Involvement of the Pleckstrin Homology Domain in the Insulin-stimulated Activation of Protein Kinase B*

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Involvement of the pleckstrin homology (PH) domain in the insulin-stimulated activation of protein kinase B (PKB) was investigated in human embryonic kidney 293 cells. Different PKB constructs that contain mutations or deletions in the PH domain were transfected into cells, and the results on the basal and insulin-induced kinase activities were analyzed. Deletion of the entire PH domain (ΔPH-PKB) did not impair the kinase activity; in contrast, the basal activity was elevated with respect to wild-type PKB. In addition, ΔPH-PKB was responsive to insulin, and as for wild-type PKB, this was dependent on phosphoinositide 3-kinase. By contrast, a point mutation within the PH domain that impairs phospholipid binding (R25C) resulted in a construct that was not responsive to insulin. However, this defect was overcome by mutations that mimic the phosphorylation state of the active kinase. The increase in the basal activity of ΔPH-PKB was shown to be due to an elevation in the level of phosphorylation of this construct. In addition, the subcellular localization of ΔPH-PKB, as determined by both immunofluorescence and fractionation, was predominately cytosolic, and ΔPH-PKB was present in the plasma membrane at much lower levels compared with wild-type PKB. These data show that phosphorylation is the major factor regulating the activity of PKB and that either removal of the PH domain or binding of phospholipids is required to permit this phosphorylation. In addition, membrane localization does not appear to be required for the activation process, but instead, binding of PKB to membrane phospholipids permits a conformational change in the molecule that allows for phosphorylation.

Protein kinase B (PKB),1 also referred to as Akt or RAC kinase, is a serine/threonine protein kinase that was cloned by virtue of its homology to protein kinases A and C and is the cellular homolog of the product of the v-akt oncogene (1–4). The kinase is activated in response to treatment of cells with agonists that bind to receptors possessing tyrosine kinase activity such as those of platelet-derived growth factor and insulin (5–7). The activity of PKB in response to these agents is strictly dependent on the activity of phosphoinositide 3-kinase (PI3K) since (i) it is sensitive to wortmannin, an inhibitor of PI3K; (ii) mutants of the platelet-derived growth factor receptor that cannot interact with PI3K are incapable of PKB activation; and (iii) constitutively active forms of PI3K are able to activate PKB in intact cells (5–7). The effect of 3'-phosphoinositides is believed to be mediated in part by the N-terminal pleckstrin homology (PH) domain of PKB. In addition, PI3K-independent activation of PKB has been demonstrated in response to heat shock, β-adrenergic receptor activation, and cAMP (8–10).

To date, PKB has been implicated in physiological processes regulating both cellular growth and metabolism. The first identified substrate of PKB was glycogen-synthase kinase-3, which is inhibited in vivo after phosphorylation by PKB (11, 12). In addition, PKB can phosphorylate phosphofructo-2-kinase (13) and is involved in the regulation of glucose transport (14, 15). Membrane-targeted constitutively active PKB has been shown to promote the activation of p70S6K (5, 16), although the physiological relevance of this is questionable. More recently, evidence has been accumulating that indicates that PKB plays a major role in protection of cells from apoptosis (17–23). This may be mediated in part by the ability of PKB to phosphorylate BAD, a protein implicated in cell survival decisions (24, 25).

Currently, a two-step model exists to explain stimulation of PKB in response to growth factors. First, the activation of PI3K subsequent to engagement of growth factor receptors leads to the production of PtdIns3,4,5-P3, which can then be dephosphorylated by a specific phospholipid phosphatase to PtdIns-3,4-P2 (26). This phospholipid product has been shown to bind PKB via the PH domain and may serve to localize PKB to the plasma membrane and/or to directly activate the kinase (27–29). Second, the activation of PKB requires phosphorylation of threonine 308 and serine 473 by two separate kinases (30). Recently, a kinase that phosphorylates PKB at Thr-308 has been identified by two independent groups (31, 32). This kinase requires PtdIns3,4,5-P3 to phosphorylate PKB and thus has been called phospholipid-dependent kinase-1 (PDK1) (31, 33). This describes a direct link between the products of PI3K and activation of a downstream event.

At present, the involvement of the PH domain in the activation of PKB remains controversial. In addition to PKB, PH domains have been found in a large number of proteins; however, to date, their physiological role is not entirely clear (for reviews, see Refs. 34 and 35). Generally, they interact with phospholipids, although in some cases, they have also been demonstrated to interact with proteins, most notably G protein βγ subunits (36). Because membrane localization has been shown to be important in regulating the function of many PH
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In this report, we attempt to further clarify the role of the PH domain in the activation of PKB. We show that a construct lacking the PH domain has an increase in its activity prior to stimulation that can be ascribed to a high level of basal phosphorylation. Furthermore, in contrast to wild-type PKB, ∆PH-PKB is found predominately in the cytosol, indicating that membrane localization is not necessary for the activation of PKB. These findings show that the PH domain of PKB plays a unique role in the activation process, and rather than localization, this domain appears to be involved in regulating the phosphorylation of PKB by upstream activating kinases.

EXPERIMENTAL PROCEDURES

Materials—Culture media and Genetin were from Life Technologies, Inc. Reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. Enzymes for molecular biology were from New England Biolabs Inc. (Beverly, MA). All chemicals not otherwise noted were from Sigma. Insulin was a kind gift from Novo-Nordisk (Copenhagen, Denmark). ϴ-32P]ATP was purchased from ICN (Orsay, France). The QuickChange™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA). All oligonucleotides were from Eurogentec (Seraing, Belgium). The T7 Sequencing™ kit was from Amersham Pharmacia Biotech (Uppsala, Sweden), and plasmid purification kits described previously (43). Site-directed mutagenesis was performed using the QuickChange™ mutagenesis kit. The GFP-PKB fusion protein was created by cloning PKB into EcoRI/BamHI sites within the pEGFP-N1 vector (CLONTECH, Palo Alto, CA).

Activity of Various PH Domain Mutants Expressed in 293-EBNA Cells—To study the role of the PH domain in the activation of PKB, we tested the kinase activity of two different PH domain mutants when overexpressed in human embryonic kidney 293 cells (Fig. 1). Consistent with other reports, we found that the kinase activity was completely abolished in the R25C mutant of PKB, but due to the elevation in the basal activity of PKB, stimulation was not as pronounced as in the wild-type construct (11-fold for wild-type PKB). However, due to the elevation in the basal activity of ∆PH-PKB, the enhancement of the kinase activity after insulin stimulation was not as pronounced as in the wild-type construct (11-fold for wild-type PKB versus 5.7-fold for ∆PH-PKB). These differences were not due to different levels of protein expression as judged by Western blot analysis (data not shown). The ability of this mutant to respond to insulin is consistent with other reports (7, 16) and demonstrates that the

DNA/peptide by the calcium phosphate method. Two days after transfection, cells were analyzed by immunofluorescence.

Immunoprecipitation and In Vitro Kinase Assay—Transfected 293-EBNA cells were stimulated or not with 1 μM insulin for 5 min. When appropriate, cells were preincubated with 100 nM wortmannin for 15 min at 4 °C. Cell lysate was prepared by lysing cells in buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM EDTA, 10 mM Na3PO4, 0.5 mM sodium orthovanadate, 100 μM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 1% (v/v) Triton X-100 for 15 min at 4 °C. The lysates were clarified by centrifugation at 15,000 × g for 15 min at 4 °C and immunoprecipitated using an anti-hemagglutinin antibody (12CA5) coupled to protein G-Sepharose. After washing, the immunoprecipitates were assayed using 3.7% paraformaldehyde for 15 min at room temperature. Cells were then washed, treated with 50 mM ammonium chloride, and rewarmed. Staining of the membrane was accomplished by incubating the cells in a humid chamber for 30 min with rhodamine-conjugated WGA (10 μM). Coverslips were mounted onto slides using Mowiol and viewed using a Leica upright confocal microscope equipped with a Leica 100 × objective (1.4 numerical aperture). The molecules were excited with the 600 line of an argon-krypton laser and imaged using either a 530-nm (GFP) or 600-nm (rhodamine) band-pass filter. Images were acquired with a scanning mode format of 256 × 256 pixels and recorded in the range of 0–255. The two confocal emission images were superimposed on a high-resolution monitor and photographed in pseudocolor mode using Kodak Ektachrome 100 ASA film.

Subcellular Fractionation of 293 Cells—293 cells transfected with PKB constructs were washed three times with phosphate-buffered saline and then lysed in 1 ml of 20 mM Tris, pH 7.4, 250 mM sucrose, and 1 mM EDTA including protease and phosphatase inhibitors using a Thomas-Potter type C homogenizer. Subcellular fractions were prepared by differential centrifugation as described (15). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene difluoride membranes and immunoblotting with an anti-PKB antibody raised against a peptide containing amino acids 469–480 of PKB (provided by one of us (B. A. H.). Proteins were revealed using incubation with 125I-protein A followed by autoradiography.

RESULTS

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EXPERIMENTAL PROCEDURES

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PH domain is dispensable for activation of PKB in response to insulin.

We next wanted to determine whether the activation of ΔPH-PKB proceeds by mechanisms that are similar to those that also stimulate wild-type PKB. We first tested whether the activation of ΔPH-PKB was also PI3K-dependent by examining the effect of wortmannin on the kinase activity of this mutant in response to insulin. As shown in Fig. 2A, wortmannin can completely inhibit the insulin-induced kinase activity of both wild-type PKB and ΔPH-PKB. These differences were not due to variation in the level of protein expression (Fig. 2A, lower panel). However, wortmannin had no effect on the high basal activity of ΔPH-PKB. These results indicate that PKB can be activated by insulin in a PI3K-dependent manner via a mechanism that does not rely on the integrity of the PH domain.

To further characterize the activity of ΔPH-PKB, we compared its kinetics of activation with those of wild-type PKB. Although ΔPH-PKB can be activated in response to insulin stimulation, we hypothesized that this process may be less efficient than for the wild-type enzyme, resulting in a delay in the kinetics. Previous reports have shown that PKB is activated rapidly in response to insulin and reaches a maximum by 5 min (11). We also observed that, in our system, PKB was rapidly activated by insulin (Fig. 2B). The time to maximal activation of ΔPH-PKB was similar to that of wild-type PKB, indicating that the stimulation occurs through similar mechanisms. Therefore, it is likely that ΔPH-PKB is phosphorylated by activating kinases equally efficiently as wild-type PKB and that the PH domain is not critical for recognition by this kinase(s).

Effect of Activating Mutations on the Kinase Activity of R25C PKB—To date, the role of phospholipids in the activation of PKB is still not entirely clear. Since deletion of the PH domain of PKB does not impair the kinase activity, it appears that the interaction of phospholipids with PKB is not necessary for activation. However, R25C PKB lacks kinase activity (Fig. 1); this could be potentially due to a conformational change resulting from the mutation that renders the kinase inactive. To test whether this mutant is catalytically active, the two residues that are phosphorylated in response to insulin stimulation were mutated to acidic amino acids to mimic the effect of phosphorylation (T308D and S473D). These mutations have been shown to result in a PKB that is constitutively active (30).

We made these activating mutations within R25C PKB (R25C-CA) and tested the effect on the kinase activity (Fig. 3A). The result of the mutations was an increase (4.5-fold) in the basal kinase activity when compared with wild-type PKB, which could not be further increased by insulin. In contrast with other reports, we found that these mutations in wild-type PKB did not result in a kinase that possessed activity equivalent to that after stimulation by insulin (Fig. 3A). This could be due in part to the lower levels of expression of this mutant. Therefore, we also determined the kinase activity of the different constructs normalized to the protein expression (Fig. 3B). From this, it is evident that the activities of constitutively active PKB and R25C-CA are similar and that R25C-CA is significantly more active than wild-type PKB. These data indicate that, once phosphorylated, PKB activity is no longer dependent upon interaction of the PH domain with phospholipids. This implies that the binding of phospholipids to the PH domain of PKB is a prerequisite for phosphorylation of the enzyme.

The Increased Basal Activity of the ΔPH PKB Mutant Results from an Increased Level of Phosphorylation—We hypothesized that binding of phospholipids to the PH domain of PKB changes the conformation, thus permitting phosphorylation and activation of PKB. Deletion of the PH domain could potentially mimic the effect of phospholipid binding and thereby also relieve the constraint on PKB phosphorylation. Therefore, the increased basal activity of the ΔPH mutant could be due to an increase in its level of phosphorylation in nonstimulated cells. To test this, we determined whether the kinase activity of ΔPH-PKB could
be prevented by dephosphorylation. We treated PKB immunoprecipitates from overexpressing cells with calf intestinal phosphatase prior to the kinase reaction to dephosphorylate the PKB constructs. To ensure that the phosphatase was indeed having an effect on the phosphorylation of PKB and not inhibiting the subsequent kinase assay, we also treated the constitutively active PKB mutant with calf intestinal phosphatase. As shown in Fig. 4A, the insulin-stimulated kinase activation of both the wild-type and ΔPH forms of PKB was abolished by treating the immunoprecipitates with calf intestinal phosphatase. Phosphatase treatment had no effect on constitutively active PKB, indicating that the phosphatase was most likely affecting the phosphorylation state of wild-type PKB and ΔPH-PKB. In addition, the basal activity of ΔPH-PKB was reduced after treatment with phosphatase. These results indicate that the increase in the kinase activity in nonstimulated cells is due to an increased level of phosphorylation of ΔPH-PKB.

Localization of ΔPH-PKB in 293 Cells—It has been proposed that the role of the PH domain is to localize the kinase on the membrane, where in can be activated, possibly by membrane-associated kinases. However, our observation that the PH domain prevents the increase in basal activity of the ΔPH mutant brings up the question of whether membrane localization is necessary for the activation of PKB. We therefore examined the subcellular localization of PKB and ΔPH-PKB in cells using both subcellular fractionation and immunolocalization.

We first studied the distribution of wild-type PKB and compared it with that of ΔPH-PKB in 293 cells overexpressing these two proteins. Cells were fractionated as described under “Experimental Procedures.” As shown in Fig. 5, both the wild-type and ΔPH forms of PKB were present in the fractions representing the cytosol (cyto), nucleus (P1), and higher density membrane components (P2). However, although wild-type PKB was present in plasma membrane fractions (PM), ΔPH-PKB exhibited a reduction in the basal kinase activity. This substantiates the findings from the phosphatase experiments showing that the increase in the basal activity of the ΔPH mutant is due to an increase in the level of phosphorylation.

Localization of ΔPH-PKB in 293 Cells—It has been proposed that the role of the PH domain is to localize the kinase on the membrane, where in can be activated, possibly by membrane-associated kinases. Much of this view stems from the observation that association of PKB with the membrane results in constitutive activation of the kinase. However, our observation that the PH domain prevents the increase in basal activity of the ΔPH mutant brings up the question of whether membrane localization is necessary for the activation of PKB. We therefore examined the subcellular localization of PKB and ΔPH-PKB in cells using both subcellular fractionation and immunolocalization.

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Although much has been learned recently regarding the activation of PKB, the exact mechanisms still remain controversial. At present, the role of phospholipid products of PI3K are not clearly known, although they are proposed to contribute to the stimulation of the kinase by two separate, but not mutually exclusive, mechanisms. First, it has been described that products of PI3K, most notable PtdIns-3,4-P$_2$, can bind to the PH domain of PKB and directly stimulate the kinase activity. Second, it is proposed that phospholipids bind to the PH domain and serve to localize PKB to the plasma membrane, which would be necessary for the activation process. In this report, we have sought to further clarify the role of the PH domain in the activation of PKB.

We first examined the effect of mutations in the PH domain of PKB on the insulin-induced activation of the kinase. Removal of the entire PH domain of PKB results in a kinase that retains its ability to be activated in response to the engagement of growth factor receptors. This activation is mechanistically similar to that of the wild-type kinase with regard to both its kinetics and its dependence on PI3K. However, this mutant is unique in that it has an ~4-fold higher basal level of activity than the wild-type construct. By contrast, a point mutation (R25C) that has been demonstrated to prevent binding to phospholipids results in an inactive kinase. From this, it is evident that binding of phospholipids to the PH domain per se is not required for activation of PKB, and perhaps removal of the PH domain somehow mimics the effect of phospholipid binding. Potentially, this could be due to two different effects of either phospholipid binding or removal of the PH domain: (i) the induction of a conformational change that directly activates the kinase and/or (ii) a structural change that permits the phosphorylation and thereby activation of the kinase. These two possibilities are not mutually exclusive, and both could account for the increase in the basal activity of ΔPH-PKB.

Several recent reports have demonstrated that addition of phospholipids, most specifically PtdIns-3,4-P$_2$, can directly activate PKB (27–29). This suggests that phospholipid binding to the PH domain can change the conformation of the enzyme, resulting in a partially active kinase. This structural change could be mimicked by removing the PH domain and thereby could account for the elevated basal activity of ΔPH-PKB. However, others have not been able to show that phospholipids can directly activate the kinase and propose that addition of phospholipids to the kinase reaction permits the phosphorylation and subsequent partial activation by a contaminating kinase (31, 44). This could also explain the high basal activity of ΔPH-PKB if removal of the PH domain allows for the phosphorylation of the kinase in a basal state. To test which of these possibilities is correct, we made several mutations in both wild-type PKB and ΔPH-PKB and determined the effects on the resulting kinase activity.

Activating mutations, in which the residues phosphorylated after insulin stimulation are changed to acidic amino acids to mimic phosphorylation, have been shown to result in a constitutively active PKB (30). In R25C PKB, mutation of Thr-308 and Ser-473 to aspartic acid also resulted in a kinase with a basal activity that was significantly higher than that of wild-type PKB. However, these mutations, in either wild-type PKB or R25C PKB, were never able to activate the kinase to the same extent as seen after insulin stimulation. Therefore, we cannot conclusively state that all of the defects of R25C could be overcome by mimicking phosphorylation. However, it is evident that at least part of the lack of phospholipid binding in R25C PKB can be compensated for by simulating phosphorylation.

Next, we determined whether phosphorylation could account for the elevated basal activity of ΔPH-PKB. We first treated immunoprecipitated ΔPH-PKB with phosphatase prior to performing a kinase assay. This treatment reduced the elevated basal kinase activity of ΔPH-PKB, suggesting that the increase is due to a higher phosphorylation state of the mutant. To confirm this finding, the two residues that are phosphorylated in response to insulin stimulation were mutated to alanines. These mutations prevented the activation of the kinase in response to insulin. In addition, the basal activity of the ΔPH-PKB alanine mutant was reduced to the levels of nonstimulated wild-type PKB. This confirms the finding showing that...
FIG. 6. Localization of GFP-PKB and GFP-ΔPH-PKB in overexpressing HeLa cells. HeLa cells were transfected with 8 μg of PKB constructs as described under “Experimental Procedures.” 48 h later, cells were stimulated (B and D) or not (A and C) with 1 μM insulin for 5 min. Cells were washed and fixed with paraformaldehyde (3.7%) prior to incubation with rhodamine-conjugated WGA to label the plasma membrane. Slides were mounted and analyzed by confocal microscopy. Images represent the center section of the X-Y plane. Membrane staining is in red, and GFP is in green. Areas of colocalization are visualized by yellow. A, GFP-PKB, nonstimulated; B, GFP-PKB, insulin-stimulated; C, GFP-ΔPH-PKB, nonstimulated; D, GFP-ΔPH-PKB, insulin-stimulated.

the high basal activity of ΔPH-PKB is due to an increased level of phosphorylation.

Our observations are consistent with recent findings of Alessi et al. (33) describing the activation of PKB by PDK1. They have shown that PDK1 requires phospholipids to phosphorylate wild-type PKB, but not ΔPH-PKB. In addition, they have shown no change in the activity of PDK1 after stimulation by insulin or insulin-like growth factor 1. This indicates that the insulin-dependent phosphorylation of PKB at Thr-308 by PDK1 requires only the phospholipid products of PI3K. Since phosphorylation of ΔPH-PKB by PDK1 occurs independently of phospholipids, it would follow that ΔPH-PKB should be phosphorylated under basal conditions by PDK1. Because phosphorylation of Thr-308 alone can partially activate the kinase (31), this could explain the increased basal activity of ΔPH-PKB.

More recently, it has been shown that PDK1 can phosphorylate p70S6K (45); however, in contrast to phosphorylation of PKB, phospholipids are not required. However, phosphorylation or deletion of the C terminus of p70S6K was a prerequisite to PDK1 phosphorylation of the kinase. Thus, it appears that activation of substrates by PDK1 is controlled at the level of access of the kinase to the substrate, rather than modulation of PDK1 itself.

In addition to PDK1 phosphorylation of Thr-308, PKB is regulated by phosphorylation of Ser-473 by another kinase. To date, this kinase has not been identified; however, since activation of PKB is completely blocked by wortmannin, it is evident that this kinase is also localized downstream of PI3K. We found that stimulation of ΔPH-PKB by insulin is also sensitive to wortmannin. Taking our findings together, we propose that ΔPH-PKB is phosphorylated under basal conditions by PDK1 in the absence of phospholipids. This accounts for the elevated activity of ΔPH-PKB in the absence of stimulation. After insulin treatment, there is an increase in ΔPH-PKB kinase activity, which is inhibited by wortmannin. We propose that this is due to phosphorylation of Ser-473 by a kinase that is dependent on PI3K. Our attempts to identify the residue that is phosphorylated in ΔPH-PKB under basal conditions were unsuccessful. Mutation of either Thr-308 or Ser-473 to alanine could reduce the basal activity of ΔPH-PKB; however, these mutants do not respond normally to insulin or platelet-derived growth factor (46),2 making it difficult to draw conclusions using these mutants. Therefore, at this time, we cannot definitively state which residue is basally phosphorylated in ΔPH-PKB, and instead, a small proportion of doubly phosphorylated molecules may account for the increased basal activity.

Translocation of PKB to the plasma membrane occurs in response to growth factor stimulation, although the function of this is still not well understood. This translocation most likely occurs via binding of the phospholipid products of PI3K to the PH domain of PKB. This redistribution is thought to activate the kinase since constitutive localization to the membrane by either fusion of the viral gag gene, as in v-akt, or addition of a myristoylation signal activates PKB (15, 16), and indeed, membrane-localized PKB is phosphorylated. How this membrane targeting results in increased phosphorylation of PKB is mechanistically not understood. It has been hypothesized that membrane targeting of PKB brings it in contact with kinases that allow for activation. However, at least one of the kinases that are responsible for the phosphorylation of PKB (PDK1) has been purified from the cytosol (31). Since ΔPH-PKB obviously lacks a PH domain and therefore is not likely to localize to the plasma membrane by this means, we questioned whether this localization was really necessary for activation. We examined the subcellular distribution of wild-type PKB and ΔPH-PKB

2 C. L. Sable, N. Filippa, C. Filloux, B. A. Hemmings, and E. Van Obberghen, unpublished observations.
using both subcellular fractionation and immunofluorescent localization. Both techniques showed that ΔPH-PKB was not localized to the plasma membrane. In addition, Andjelkovic et al. (42) have cited that they also failed to detect ΔPH-PKB in the membrane, although it is activated in response to insulin-like growth factor 1. From this, it is evident that membrane targeting is not required for the activation process, showing that the kinase(s) responsible must also exist in the cytosol.

The results presented here allow us to propose a modified model to explain the activation of PKB in response to insulin stimulation, but it may be necessary for downstream signaling events such as glucose transport. This model proposes a novel role for the PH domain: rather than acting as a membrane-targeting domain, it is instead involved in regulating the accessibility of target phosphorylation sites to upstream activators.

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