

# High Affinity Binding of Inositol Phosphates and Phosphoinositides to the Pleckstrin Homology Domain of RAC/Protein Kinase B and Their Influence on Kinase Activity\*

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The influence of inositol phosphates and phosphoinositides on the  $\alpha$  isoform of the RAC-protein kinase B (RAC/PKB) was studied using purified wild type and mutant kinase preparations and a recombinant pleckstrin homology (PH) domain. Binding of inositol phosphates and phosphoinositides to the PH domain was measured as the quenching of intrinsic tryptophan fluorescence. Inositol phosphates and D3-phosphorylated phosphoinositides bound with affinities of 1–10  $\mu$ M and 0.5  $\mu$ M, respectively. Similar values were obtained using RAC/PKB expressed and purified from baculovirus-infected Sf9 cells in the fluorescence assay. The influence of synthetic dioctanoyl derivatives of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate on the activity of RAC/PKB purified from transfected COS-1 cells was studied. Phosphatidylinositol 3,4,5-trisphosphate was found to inhibit the RAC/PKB kinase activity with half-maximal inhibition at 2.5  $\mu$ M. In contrast, phosphatidylinositol 3,4-bisphosphate stimulated kinase activity (half-maximal stimulation at 2.5  $\mu$ M). A mutant RAC/PKB protein lacking the PH domain was not affected by D3-phosphorylated phosphoinositides. These results demonstrate that the PH domain of RAC/PKB binds inositol phosphates and phosphoinositides with high affinity, and suggest that the products of the phosphatidylinositide 3-kinase can act as both a membrane anchor and modulator of RAC/PKB activity. The data also provide further evidence for a link between phosphatidylinositide 3-kinase and RAC/PKB regulation.

The stimulation of receptor tyrosine kinases (RTK)<sup>1</sup> by agonists leads to immediate activation of intracellular signal

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<sup>1</sup> The abbreviations used are: RTK, receptor tyrosine kinase; PI3-K, phosphoinositide 3-kinase; RAC/PKB, related to A and C kinases/protein kinase B; PH, pleckstrin homology; PKC, protein kinase C; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P<sub>2</sub>, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; PtdIns, phosphatidylinositide; Ins(1)P, *myo*-D-inositol 1-monophosphate; Ins(2)P, *myo*-D-inositol 2-monophosphate; Ins(1,4)P<sub>2</sub>, *myo*-D-inositol 1,4-bisphosphate; Ins(1,4,5)P<sub>3</sub>, *myo*-D-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P<sub>4</sub>, *myo*-D-inositol 1,3,4,5-tetrakisphosphate; Ins(3,4,5,6)P<sub>4</sub>, *myo*-D-inositol 3,4,5,6-tetrakisphosphate; Ins(1,2,3,5)P<sub>4</sub>, *myo*-D-inositol 1,2,3,5-tetrakisphosphate; Ins(1,2,3,4,5,6)P<sub>6</sub>, *myo*-D-inositol 1,2,3,4,5,6-hexakisphosphate; DTT, dithio-

transduction pathways. The assembly of multiprotein complexes at the plasma membrane is one important feature of RTK signal transduction mechanisms (reviewed in Refs. 1 and 2). Numerous studies suggest that the activation of phosphatidylinositide 3-kinase (PI3-K) by growth factors is also involved (3, 4), leading to the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P<sub>2</sub>); these metabolites are assumed to act as second messengers (5). Recently RAC-protein kinase B (RAC/PKB) has emerged as a key player in the PI3-K-stimulated signaling pathway, based on the inhibition of its activation by wortmannin (6–11).

RAC/PKB is a subfamily of the second messenger-regulated serine/threonine kinases (12). Three isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) have been identified, each consisting of an amino-terminal pleckstrin homology (PH) domain, a central kinase domain, and a serine/threonine-rich carboxyl-terminal region (13–17). Various stimuli, such as insulin, PDGF, epidermal growth factor, basic fibroblast growth factor, serum (6–10), and protein phosphatase inhibitors (9), lead to activation of RAC/PKB kinase activity. The activation is promoted by signals emanating from RTK-regulated PI3-K and is accompanied by an increase in serine/threonine phosphorylation of RAC/PKB (6, 9). The phosphorylation of two sites has been shown to be critical for activation by insulin and insulin growth factor-1 (18): Thr-308 in the T-loop and Ser-473 in the Ser/Thr-rich carboxyl-terminal region. Interestingly, the Ser-473 site is conserved in many of the second messenger subfamily of protein kinases. The inactivation of kinase activity by *in vitro* phosphatase treatment suggest that protein phosphatase 2A is also involved in the regulation of RAC/PKB (9).

The inhibition of growth factor-mediated RAC/PKB activation by wortmannin and LY 294002 suggests that RAC/PKB plays a crucial role in perceiving and transmitting signals from RTK-activated PI3-K (6–11). In particular, the amino-terminal PH domain of RAC/PKB appears to be important in kinase regulation because its deletion abrogates activation by PDGF and serum (7, 9). The PH domain is a module of approximately 100 amino acids identified in over 90 different proteins (19–21). The primary structure of the PH domain varies in different proteins. The three-dimensional structures have been determined for pleckstrin (22), dynamin (23),  $\beta$ -spectrin (24), and phospholipase C $\delta$ -1 (25) and all are similarly folded. It was noted that the three-dimensional structure of the pleckstrin PH domain had similarities to lipid-binding proteins (22). Subsequently, inositol phosphates and phosphoinositides were found to bind to PH domains from pleckstrin (26),  $\beta$ -spectrin

threitol; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PLC, phospholipase C; HA, hemagglutinin.

(27), and phospholipase C $\delta$ -1 (28, 29).

Structural studies have revealed the critical amino acid residues involved in the binding of inositol phosphates. The structures of the  $\beta$ -spectrin and the phospholipase C $\delta$ -1 (PLC $\delta$ -1) PH domains complexed with inositol (1,4,5)-trisphosphate (Ins(1,4,5)P<sub>3</sub>), indicated that Trp-23 of  $\beta$ -spectrin and Trp-36 of PLC $\delta$ -1 are hydrogen bonded to a phosphate group of the ligand (27, 25). The existence of an homologous Trp-22 in RAC/PKB prompted us to use the intrinsic tryptophan fluorescence of the RAC/PKB PH domain to monitor interactions with phosphoinositides and inositol phosphates. Here we demonstrate that this PH domain binds with high affinity to certain inositol phosphates and to PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub>. Conflicting results on the influence of D3-phosphorylated phosphoinositides on RAC/PKB activity (7, 30) also led us to examine the effect of these ligands on RAC/PKB kinase activity. The data obtained show that these compounds can both inhibit and activate the kinase in a PH domain-specific manner.

#### MATERIALS AND METHODS

**Phosphoinositides and Inositol Phosphates**—The dioctanoyl derivatives of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> were synthesized according to the method of Reddy *et al.* (31). PtdIns(4,5)P<sub>2</sub> and inositol phosphates were purchased from Fluka, Switzerland.

**Construction of Expression Vectors**—The RAC/PKB PH domain consisting of amino acids 1–131 was subcloned as a 5'-BamHI/3'-EcoRI PCR fragment into pGEX-2T (Pharmacia) vector. The hemagglutinin (HA) epitope-tagged constructs (HA-RAC/PKB $\alpha$  and HA- $\Delta$ NRAC/PKB $\alpha$ ) were previously described (9, 18).

**Construction of a Recombinant RAC/PKB Producing Baculovirus**—The 1.6-kilobase pair EcoRI fragment of human RAC/PKB $\alpha$  (13) was subcloned into the EcoRI site of the baculovirus transfer vector pVL1392 (Invitrogen) in the correct orientation. The plasmid was co-transfected into Sf9 cells together with the purified wild-type baculovirus (AcMNPV) DNA. The culture supernatant was collected and the recombinant virus was purified by limiting dilution and screened by dot-blot hybridization. The pure recombinant virus was amplified and then used for recombinant protein production.

**Expression and Purification of the RAC/PKB PH Domain**—*Escherichia coli* JM109 transformed with the pGEX2T PH131 expression construct was diluted in 2  $\times$  YT media containing 100  $\mu$ g/ml ampicillin, grown at 37  $^{\circ}$ C to a density OD<sub>600</sub> of 0.8 to 0.9, and then induced with 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 3 h at 25  $^{\circ}$ C. Bacterial pellets were lysed in 5 volumes of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride using lysozyme (1 mg/ml). After lysis, the extracts were treated with 0.1% (w/v) sodium deoxycholate and 20  $\mu$ g/ml DNase I for 30 min at 4  $^{\circ}$ C and then centrifuged at 20,000  $\times$  g for 45 min at 4  $^{\circ}$ C. Glutathione-agarose (Sigma) was added to the supernatant and incubated for 1 h at 4  $^{\circ}$ C with gentle agitation. The glutathione-agarose beads were collected by centrifugation, washed with phosphate-buffered saline, and the fusion protein eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA for 2 h at room temperature. Following cleavage with 2 units of thrombin (Sigma) per mg of fusion protein, the PH domain preparation was purified to homogeneity by Mono Q FPLC (Pharmacia). The PH domain was eluted from the column with a 20-ml linear gradient of 0–600 mM NaCl in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA with a flow rate of 1 ml/min. The PH domain eluted at 450 mM NaCl and peak fractions were examined by SDS-PAGE.

**Purification of RAC/PKB Expressed in Sf9 Cells**—The Sf9 cells were harvested 72 h after infection with recombinant baculovirus by centrifugation at 1000  $\times$  g for 10 min. The cells were washed twice with TBS (Tris-buffered saline: 20 mM Tris-HCl, pH 7.5, 120 mM NaCl) and then lysed with extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20  $\mu$ M leupeptin, 20  $\mu$ M aprotinin) at 4  $^{\circ}$ C. The lysate was centrifuged at 14,000  $\times$  g for 10 min, the supernatant was diluted 5-fold with the appropriate column buffer and applied directly to the Mono Q column (HR 5/5, Pharmacia). The column was equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT. The sample was applied at a flow rate of 0.5 ml/min and the column was washed with 2 bed volumes before a linear ascending gradient from 0 to 0.4 M NaCl (20 ml) and then from 0.4 to 1.0 M NaCl (10 ml) in column buffer was applied at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. Fractions were analyzed for protein content and by SDS-PAGE and immunoblotting for RAC/PKB. The

fractions containing RAC/PKB were pooled and concentrated using a Centricon-30 to 200  $\mu$ l and subjected to further purification by gel filtration chromatography on a Superose 12 (HR 10/30) column (Pharmacia). The column was equilibrated in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.02% NaN<sub>3</sub>. The protein was eluted in the above buffer at a flow rate of 0.2 ml/min. Fractions of 0.5 ml were collected and analyzed for protein content by SDS-PAGE.

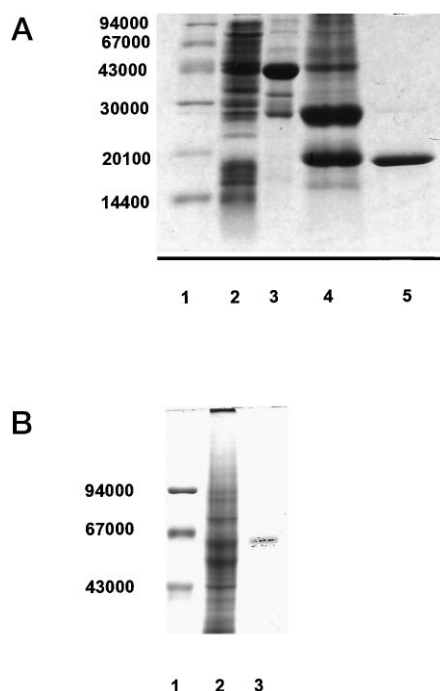
**Purification of HA-tagged RAC/PKB**—Epitope-tagged HA-RAC/PKB was purified from 40  $\times$  10-cm dishes of transfected COS-1 cells without treatment or following stimulation with 0.1 mM pervanadate as described previously (9). The cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 120 mM NaCl, 1 mM EDTA, 50 mM sodium fluoride, 40 mM  $\beta$ -glycerophosphate, 0.1 mM pervanadate, 100 mM okadaic acid, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, 1 mM benzamide, and 0.5 mM phenylmethylsulfonyl fluoride. The extract was centrifuged for 15 min at 12,000  $\times$  g and 4  $^{\circ}$ C, and the supernatant applied to a 1-ml affinity column of the HA-specific monoclonal antibody 12CA5 covalently attached to protein A-Sepharose according to the supplier's protocol (Pierce). The bound protein was eluted stepwise with 1 mg/ml HA-epitope peptide (AAYPYDVPDYA) for 1 h at 4  $^{\circ}$ C. The pooled fractions were dialyzed for 3 h at 4  $^{\circ}$ C against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM okadaic acid. The dialyzed protein preparation was loaded onto a HR5/5 Mono Q column (FPLC Pharmacia) at a flow rate of 1 ml/min and protein eluted with a 20-ml gradient of 50–550 mM NaCl in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM okadaic acid. The kinase eluted at about 350 mM NaCl and the fractions were analyzed by Western blot and assayed for kinase activity (as described below). Fractions were pooled and spin concentrated with a Centricon-30 (Amicon). HA- $\Delta$ NRAC/PKB was purified in the same way from pervanadate-stimulated COS-1 cells, but the kinase eluted from Mono Q at 300 mM NaCl. Protein concentrations were determined by the Bradford assay using the Bio-Rad kit.

**RAC/PKB Kinase Assay**—Kinase assays were performed at 30  $^{\circ}$ C in a total reaction volume of 50 or 100  $\mu$ l consisting of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1  $\mu$ M cAMP-dependent protein kinase inhibitor, 30  $\mu$ M Crosstide (GRPRSSFAEG) (10), 50  $\mu$ M ATP with [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 4000 cpm/pmol). Where indicated, PtdIns(3,4)P<sub>2</sub> or PtdIns(3,4,5)P<sub>3</sub> or other inositol phosphates were added. The reactions were equilibrated at 30  $^{\circ}$ C for 5 min and initiated by addition of purified protein. At appropriate time points, 10- $\mu$ l aliquots of the reaction solution were spotted onto P81 filters (Whatman) and washed 4 times with 1% phosphoric acid. Filter-bound radioactivity was determined by scintillation counting. Under the conditions used the assay is linear for 60 min (see Fig. 4A).

**Fluorescence Spectroscopy**—The fluorescence measurements were recorded using a Perkin Elmer Luminescence Spectrometer LS 50 B at 25  $^{\circ}$ C in 20 mM Hepes-NaOH, pH 7.0, 10 mM MgCl<sub>2</sub> at a protein concentration of 1.6–3.2  $\mu$ g/ml (0.1–0.2  $\mu$ M) in a volume of 1 ml. The purified protein was excited at a wavelength of 290 nm and fluorescence measured at 345 nm. Titration data were fitted using simple weighting to a rectangular hyperbolic function by nonlinear regression analysis (Grafit 3.0 Erithacus software).

#### RESULTS

**Expression and Purification of Proteins**—To study the function of the PH domain of RAC/PKB we expressed the PH domain (amino acids 1–131 of the RAC/PKB $\alpha$  sequence) as a soluble glutathione S-transferase fusion protein. The fusion protein was isolated by affinity chromatography, cleaved by thrombin, and purified to homogeneity by Mono Q ion-exchange chromatography (Fig. 1A). Full-length wild type RAC/PKB was expressed in baculovirus-infected Sf9 cells and purified as described under "Materials and Methods." The final purification step involved gel filtration on Superose 12; the purity of the protein obtained by this procedure is shown in Fig. 1B. The RAC/PKB obtained from Sf9 cells had a low specific activity and therefore was probably only phosphorylated on Ser-124 and Thr-450 (18). Phosphorylation of Thr-308 and Ser-473 is required for activation of RAC/PKB (18). In order to produce activated RAC/PKB we therefore chose to express the kinase in COS-1 cells. The hemagglutinin-tagged RAC/PKB protein was overexpressed in COS-1 cells and purified as outlined under "Materials and Methods." The different purified enzyme preparations were used for the characterization of their



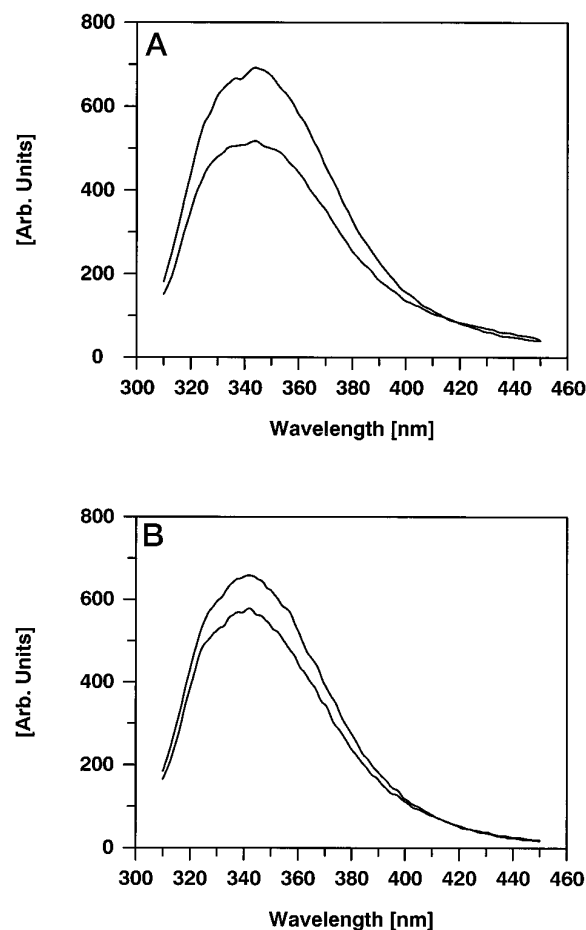
**FIG. 1. Purification of RAC/PKB PH domain and RAC/PKB.** A, 15% SDS-PAGE showing the purification of the RAC/PKB PH131 domain. Lane 1, molecular weight markers (Bio-Rad). Lane 2, soluble extract of JM109 expressing the fusion protein GST-PH131. Lane 3, GST-PH131 after elution from the glutathione-agarose. Lane 4, glutathione S-transferase (GST) and PH131 after thrombin cleavage. Lane 5, the protein after Mono Q chromatography. Molecular masses in Da are shown at the left of the gel. B, 10% SDS-PAGE of RAC/PKB purified from baculovirus infected Sf9 cells. Lane 1, molecular weight markers (Bio-Rad). Lane 2, extract of infected Sf9. Lane 3, RAC/PKB after gel filtration on Superose 12. Molecular masses in Da are shown on the left of the gel.

interaction with inositol phosphates and phosphoinositides.

**Binding of Inositol Phosphates to RAC/PKB**—We examined the binding of inositol phosphates to the bacterially expressed PH domain and with RAC/PKB purified from baculovirus-infected Sf9 cells. Following excitation at 290 nm, the PH domain exhibited an emission spectra typical for tryptophan with a maximum fluorescence at 345 nm; the fluorescence was quenched by approximately 30% upon addition of ligands (Fig. 2A). Similar changes in the fluorescence were detected for the full-length native protein isolated from Sf9 cells, but the fluorescence was only quenched maximally 15% upon addition of ligands (Fig. 2B). No shift of the maximum of the emitted fluorescence was observed with both purified preparations in the presence of ligands. Changes in the fluorescence in the presence of inositol phosphates were used to determine the dissociation constants ( $K_d$ ) for the complexes.

Inositol phosphates bound to the PH domain with micromolar affinities (Table I). For Ins(1)P and Ins(2)P the  $K_d$  values were 7.0 and 8.4  $\mu$ M, respectively (Table I). With Ins(1,4)P<sub>2</sub> the affinity was about 3-fold higher with a  $K_d$  of 2.4  $\mu$ M. The highest affinities for inositol phosphates were found with Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub>, with  $K_d$  values of 1.2  $\mu$ M and 1.5  $\mu$ M, respectively.

In contrast the affinities for other inositol tetrakisphosphates were lower, and  $K_d$  values for Ins(3,4,5,6)P<sub>4</sub> and Ins(1,2,3,5)P<sub>4</sub> were 6.4 and 5.0  $\mu$ M, respectively. The affinity of the PH domain for Ins(1,2,3,4,5,6)P<sub>6</sub> was slightly higher with a  $K_d$  of 8.7  $\mu$ M. The observed affinity for Ins(1,4,5)P<sub>3</sub> is about 30-fold higher than values previously reported for  $\beta$ -spectrin (27), and 3500-fold higher than the affinity of 1-( $\alpha$ -glycerophosphoryl)-inositol(4,5)-bisphosphate for dynamin (32). The



**FIG. 2. Emission spectra of RAC/PKB PH domain and RAC/PKB.** A, the fluorescence spectrum of the RAC/PKB PH domain was obtained at a protein concentration of 2.5  $\mu$ g/ml (0.16  $\mu$ M) in 20 mM Hepes/NaOH, pH 7.0, 10 mM MgCl<sub>2</sub>, at 25 °C. Excitation was carried out at 290 nm with a bandwidth of 5 nm and the emission spectra recorded a bandwidth of 15 nm. Upper curve, emission spectrum of the PH domain alone; lower curve, emission spectrum of the PH domain in the presence of 10  $\mu$ M PtdIns(3,4,5)P<sub>3</sub> expressed in arbitrary units. B, the fluorescence spectrum of RAC/PKB expressed in baculovirus-infected Sf9 cells. Protein concentration was 12  $\mu$ g/mg (0.22  $\mu$ M) in 20 mM Hepes/NaOH, pH 7.0, 10 mM MgCl<sub>2</sub>, at 25 °C. Excitation was carried out at 290 nm, with a bandwidth of 5 nm, and the emission spectra were recorded with a bandwidth of 9 nm. Upper curve, emission spectrum of the protein alone; lower curve, emission spectrum of the protein in the presence of 10  $\mu$ M PtdIns(3,4,5)P<sub>3</sub> expressed in arbitrary units.

PLC $\delta$ -1 PH domain bound Ins(1,4,5)P<sub>3</sub> 6-fold better than the RAC/PKB PH domain, showing a clear preference for Ins-(1,4,5)P<sub>3</sub> and PtdIns(4,5)P<sub>2</sub> (28). In contrast to the PH domain of PLC $\delta$ -1, we found specific binding of D3-phosphorylated inositol phosphates by the RAC/PKB PH domain; however, the RAC/PKB PH domain distinguished poorly between Ins-(1,3,4,5)P<sub>4</sub> and Ins(1,4,5)P<sub>3</sub>. Furthermore, the phosphate groups at positions 3, 4, and 5 of the inositol ring were important for the interaction with the RAC/PKB PH domain, as additional phosphate groups interfered with binding, as observed with Ins(3,4,5,6)P<sub>4</sub>, Ins(1,2,3,5)P<sub>4</sub>, and Ins(1,2,3,4,5,6)P<sub>6</sub>.

To examine whether there are dramatic differences in the binding characteristics between the isolated PH domain and the full-length protein, we further determined the affinities for several inositol phosphates to the purified RAC/PKB expressed in Sf9 cells (Table I). Here, we measured a dissociation constant of 11.8  $\mu$ M for Ins(1)P and 4.1  $\mu$ M for Ins(1,4)P<sub>2</sub>, for Ins(1,2,3,4,5,6)P<sub>6</sub> the dissociation constant was 7.5  $\mu$ M. As for the isolated PH domain the inositol phosphates with the highest affinities were found with Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub>

TABLE I  
Dissociation constants for RAC/PKB inositol phosphate or phosphoinositide complexes

The dissociation constants were determined as indicated under "Materials and Methods" at 25 °C by tryptophan fluorescence quenching. Values are means of at least three independent experiments. Standard errors are given in the parentheses. The proteins used for these assays were the isolated PH domain and the RAC/PKB expressed in Sf9 cells, purified as described under "Materials and Methods." The determination of the  $K_d$  for PtdIns(4,5)P<sub>2</sub> was not possible by this assay due to nonspecific interaction of this compound with RAC/PKB.

| Compound                       | PH domain             |               | RAC/PKB               |               |
|--------------------------------|-----------------------|---------------|-----------------------|---------------|
|                                | Dissociation constant | Stoichiometry | Dissociation constant | Stoichiometry |
|                                | $\mu\text{M}$         |               | $\mu\text{M}$         |               |
| Ins(1)P                        | 7.0 ( $\pm 0.42$ )    |               | 11.8 ( $\pm 0.6$ )    |               |
| Ins(2)P                        | 8.4 ( $\pm 0.64$ )    |               | ND <sup>a</sup>       |               |
| Ins(1,4)P <sub>2</sub>         | 2.4 ( $\pm 0.3$ )     |               | 4.1 ( $\pm 0.4$ )     |               |
| Ins(1,4,5)P <sub>3</sub>       | 1.2 ( $\pm 0.1$ )     |               | 3.2 ( $\pm 0.4$ )     |               |
| Ins(1,3,4,5)P <sub>4</sub>     | 1.5 ( $\pm 0.2$ )     | 0.95          | 2.5 ( $\pm 0.3$ )     |               |
| Ins(3,4,5,6)P <sub>4</sub>     | 6.4 ( $\pm 1.0$ )     |               | ND <sup>a</sup>       |               |
| Ins(1,2,3,5)P <sub>4</sub>     | 5.0 ( $\pm 0.5$ )     |               | ND <sup>a</sup>       |               |
| Ins(1,2,3,4,5,6)P <sub>6</sub> | 8.7 ( $\pm 0.4$ )     |               | 7.5 ( $\pm 0.1$ )     |               |
| PtdIns(4,5)P <sub>2</sub>      | 2.5 ( $\pm 0.5$ )     |               | NP <sup>b</sup>       |               |
| PtdIns(3,4)P <sub>2</sub>      | 0.57 ( $\pm 0.03$ )   | 0.90          | 1.0 (0.2)             | 1.02          |
| PtdIns(3,4,5)P <sub>3</sub>    | 0.40 ( $\pm 0.03$ )   | 0.92          | 0.6 (0.05)            | 1.1           |

<sup>a</sup> ND, not determined.

<sup>b</sup> NP, not possible.

with  $K_d$  values of 3.2 and 2.5  $\mu\text{M}$ , respectively. The overall picture of the affinities for the full-length protein was therefore similar to that of the isolated PH domain.

**Binding of Phosphoinositides to RAC/PKB**—To study the interaction of the RAC/PKB PH domain with phosphoinositides we used PtdIns(4,5)P<sub>2</sub> and the dioctanoyl derivatives (31) of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> (Fig. 3A). The  $K_d$  determined for PtdIns(4,5)P<sub>2</sub> was 2.5  $\mu\text{M}$ , which is in the same range as values reported for dynamin (32) and PLC $\delta$ -1 (28). The values determined for PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, 570 and 400 nM, respectively, reflect the preference of the RAC/PKB PH domain for D3-phosphorylated phosphoinositides. The stoichiometry of binding examined by Scatchard analysis indicated a 1:1 complex for PtdIns(3,4,5)P<sub>3</sub> (Fig. 3B).

With the dioctanoyl derivatives of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> we were able to determine their affinities for the full-length RAC/PKB; the  $K_d$  values for PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> were 0.7 and 1.0  $\mu\text{M}$ , respectively, and both formed 1:1 complexes as determined by Scatchard analysis.

**Influence of Phosphoinositides on RAC/PKB Activity**—To further characterize the effects of inositol phosphates and phosphoinositides on RAC/PKB, we examined the influence of these compounds on the activities of purified wild type HA-RAC/PKB protein and of a construct lacking the PH domain. HA-RAC/PKB and HA- $\Delta$ NRAC/PKB were overexpressed in COS-1. The wild type protein was purified from both untreated and pervanadate stimulated cells in order to obtain enzyme preparations which were dephosphorylated or phosphorylated on the activating sites Thr-308 and Ser-473 (9, 18). Kinase preparations were purified by immunoaffinity chromatography using the 12CA5 monoclonal antibody immobilized on protein A-Sepharose (see "Materials and Methods") followed by anion exchange chromatography over Mono Q. The *in vitro* kinase activity of RAC/PKB was assayed using Crosstide (Fig. 4A), a peptide derived from GSK-3 (see "Materials and Methods"), which is efficiently phosphorylated by RAC/PKB (10). The specific activity of HA-RAC/PKB from pervanadate-stimulated cells was 350 pmol/min/mg compared to 18 pmol/min/mg of the enzyme obtained from unstimulated cells. The specific activity of HA- $\Delta$ NRAC/PKB was 14 pmol/min/mg indicating that this protein was not activated by pervanadate treatment, as re-

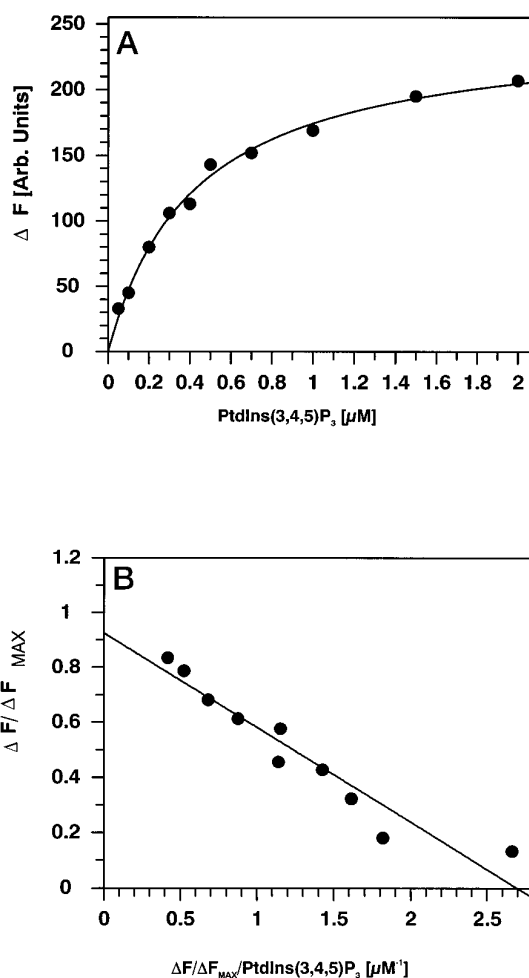
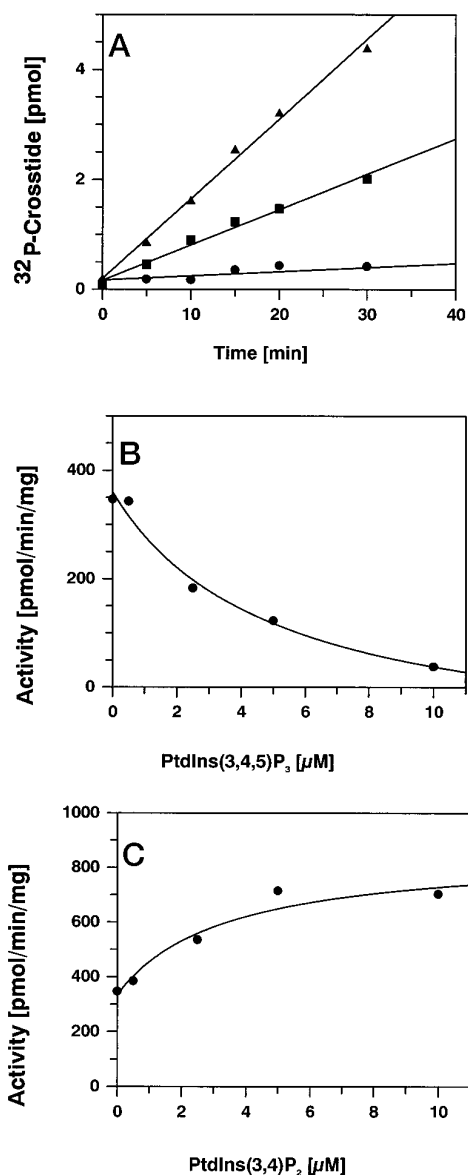


Fig. 3. **Binding of PtdIns(3,4,5)P<sub>3</sub> to the RAC/PKB PH domain.** A, titration of the RAC/PKB PH domain with increasing concentrations of PtdIns(3,4,5)P<sub>3</sub>; fluorescence was measured under the same conditions as above. Excitation was carried out at 290 nm with a bandwidth of 5 nm and emission measured at 345 nm with a bandwidth of 15 nm. The non-linear fit to a hyperbolic function resulted in a  $\Delta F_{\text{max}}$  of 250 arbitrary units and a dissociation constant of 420 nM. One representative experiment of three is shown. B, Scatchard plot of the data from panel A, indicating a stoichiometry of 1:0.92 for the PH domain-PtdIns(3,4,5)P<sub>3</sub> complex.

ported previously (9).

The initial rates of peptide phosphorylation by RAC/PKB were measured in the presence of increasing concentrations of the dioctanoyl derivatives of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. Surprisingly, we found that PtdIns(3,4,5)P<sub>3</sub> inhibited HA-RAC/PKB activity in a concentration-dependent manner, with half-maximal inhibition at 2.5  $\mu\text{M}$  and 95% inhibition at 10  $\mu\text{M}$  (Fig. 4B). In contrast, PtdIns(3,4)P<sub>2</sub> stimulated HA-RAC/PKB activity up to 2-fold (Fig. 4C). PtdIns(4,5)P<sub>2</sub> did not influence the activity of HA-RAC/PKB. However, PtdIns(3,4)P<sub>2</sub> at 6  $\mu\text{M}$  appeared to prevent inhibition of HA-RAC/PKB activity by 2.5  $\mu\text{M}$  PtdIns(3,4,5)P<sub>3</sub> and was even able to revert the effect by causing a slight stimulation of the activity (Fig. 5). Addition of 15  $\mu\text{M}$  Ins(1,3,4,5)P<sub>4</sub> reduced the inhibition caused by 2.5  $\mu\text{M}$  PtdIns(3,4,5)P<sub>3</sub>, probably by competition for the same binding site on the PH domain. Significantly, at concentrations up to 40  $\mu\text{M}$ , Ins(1,3,4,5)P<sub>4</sub> did not induce the same effect with HA-RAC/PKB as PtdIns(3,4,5)P<sub>3</sub>. This result indicates a different mode of binding to the PH domain compared with PtdIns(3,4,5)P<sub>3</sub>.

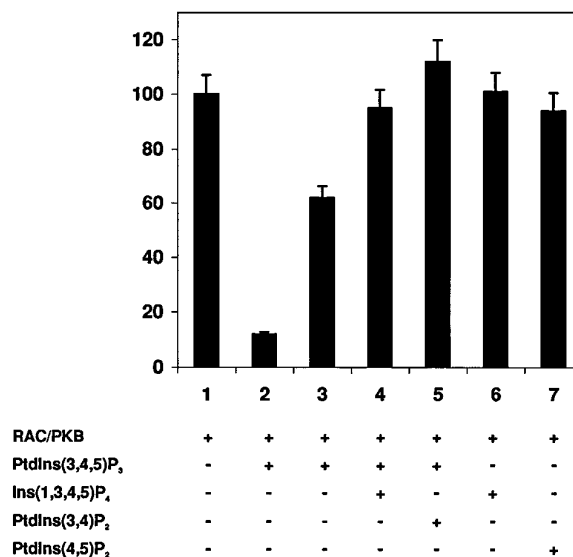
To further characterize the influence of PtdIns(3,4,5)P<sub>3</sub> or PtdIns(3,4)P<sub>2</sub> on RAC/PKB, we examined their effects on enzyme purified from unstimulated cells, which is not phospho-



**FIG. 4. Influence of PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> on HA-RAC/PKB activity.** A, time course of Crosstide phosphorylation by HA-RAC/PKB in the presence of phosphoinositides. Kinase activity was measured using Crosstide and 2.0 μg/ml purified protein in the presence of 10 μM PtdIns(3,4,5)P<sub>3</sub> (●) or 10 μM PtdIns(3,4)P<sub>2</sub> (▲), or without additions (■) as described under "Materials and Methods." B, inhibition of RAC/PKB activity by PtdIns(3,4,5)P<sub>3</sub>. C, stimulation of the HA-RAC/PKB activity by PtdIns(3,4)P<sub>2</sub>. The initial rates of the reactions were determined and plotted against the concentrations of phosphoinositides. In each case, one representative experiment is shown of three performed.

rylated on the activating residues. The effects of the two compounds were comparable to the activated RAC/PKB. PtdIns(3,4)P<sub>2</sub> increased the activity 2.5-fold and PtdIns(3,4,5)P<sub>3</sub> inhibited kinase activity with a half-maximal concentration of 2.2 μM (data not shown). Thus the phosphorylation state of the kinase had little influence on phosphoinositide interactions under the conditions used.

To determine whether the PH domain was involved in mediating these effects on kinase activity, we purified a mutant RAC/PKB lacking the NH<sub>2</sub>-terminal 116 amino acids (HA-ΔNRAC/PKB). As expected, the kinase activity of HA-ΔNRAC/PKB was not affected by phosphoinositides (Fig. 6). Neither the addition of 10 μM PtdIns(3,4,5)P<sub>3</sub> nor 10 μM PtdIns(3,4)P<sub>2</sub> had any significant influence on the HA-ΔNRAC/PKB activity *in*



**FIG. 5. Influence of inositol phosphates and phosphoinositides on the activity of HA-RAC/PKB.** The RAC/PKB activity was measured as described in the legend to Fig. 4. The activity of RAC/PKB was assayed without phosphoinositide or inositol phosphates (1), and in the presence of the following: 10 μM PtdIns(3,4,5)P<sub>3</sub> (2), 2.5 μM PtdIns(3,4,5)P<sub>3</sub> (3), 2.5 μM PtdIns(3,4,5)P<sub>3</sub> and 15.0 μM Ins(1,3,4,5)P<sub>4</sub> (4), 2.5 μM PtdIns(3,4,5)P<sub>3</sub> and 6.0 μM PtdIns(3,4)P<sub>2</sub> (5), 15 μM Ins(1,3,4,5)P<sub>4</sub> (6), or 10 μM PtdIns(4,5)P<sub>2</sub> (7). The bars indicate the measured RAC/PKB activities expressed in percent compared to RAC/PKB activity without phosphoinositide or inositol phosphates.

*vitro*. Furthermore, 10 μM PtdIns(4,5)P<sub>2</sub> had no effect on the activity. Thus, the PH domain apparently influenced kinase activity and the overall effect was dependent on the ligand bound.

Taken together the results so far available indicate that the PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> derivatives influence kinase activity via an intramolecular interaction. Alternatively they could induce dimerization and this ultimately affects kinase activity. These possibilities are currently being investigated.

#### DISCUSSION

Recently we demonstrated that RAC/PKB activation by insulin and insulin growth factor-1 is mediated by phosphorylation of Thr-308 in the T-loop, and Ser-473 in the carboxyl-terminal regulatory domain (18). The implication of these data is that RAC/PKB is regulated by an upstream kinase. However, the fact that the activation is potently inhibited by wortmannin suggests that PI3-K plays a pivotal role in this process. We suggested previously that a putative RAC/PKB upstream kinase could be directly regulated by phosphoinositides (18). Moreover, it is also possible that phosphoinositides directly interact with RAC/PKB during the activation process.

In this article we have investigated the interaction of inositol phosphates and phosphoinositides with RAC/PKB. Our results show that the RAC/PKB PH domain binds inositol phosphates and phosphoinositides, and that the activity of RAC/PKB is influenced *in vitro* by PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub>. The affinities for these compounds were higher than those recently determined for pleckstrin, β-spectrin, and dynamin (26, 27, 32). The highest affinities were found for Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> and for the D3-phosphorylated phosphoinositides PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. Phosphate groups at positions 3, 4, and 5 of the inositol ring were found to be important for the interaction of the phosphoinositides and inositol phosphates with the RAC/PKB PH domain. Studies with Ins(3,4,5,6)P<sub>4</sub>, Ins(1,2,3,5)P<sub>4</sub>, and Ins(1,2,3,4,5,6)P<sub>6</sub> showed that additional phosphates interfered with binding to the PH

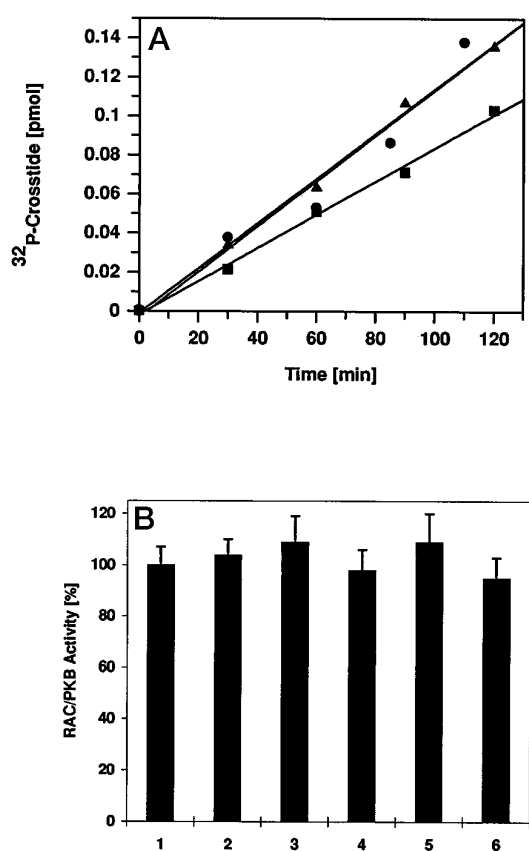


FIG. 6. Influence of phosphoinositides on the activity of HA- $\Delta$ NRAC/PKB. Kinase activity was measured as described in the legend to Fig. 4. A, time course of Crosstide phosphorylation by HA- $\Delta$ NRAC/PKB. Kinase activity was measured using Crosstide and 7  $\mu$ g/ml purified protein in the presence of 10  $\mu$ M PtdIns(3,4,5)P<sub>3</sub> (▲) or 10  $\mu$ M PtdIns(3,4)P<sub>2</sub> (■), or without additions (●) as described under "Materials and Methods." B, the activity of HA- $\Delta$ NRAC/PKB was assayed without phosphoinositide (1), and in the presence of the following: 5.0 (2) and 10 (3)  $\mu$ M PtdIns(3,4)P<sub>2</sub>; 5 (4) and 10 (5)  $\mu$ M PtdIns(3,4,5)P<sub>3</sub>; or 10  $\mu$ M PtdIns(4,5)P<sub>2</sub> (6). The bars indicate the measured RAC/PKB activities expressed in percent compared to HA- $\Delta$ NRAC/PKB activity without phosphoinositides.

domain. In contrast to the PH domain of PLC $\delta$ -1, we found specific binding of D3-phosphorylated inositol phosphates and phosphoinositides by the RAC/PKB PH domain. However, the discrimination between the tested ligands was less pronounced than for phospholipase C $\delta$ -1 (28).

Obviously the mechanism and/or the site for binding inositol phosphates are different compared to PLC $\delta$ -1 where structural restraints hinder the binding of D3-phosphorylated inositol phosphate (25). Ins(1,4,5)P<sub>3</sub> seems to be buried in a binding pocket of PLC $\delta$ -1, which is formed by the  $\beta$ 1/ $\beta$ 2,  $\beta$ 3/ $\beta$ 4, and  $\beta$ 5/ $\beta$ 6 loops, where the ligand makes 12 hydrogen bonds with nine amino acids contributed by the loops. The interactions of ligands with the loop  $\beta$ 3/ $\beta$ 4 seems to be unique for this PH domain and could explain the high affinity of PLC $\delta$ -1 for Ins(1,4,5)P<sub>3</sub> and PtdIns(4,5)P<sub>2</sub> (25). In the crystal structure of the complex of Ins(1,4,5)P<sub>3</sub> and  $\beta$ -spectrin, the Ins(1,4,5)P<sub>3</sub> seems to bind to a different location compared to PLC $\delta$ -1 and is more exposed to solvent (25, 27). Here the binding pocket is formed by the  $\beta$ 1/ $\beta$ 2 and  $\beta$ 5/ $\beta$ 6 loops (27). The number of hydrogen bonds is decreased, such that five amino acids form seven hydrogen bonds to bind the ligand, which may account for the decreased affinity. The phosphates at the D-4 and D-5 positions are important for the interaction. Inspection of the  $\beta$ -spectrin PH domain structure does not reveal candidate residues that would potentially interact with a phosphate group at

the D-3 position, or occlude ligand binding (27). Indeed a change in the circular dichroic spectrum was observed with Ins(1,3,4,5)P<sub>4</sub> but not for compounds lacking the phosphates at D-4 or D-5 positions (27). For other PH domains it is not yet known in detail whether they bind to D3-phosphorylated inositol phosphates. Presumably the diverse primary structures lead to variations in the binding site resulting in different affinities and specificities for inositol phosphates.

The primary sequence of the potential inositol phosphate binding site of RAC/PKB is likely to be more closely related to PLC $\delta$ -1 than  $\beta$ -spectrin, as shown in Fig. 7. Analysis of the affinities of inositol phosphates for the PH domain of RAC/PKB predicts a binding site with high affinities for D3-phosphorylated compounds and poor discrimination against other inositol phosphates. RAC/PKB shows amino acid residues homologous to PLC $\delta$ -1 in the presumed variable loop  $\beta$ 3/ $\beta$ 4. The loop  $\beta$ 3/ $\beta$ 4 in PLC $\delta$ -1 is involved in the binding of Ins(1,4,5)P<sub>3</sub> (25) and it is possible that the homologous region of RAC/PKB is also involved in binding of the ligand, as well as the  $\beta$ 1/ $\beta$ 2 and  $\beta$ 4/ $\beta$ 5 loops. This could explain the higher affinities compared to dynamin and  $\beta$ -spectrin. Furthermore, our results suggest that there should be specific residues in RAC/PKB that interact with the D3 phosphate group of the ligand. We are currently in the process of determining the structure of the kinase PH domain complexed with different inositol phosphates.

Several studies have shown that growth factor receptors stimulate PI3-K activity, leading to the activation of RAC/PKB (6–10). Activation of PI3-K leads to an elevation in the levels of D3-phosphorylated phosphoinositides in cells (reviewed in Refs. 3–5). The specific binding of these phosphoinositides to the RAC/PKB PH domain could provide the link between PI3-K and RAC/PKB. In accordance with this suggestion previous studies revealed that activation of RAC/PKB by serum and PDGF was ablated with constructs lacking a functional PH domain (7, 9). However, it should be noted that insulin is able to stimulate RAC/PKB mutants which lack the PH domain (34).<sup>2</sup> The mechanistic reasons for these differences remain to be established.

Franke *et al.* (7) showed that D3-phosphorylated PtdIns produced by immunoprecipitated PI3-K activated RAC/PKB *in vitro*. However, *in vivo* it is not clear whether PtdIns is a substrate of PI3-K; PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> are more likely physiological substrates (3, 4). Here, we demonstrate that the D3-phosphorylated phosphoinositides PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> bind to the PH domain of RAC/PKB and have opposing effects on RAC/PKB activity. PtdIns(3,4,5)P<sub>3</sub> completely inhibits the activity of RAC/PKB *in vitro* and PtdIns(3,4)P<sub>2</sub> stimulates the activity of RAC/PKB about 2-fold. Depending on the turnover of PtdIns(3,4,5)P<sub>3</sub> after cellular stimulation it is possible that the interaction of RAC/PKB with PtdIns(3,4,5)P<sub>3</sub> has a minor function in the cellular environment. In thrombin- or fMet-Leu-Phe-stimulated platelets the peak response of PtdIns(3,4,5)P<sub>3</sub> already declined after 1 min whereas the level of PtdIns(3,4)P<sub>2</sub> increased and was maintained over several minutes (35, 36). In PDGF-stimulated smooth muscle cells, or insulin-stimulated Chinese hamster ovary cells the levels of both PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> are increased over several minutes (37, 38). The conversion from PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> could possibly be mediated by the mitogen-stimulated inositol polyphosphate 5-phosphatase SHIP (39). It is conceivable that in some cell types, RAC/PKB interacts predominantly with PtdIns(3,4)P<sub>2</sub> and that the ratio of PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> may modulate the

<sup>2</sup> M. Andjelkovic, D. R. Alessi, R. Mayer, P. Cohen, and B. A. Hemmings, unpublished data.

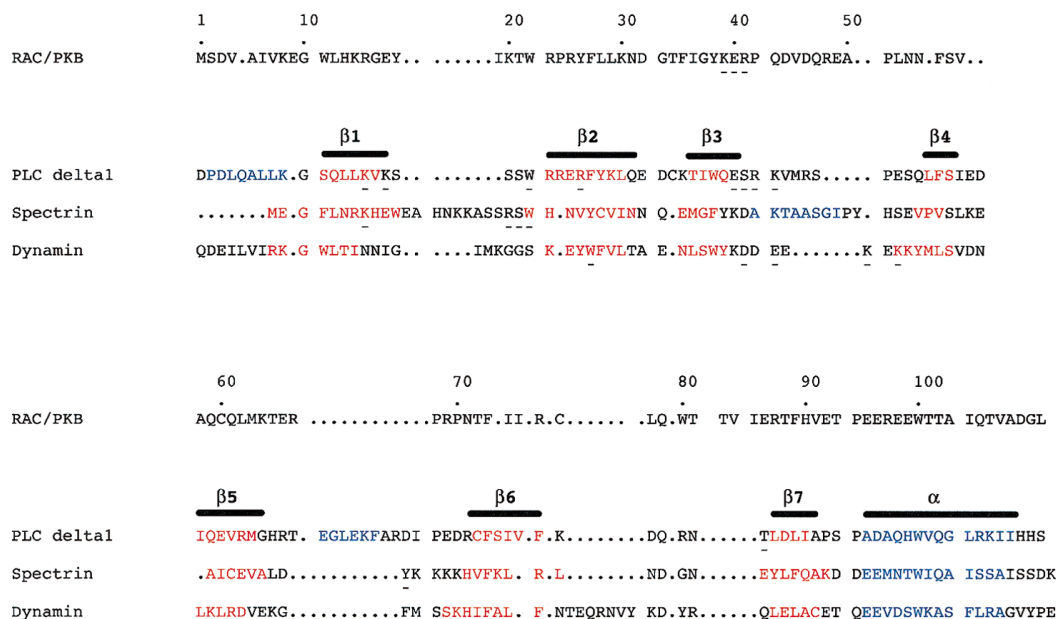


FIG. 7. **Sequence alignment of different PH domains.** Structure based sequence alignment of PLC $\delta$ -1,  $\beta$ -spectrin, and dynamin (according to Ferguson *et al.* (25)) compared to the sequence of the RAC/PKB PH domain. The secondary structure elements are shown in red letters for  $\beta$ -strands and blue letters for  $\alpha$ -helices. The amino acids interacting with Ins(1,4,5)P<sub>3</sub> are *underlined*. For PLC $\delta$ -1 and  $\beta$ -spectrin the amino acid residues which interact with the ligand were identified in the crystallographic structures of the respective PH domains complexed with Ins(1,4,5)P<sub>3</sub> (24, 26). For dynamin the amino acid residues interacting with the ligand were identified by NMR spectroscopy (32). The *dotted line* in the RAC/PKB sequence indicates residues in the presumed  $\beta$ 3/ $\beta$ 4 loop which could be involved in the binding of ligands.

activation of RAC/PKB.

The high affinities of D3-phosphorylated phosphoinositides for the RAC/PKB PH domain is consistent with this domain being important for membrane localization. Given the *in vivo* concentrations of PtdIns(4,5)P<sub>2</sub> and D3-phosphorylated phosphoinositides reported (40), the affinities of the RAC/PKB PH domain for phosphoinositides are sufficient for membrane attachment. Discrimination by the PH domain between PtdIns(3,4)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>, and PtdIns(4,5)P<sub>2</sub> would depend on their concentrations in the cell membrane. The influence of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> on RAC/PKB activity is reminiscent of the allosteric behavior of phospholipase C- $\delta$ 1, which is activated by binding of PtdIns(4,5)P<sub>2</sub> to its PH domain (33, 41). It is not known whether the PH domain of RAC/PKB undergoes a conformational change upon ligand binding. The fact that we see no shift in the emission maximum of the isolated PH domain and the full-length protein suggests no conformational changes in the environment of the tryptophan involved in binding. James and colleagues (30) showed binding of RAC/PKB to phosphoinositide containing vesicles with similar affinities as we found with our fluorescence assay, but they did not observe any influence on the activity of RAC/PKB in the presence of PtdIns(3,4)P<sub>2</sub> or PtdIns(3,4,5)P<sub>3</sub> under their experimental conditions. They did not investigate the binding mechanism or possible conformational changes involved upon ligand binding. Clearly, the kinetics of the association and dissociation and possible accompanying conformational changes has to be studied in more detail. The fact that RAC/PKB isolated from unstimulated cells showed the same behavior, with similar half-maximal concentrations of inhibition and activation by PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> argues that for this interaction the phosphorylation state of the protein has a minor influence. The maximal activation induced by PtdIns(3,4)P<sub>2</sub> *in vitro* is lower than the activation of RAC/PKB achieved by phosphorylation *in vivo*. Therefore, a conformational change induced by PtdIns(3,4)P<sub>2</sub> alone would not be sufficient to cause maximal activation as proposed by Franke *et al.* (7).

In order to more fully understand the role of the PH domain

in kinase activation we have recently prepared a series of membrane targeted RAC/PKB constructs.<sup>2</sup> The rationale for these experiments was that if the PH domain were important for kinase activation by localizing the protein at the membrane, artificially targeting the RAC/PKB should promote activation. In summary, our results show that membrane targeting was sufficient to promote kinase activation by phosphorylation on Thr-308 and Ser-473. These results therefore suggest that an upstream RAC/PKB kinase or kinases are localized on the membrane. The fact that a “kinase-dead” RAC/PKB construct was also phosphorylated on the same residues supports this suggestion. During the preparation of this manuscript Kohn *et al.* (34) reported that membrane targeting of RAC/PKB in TRMP canine kidney epithelial cells overexpressing the PDGF receptor and CHO.IR cells overexpressing the insulin receptor lead to a substantial increase of kinase activity. With both studies (34)<sup>2</sup> deletion of the PH domain from the membrane targeted construct did not block or inhibit activation. These results illustrate that phosphorylation of RAC/PKB is the principal mechanism for kinase activation following stimulation by growth factors or insulin (9, 18).

The interaction of RAC/PKB with PtdIns(3,4)P<sub>2</sub> could therefore promote both membrane localization and cause a conformational change which allows efficient phosphorylation by an as yet unidentified kinase. It is also possible that PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> generated after cell stimulation activates this upstream kinase which phosphorylates Thr-308 and Ser-473, working in concert with the PtdIns(3,4)P<sub>2</sub> induced conformational change to fully activate RAC/PKB. Isolation and characterization of the upstream kinase will be required to fully delineate the mechanism of RAC/PKB activation, and further study of RAC/PKB regulation by RTK-activated PI3-K should provide significant insights into lipid signaling mechanisms and the involvement of PH domains.

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## High Affinity Binding of Inositol Phosphates and Phosphoinositides to the Pleckstrin Homology Domain of RAC/Protein Kinase B and Their Influence on Kinase Activity

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