin for 10 min before IGF-1 stimulation. A and B, 7 nm protein A gold; C, 5 nm protein A gold. Bars, 100 nm.

**FIG. 11.** Changes in subcellular distribution of HA-PKBα during IGF-1 stimulation. 293 cells grown and transfected on coverslips were serum-starved for 12 h before stimulation with buffer or 100 ng/ml IGF-1. Cells were stained by the anti-HA epitope 12CA5 monoclonal antibody, followed by a biotinylated secondary antibody, and streptavidin-coupled to Texas Red and visualized by laser scanning microscopy. A, unstimulated cells showing mainly cytosolic staining. B and C, cells stimulated with IGF-1 for 2 and 5 min, respectively, with clear membrane staining. D, nuclear staining of cells stimulated with IGF-1 for 30 min.
activation, PKB detaches from the membrane, which, in turn, enables it to phosphorylate its cytosolic, as well as nuclear substrates. Correct subcellular localization is therefore crucial for the activation of the kinase, and it may also allow its appropriate inactivation by phosphatases.

Unlike the wild-type kinase, the form of PKBo that is constitutively localized to the membrane escapes the physiological spatial regulation. The finding that PKBo is activated by membrane targeting may explain the oncogenic potential of v-akt, which encodes a fusion protein between the viral Gag and PKBo (2). Significantly, 40% of the v-Akt protein is membrane-associated since it is myristoylated at the N terminus (17). It was reported that the Gag-PKBo fusion protein, which mimics v-Akt, has increased activity and stimulates p70S6K activity (5).
Furthermore, expression of this form of the kinase in 3T3-L1 preadipocytes is sufficient to elicit cellular responses that normally require insulin/IGF-1, such as glucose transport and differentiation (12, 48). The constitutively active, membrane-targeted form of PKB may also induce other insulin/IGF-1-evoked responses, such as cell survival caused by the inhibition of apoptosis (13, 45, 49, 50).

In summary, we have demonstrated that forced membrane localization is sufficient for its full activation as a result of the essentially stoichiometric phosphorylation of Thr^328 and Ser^473 by upstream kinases. Transient membrane association is required for the physiological activation of PKB. The characterization of cytosolic and nuclear substrates of PKB is now critical to understand how the kinase mediates physiological responses to insulin and IGF-1, as well as its role in cell survival and transformation.

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Note Added in Proof—Recently we have shown that PKBα is also constitutively activated by membrane targeting. Furthermore, stimulation of cells expressing PKBα also leads to nuclear translocation (Meier, R., Alessi, D. R., Cron, B., Andjelkovic, M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 30491–30497).