# Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) interacts stereoselectively with the D-enantiomer of PtdIns(3,4,5)$P_3$ ($K_D$ 1.6 nM) and PtdIns(3,4)$P_2$ ($K_D$ 5.2 nM), but binds with lower affinity to PtdIns3$P$ or PtdIns(4,5)$P_2$. The binding of PtdIns(3,4,5)$P_3$ to PDK1 was greatly decreased by making specific mutations in the pleckstrin homology (PH) domain of PDK1 or by deleting it. The same mutations also greatly decreased the rate at which PDK1 activated protein kinase B$\alpha$ (PKB$\alpha$) *in vitro* in the presence of lipid vesicles containing PtdIns(3,4,5)$P_3$, but did not affect the rate at which PDK1 activated a PKB$\alpha$ mutant lacking the PH domain in the absence of PtdIns(3,4,5)$P_3$. When overexpressed in 293 or PAE cells, PDK1 was located at the plasma membrane and in the cytosol, but was excluded from the nucleus. Mutations that disrupted the interaction of PtdIns(3,4,5)$P_3$ or PtdIns(4,5)$P_2$ with PDK1 abolished the association of PDK1 with the plasma membrane. Growth-factor stimulation promoted the translocation of transfected PKB$\alpha$ to the plasma membrane, but had no effect on the subcellular distribution of PDK1 as judged by immunoelectron microscopy of fixed cells. This conclusion was also supported by confocal microscopy of green fluorescent protein–PDK1 in live cells. These results, together with previous observations, indicate that PtdIns(3,4,5)$P_3$ plays several roles in the PDK1-induced activation of PKB$\alpha$. First, it binds to the PH domain of PKB, altering its conformation so that it can be activated by PDK1. Secondly, interaction with PtdIns(3,4,5)$P_3$ recruits PKB to the plasma membrane with which PDK1 is localized constitutively by virtue of its much stronger interaction with PtdIns(3,4,5)$P_3$ or PtdIns(4,5)$P_2$. Thirdly, the interaction of PDK1 with PtdIns(3,4,5)$P_3$ facilitates the rate at which it can activate PKB.

Key words: lipid binding, protein kinase B, signal transduction, surface plasmon resonance.

## INTRODUCTION

Phosphoinositide (PI) 3-kinases are a family of enzymes capable of phosphorylating inositol phospholipids on the 3-hydroxy position of the inositol ring [1]. Although these enzymes phosphorylate PtdIns, PtdIns4$P$ and PtdIns(4,5)$P_2$ *in vitro*, they appear to phosphorylate PtdIns(4,5)$P_2$ preferentially *in vivo*, producing PtdIns(3,4,5)$P_3$ [2]. The type I subfamily of PI 3-kinases are regulated by many agonists and growth factors which therefore cause stimulated increases in the levels of PtdIns(3,4,5)$P_3$. PtdIns(3,4,5)$P_3$ is rapidly metabolized and inactivated by a family of 5-phosphatases [3] or by a 3-phosphatase [2,4] to give PtdIns(3,4)$P_2$ and PtdIns(4,5)$P_2$ respectively. Stimulation of PI 3-kinases therefore also increases the level of PtdIns(3,4)$P_2$ after a short delay [2]. PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ are potential lipid second messengers, although the effectors of these signals are still not fully defined.

Protein kinase B (PKB) was identified independently by three groups on the basis of similarity to protein kinases A and C and as the cellular homologue of the v-Akt oncogene [5–7]. PKB is activated within minutes in response to growth factors or insulin [8–11] and is thought to regulate a number of cellular processes, including glycogen metabolism [10], protein synthesis [12] and apoptosis [13,14]. The activation of PKB is mimicked by constitutively active PI 3-kinases [15,16] and blocked by pharmacological inhibitors of PI 3-kinase [8] or by dominant-negative constructs of the p85 regulatory subunit of PI 3-kinase [9]. The activation of PKB$\alpha$ requires its phosphorylation at Thr$^{308}$ and Ser$^{473}$ [17] and both phosphorylations are blocked by PI 3-kinase inhibitors. These observations indicated that the phosphorylation and activation of PKB$\alpha$ by one or more 'upstream' kinases is dependent on PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$.

A protein kinase that phosphorylates PKB$\alpha$ on Thr$^{308}$ in the presence of PtdIns(3,4,5)$P_3$ or PtdIns(3,4)$P_2$ has been identified [18–21] and called '3-phosphoinositide-dependent protein kinase-1' (PDK1). The above findings imply that Ser$^{473}$ is phosphorylated by a distinct protein kinase that has still to be identified, provisionally termed PDK2. PDK1 comprises an N-terminal catalytic domain and a C-terminal pleckstrin homology (PH) domain. This contrasts with PKB, which contains an N-terminal PH domain followed by a C-terminal catalytic domain. PDK1 [21] and PKB$\alpha$ [22] bind (presumably via their PH domains) to lipid vesicles containing PtdIns(3,4,5)$P_3$ or PtdIns(3,4)$P_2$ [21]. Although initial studies suggested that PKB$\alpha$ might be activated directly by PtdIns(3,4)$P_2$ [23–25], subsequent work failed to reproduce the results of these experiments and instead showed that PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ exert their effects by promoting the phosphorylation and activation of PKB$\alpha$ by

Abbreviations used: PtdCho, phosphatidylcholine; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; DiIC$_{16}$(3), 3-*H*-indolinium hexadec-1-yl-2-[3-(hexadec-1-yl-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-ylidine)propen-1-yl-3,3-dimethyl; PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology; IGF-1, insulin-like growth factor-1; PKI, TTYADFIASGRTGRRNAIHD, the specific inhibitor of cAMP-dependent protein kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; FSG, fish skin gelatin; DMEM, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor; HPA, hydrophobic affinity.

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PDK1 [18,20]. Deletion of the PH domain of PDK1 greatly decreases its ability to phosphorylate and activate PKB$\alpha$ *in vitro*, but this phosphorylation is still PtdIns(3,4,5)$P_3$-dependent [19]. Deletion of the PH domain of PKB also greatly decreases the ability of PDK1 to phosphorylate and activate PKB, but this phosphorylation is now PtdIns(3,4,5)$P_3$-independent [19]. Taken together, these observations indicate that one role for PtdIns(3,4,5)$P_3$ in the activation of PKB is to change its conformation so that it becomes a substrate for PDK1. However, a role for the binding of PtdIns(3,4,5)$P_3$ to PDK1 in the activation of PKB was suggested by the large decrease in the rate of PKB activation that occurred when the PH domain of PDK1 was deleted [19]. This is also suggested by the finding that PKB$\alpha$ lacking a PH domain is still activated in response to insulin, activation being suppressed by inhibitors of PI 3-kinase [11].

PKB$\alpha$ translocates to the plasma membrane in response to insulin or insulin-like growth factor-1 (IGF-1), and this association requires an intact PH domain and is inhibited by wortmannin [26]. Thus the activation of wild-type PKB$\alpha$ appears to involve a PtdIns(3,4,5)$P_3$-mediated translocation to the plasma membrane, followed by its phosphorylation by PDK1 and PDK2. These observations suggest that PDK1 and PDK2 might be associated with, or enriched at, the plasma membrane. Further evidence supporting this possibility has been obtained by attaching a membrane-targeting motif to the N-terminus of PKB. When cells are transfected with this construct, PKB$\alpha$ is largely present at the plasma membrane and is maximally activated and phosphorylated at $Thr^{308}$ and $Ser^{473}$, even in unstimulated cells [26].

In the present study we have investigated whether the binding of PtdIns(3,4,5)$P_3$ to PDK1 plays a role in the activation of PKB$\alpha$ by making mutations in the PH domain of PDK1. We find that the PH domain of PDK1 binds to PtdIns(3,4,5)$P_3$ [and more weakly to PtdIns(3,4)$P_2$] and that this is required for the rapid activation of PKB$\alpha$ *in vitro*. We also find that a functional PH domain is needed for the constitutive association of PDK1 with the plasma membrane *in vivo*. We fail to observe any translocation of PDK1 from the cytosol to the membrane in response to growth-factor stimulation, in contrast with another recent report [28].

## MATERIALS AND METHODS

### Materials

Tissue-culture reagents and IGF-1 were from Life Technologies (Paisley, Refrewshire, Scotland,U.K.). 'Complete' protease cocktail was from Boehringer-Mannheim (Lewes, East Sussex, U.K.). All inositol phospholipids were from previously described sources [18], except for PtdIns3*P*, which was from Eschelon Research Laboratories, Salt Lake City, UT, U.S.A. An antibody was raised in sheep against the whole PDK1 protein and affinity-purified on PDK1 coupled to CH-Sepharose (Amersham Pharmacia Biotech, St. Albans, Herts., U.K.). The rabbit anti-sheep secondary antibody used for electron microscopy was from Southern Biotechnology Associates Inc. (Birmingham, AL, U.S.A.), and 8 nm colloidal gold coupled to Protein A was prepared as described in [36]. PBS tablets were from Oxoid Ltd. (Basingstoke, U.K.) and were dissolved according to the manufacturer's instructions. The peptides RPRAATF (a specific substrate for PKB) and TTYADFIASGRTGRRNAIHD (the specific inhibitor of cAMP-dependent protein kinase, termed PKI) were synthesized by Mr. F. B. Caudwell (MRC Protein Phosphorylation Unit, Dundee) on an Applied Biosystems 431A peptide synthesizer. Oligonucleotides were purchased from Oswel (Southampton, U.K.). All other materials were either from Sigma Chemical Co. (Poole, Dorset, U.K.) or Merck/BDH (Poole, Dorset, U.K.).

### Generation of PDK1 PH domain mutants

Conserved amino acids in the PH domain of PDK1 [19] were mutated to leucine using PCR-based methods. The resulting PCR products were sequenced and ligated into pCMV5 vector [37] as *Eco*RI–*Kpn*I inserts. In order to express recombinant proteins for biochemical studies, the same PDK1 mutants were ligated into the *Bam*HI–*Kpn*I site of pEBG-2T [38] as *Bgl*II–*Kpn*I inserts. In order to create an N-terminally tagged green fluorescent protein (GFP)–PDK1 construct, the *Bgl*II–*Kpn*I fragment of PDK1 was ligated into the corresponding sites in the pEGFP-C1 plasmid (Clontech, Palo Alto, CA, U.S.A.). In order to create an N-terminal tagged GFP–PKB-PH domain construct, a 750 bp *Bam*HI–*Apa*I fragment encoding the first 250 amino acids of PKB$\alpha$ (incorporating the PH domain) was ligated into a *Bam*HI–*Bgl*II site of the pEGFP-C1 plasmid (Clontech, Palo Alto, CA, USA). The nucleotide-sequence-database accession number for human PDK1 is AF017995 and that for human PKB$\alpha$ is M63167 (except that the nucleotide sequence differed at one position, which changed $Ser^{478}$ to a glycine residue).

### Expression of PDK1 and PKB in 293 cells

PDK1, PKB and mutants derived from these enzymes were expressed as glutathione S-transferase (GST) fusion proteins and purified by glutathione–Sepharose affinity chromatography [18]. All of the proteins were $> 80\,\%$ pure as judged by SDS/PAGE (results not shown) and 0.1–0.2 mg of wild-type or mutant PDK1 was obtained from 20 10-cm-diameter dishes of 293 cells. Human PKB$\alpha$ and PDK1 were both expressed as a haemagglutinin (HA)-tagged protein for studies of their localization by electron microscopy [26].

### Assay of wild-type and mutant PDK1

PDK1 assays were carried out as described in [18], using 3.2 ng of PDK1 per assay with either 0.5 $\mu$g of wild-type GST–PKB$\alpha$ in the presence of lipid vesicles containing PtdIns(3,4,5)$P_3$ or with 0.5 $\mu$g of GST–$\Delta$PH-PKB ($\Delta$PH indicates that PKB lacks the PH domain) in the absence of any lipids. The autophosphorylation of PDK1 was examined by incubating the enzyme (40 ng) for 10 min at 30 °C in 50 mM Tris/HCl (pH 7.5)/5 $\mu$M PKI/10 mM magnesium acetate/0.1 mM [$\gamma$-$^{32}$P]ATP (sp. radioactivity 400 c.p.m./nmol) in a reaction volume of 10 $\mu$l. The reaction was terminated by denaturation in SDS and the samples were subjected to SDS/PAGE. PDK1 autophosphorylation was detected by autoradiography of the gels.

### Binding of inositol phospholipids to PDK1 measured by surface plasmon resonance

The kinetics of binding recombinant GST fusion proteins of wild-type and mutant PDK1 to inositol phospholipids was determined in an Upgraded BiaLite biosensor (BiaCore AB, Stevenage, Herts., U.K.). Unilamellar phospholipid vesicles containing *sn*-1-palmitoyl-2-oleoyl phosphatidylcholine (PtdCho) with or without PtdIns(3,4,5)$P_3$ at up to $4\,\mathrm{mol}\,\%$ were prepared by extrusion through 100 nm-pore-size polycarbonate filters as described [22]. A supported lipid monolayer was created over an octyl glucoside-washed hydrophobic affinity (HPA) Sensorchip (BiaCore) by injecting 100 $\mu$l of vesicles at 0.5 mM with respect to PtdCho in 50 mM Hepes (pH 7.4)/150 mM NaCl at a flow rate of 5 $\mu$l/min. The binding rate of phospholipid vesicles containing the acidic inositol phospholipids was greatly

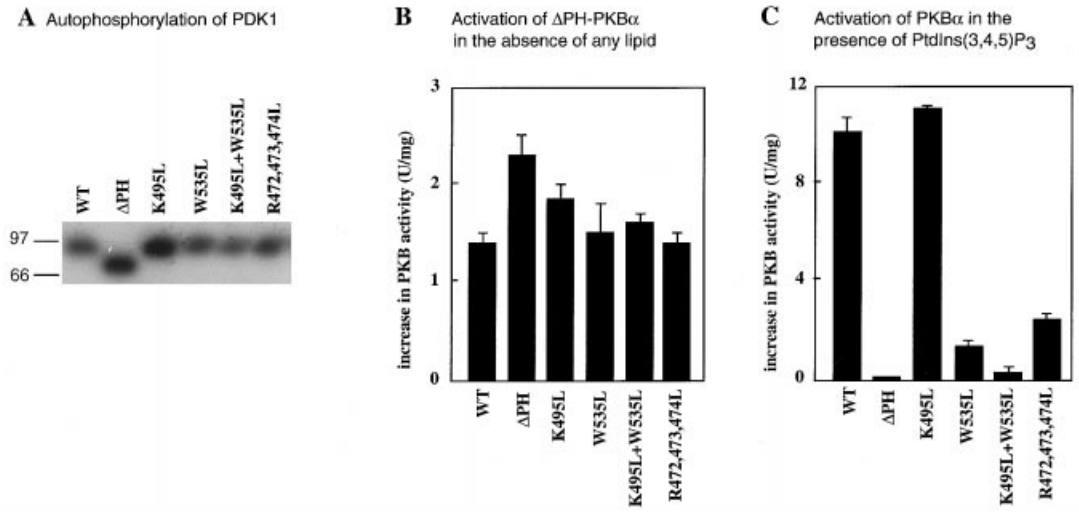


**Figure 1 Effect of mutating the PH domain on the activity of PDK1**

(**A**) Wild-type PDK1 and the PH domain mutants were incubated for 10 min at 30 °C with Mg[$\gamma$-$^{32}$P]ATP and subjected to SDS/PAGE followed by autoradiography. The positions of the molecular-mass markers glycogen phosphorylase (97 kDa) and BSA (66 kDa) are indicated. (**B**,**C**) Wild-type PDK1 and the PH domain mutants were assayed using ΔPH-PKB as a substrate in the absence of PtdIns(3,4,5)$P_3$ (**B**) or using GST–PKBα as a substrate in the presence of 1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)$P_3$ (**C**); 1 unit (U) of PKB is the activity that phosphorylates 1 nmol of the PKB peptide substrate Crosstide in 1 min. The data are presented as the increase in GST–PKBα activity compared with a control incubation in which PDK1 was omitted and are given as means ± S.E.M. for two or three separate experiments each performed in triplicate.

decreased in 50 mM Hepes (pH 7.4)/150 mM NaCl and could be enhanced by increasing the NaCl concentration to 450 mM. After several brief pulses of 10 mM NaOH, increasing the flow rate to 100 $\mu$l/min for 15 min and then decreasing it to 5 $\mu$l/min for 12 h, a stable baseline was established [loss of lipid $< 2$ RU (resonance units; BiaCore)/min]. The binding of 50–200 nM GST–PDK1 to these lipid monolayers was measured in an intracellular type buffer [22] at a flow rate of 30 $\mu$l/min. The monolayer was regenerated using brief pulses of 10 mM NaOH. Binding was corrected for background association to the PtdCho surface and the resulting sensorgram analysed using the BiaEvaluation 2 software supplied with the instrument. Sensorgrams were analysed using a simple bimolecular-interaction model; after allowing for drift, this model gave a reasonably good fit to the data (as assessed by small $\chi^2$ values relative to the data noise and random scatter of residuals). The monolayer could be removed by washing the surface with 40 mM octyl glucoside and a fresh monolayer subsequently laid down on the same HPA Sensorchip.

### Determination of the localization of PDK1 using immunoelectron microscopy

293 cells were transiently transfected with the pCMV5 mammalian expression vector containing wild-type or mutant PDK1 as described in [17]. Cells were serum-starved for 16 h before stimulation with IGF-1. At 40 h post-transfection the medium was removed from the cells, which were then fixed in 4 % (w/w) paraformaldehyde/0.1 % (w/w) glutaraldehyde in 0.2 M Pipes, pH 7.2, for 30 min and washed in PBS, pH 7.4, then scraped from the dish with a 'rubber policeman' and embedded in 10 % pig skin gelatin before cryoprotection in 2.3 M sucrose in PBS. Ultrathin sections were cut at −110 °C in a Reichert Ultracut E cryomicrotome and mounted on carbon/formvar-coated grids. Sections mounted on grids were labelled at room temperature as follows: after 10 min the grids were placed on top of drops of 0.1 M $NH_4Cl$ in PBS followed by a 10 min preincubation on 0.5 % (w/w) fish skin gelatin in PBS (FSG/PBS). The grids were then transferred on to drops of anti-PDK1 antibody or anti-HA antibody (5 $\mu$g/ml in FSG/PBS) for 30 min. The grids were washed three times (5 min per wash) in PBS then transferred on to drops of rabbit anti-sheep or anti-mouse secondary antibody (2 $\mu$g/ml in FSG/PBS) for 20 min. After a further three washes with PBS, the grids were incubated with Protein A–gold for 20 min, then washed six times (10 min per wash) with PBS and 10 times with distilled water (1 min per wash). Control experiments were carried out in which the primary antibody was replaced with FSG/PBS. Sections were embedded and contrasted in methylcellulose uranyl acetate as described in [39]. Observations were made using a Jeol 1200 EX electron microscope. To quantify immunolabelling, sections were scanned systematically and gold-labelled cells were identified. All visible parts of the plasma membrane and adjacent cytoplasm were photographed at 15 000 × magnification. Cytoplasmic areas (excluding the nucleus and other organelles) and plasma-membrane profile length were estimated using a square grid lattice with 1 cm line spacing as described in [36]. Gold particles were only assigned to the plasma membrane if they lay within two particle widths of the plasma-membrane profile. Plasma-membrane labelling was evaluated as the ratio of plasma membrane to cytosolic labelling density. This ratio takes account of any variations in cytosolic PDK1 expression, which appeared to be linearly related to the plasma-membrane labelling under the different conditions tested.

### Determination of the localization of PDK1 using confocal microscopy

293 cells were transfected with 2 $\mu$g of GFP–PDK1 or GFP–PKB-PH construct using the Fugene 6 (BCL) transfection reagent according to the manufacturer's instructions and maintained for 24 h in Dulbecco's modified Eagle's medium (DMEM) to allow expression of the constructs. Transfection efficiencies of 35–60 % were obtained routinely by this method as assessed by fluorescence microscopy. All cells were serum-starved for 3 h prior to

stimulation in serum-free DMEM. In the case of dual labelling experiments, the lipid of the plasma membrane was labelled with 3-*H*-indolinium hexadec-1-yl-2-[3-(hexadec-1-yl-1,3-dihydro-3,3-dimethyl-2*H*-indol-2-ylidine)propen-1-yl-3,3-dimethyl; [$DiIC_{16}(3)$; Cambridge Bioscience, Cambridge, U.K.] at a concentration of 5 $\mu$M by incubation for 10 min at 37 °C in serum-free DMEM. Live transfected and $DiIC_{16}(3)$-labelled cells were washed and covered with Krebs buffer [118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/1.3 mM $CaCl_2$/1.8 mM $K_2HPO_4$/25 mM Hepes (pH 7.4)/11.7 mM glucose] before observation under the confocal microscope. A single cell (or group of cells) was chosen at random and insulin was added to the Krebs buffer to a final concentration of 100 nM. Images of the cells were collected at a rate of one per minute over a 15 min time course.

## RESULTS

### Analysis of PH-domain mutants of PDK1

In order to investigate the role of the PH domain of PDK1, five mutants were generated. Residues conserved in other PI-binding PH domains were altered to leucine residues. These single mutants [K495L (one-letter code, $Lys^{495} \rightarrow Leu$ etc.) and W535L], the double mutant K495L/W535L and the triple mutant R472L/R473L/R474L), together with a further mutant lacking the PH domain (ΔPH-PDK1) were cloned, expressed and purified (see the Materials and methods section).

The PDK1-catalysed activation of a PKBα mutant lacking its PH domain (ΔPH-PKBα) occurs in the absence of 3-phosphorylated inositides, albeit very slowly [19]. All five PDK1 mutants activate ΔPH-PKBα at a similar rate to wild-type PDK1 in the absence of PtdIns(3,4,5)$P_3$ (Figure 1B) and autophosphorylate to similar extents (Figure 1A). This demonstrates that the catalytic activity of PDK1 is not compromised by the alterations made to the PH domain.

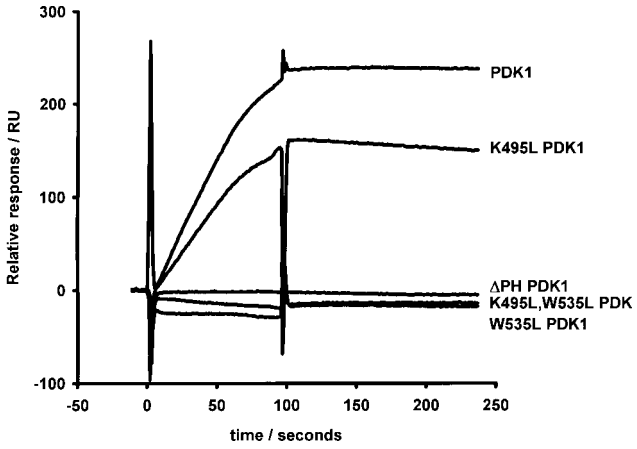


**Figure 2 Binding of PtdIns(3,4,5)$P_3$ to wild-type and mutant PDK1**

A supported phospholipid monolayer containing 1 mol% of PtdIns(3,4,5)$P_3$ was created on an HPA Sensorchip as described in the Materials and methods section. Wild-type PDK1 and the indicated PDK1 PH domain mutants were injected over the surface at 100 nM and protein binding was measured by surface plasmon resonance. The results show an overlay plot of typical sensorgrams, which were repeated six times.

**Table 1 Binding of PDK1 and PKB to supported lipid monolayers containing polyphosphoinositides measured by surface plasmon resonance**

A supported lipid monolayer was created on an HPA sensorchip by injection of extruded lipid vesicles containing *sn*-1,2-palmitoyloleoyl PtdCho and 4 mol% of the indicated poly-PI. Binding of 100 nM PDK1 or PKB was then measured as described in the Materials and methods section. Data are means ± S.E.M. from at least three experiments. No specific binding to the sensorchip is indicated by nb. In this Table and Table 2, $k_d$ is the dissociation rate constant, $k_a$ is the association rate constant and $K_D$ is the equilibrium dissociation constant, i.e. the ratio of the other two constants.

| Protein | Poly-PI | $k_a$ ($M \cdot s^{-1}$) | $k_d$ ($s^{-1}$) | Apparent $K_D$ (nM) |
|---|---|---|---|---|
| PDK1 | D-PtdIns(3,4,5)$P_3$ | $(1.41 \pm 0.09) \times 10^5$ | $(1.8 \pm 0.6) \times 10^{-4}$ | 1.6 |
| | PtdIns(3,4)$P_2$ | $(1.3 \pm 0.1) \times 10^5$ | $(6.8 \pm 0.6) \times 10^{-4}$ | 5.2 |
| | PtdIns(4,5)$P_2$ | $(7.5 \pm 0.9) \times 10^4$ | $(1.8 \pm 0.8) \times 10^{-3}$ | 24 |
| | PtdIns3$P$ | $(1.1 \pm 0.2) \times 10^5$ | $(9.2 \pm 0.8\} \times 10^{-2}$ | 84 |
| PKB | D-PtdIns(3,4,5)$P_3$ | $(3.1 \pm 0.2) \times 10^4$ | $(1.10 \pm 0.02) \times 10^{-3}$ | 35 |
| | PtdIns(4,5)$P_2$ | nb | nb | > 100 |

**Table 2 Binding of wild-type and mutant PDK1 and PKB to 1,2-stearoylarachidonyl D-PtdIns(3,4,5)$P_3$ and D-PtdIns(4,5)$P_2$ on a supported lipid monolayer**

Sensorgrams were produced and analysed as described in Table 1 and in the Materials and methods section. Data are means ± S.E.M. from at least three experiments.

| PI ... | PtdIns(3,4,5)$P_3$ | | | PtdIns(4,5)$P_2$ | | |
|---|---|---|---|---|---|---|
| Protein | $k_a$ ($M \cdot s^{-1}$) | $k_d$ ($s^{-1}$) | Apparent $K_D$ (nM) | $k_a$ ($M \cdot s^{-1}$) | $k_d$ ($s^{-1}$) | Apparent $K_D$ (nM) |
| PDK1 | $(1.41 \pm 0.05) \times 10^5$ | $(2.3 \pm 0.6) \times 10^{-4}$ | 1.6 | $(7.5 \pm 0.9) \times 10^4$ | $(1.8 \pm 0.80) \times 10^{-3}$ | 24 |
| K495L-PDK1 | $(1.22 \pm 0.09) \times 10^5$ | $(2.5 \pm 0.8) \times 10^{-3}$ | 20 | $(6.9 \pm 1) \times 10^4$ | $(3.2 \pm 1) \times 10^{-3}$ | 47 |
| W535L-PDK1 | nb | nb | > 100 | nb | nb | > 100 |
| W535L/K495l-PDK1 | nb | nb | > 100 | nb | nb | > 100 |
| R472/473/474L-PDK1 | nb | nb | > 100 | nb | nb | > 100 |
| ΔPH-PDK1 | nb | nb | > 100 | nb | nb | > 100 |
| PKB | $(3.1 \pm 0.2) \times 10^4$ | $(1.10 \pm 0.02) \times 10^{-3}$ | 35 | nb | nb | > 100 |
| ΔPH-PKB | nb | nb | > 100 | nb | nb | > 100 |

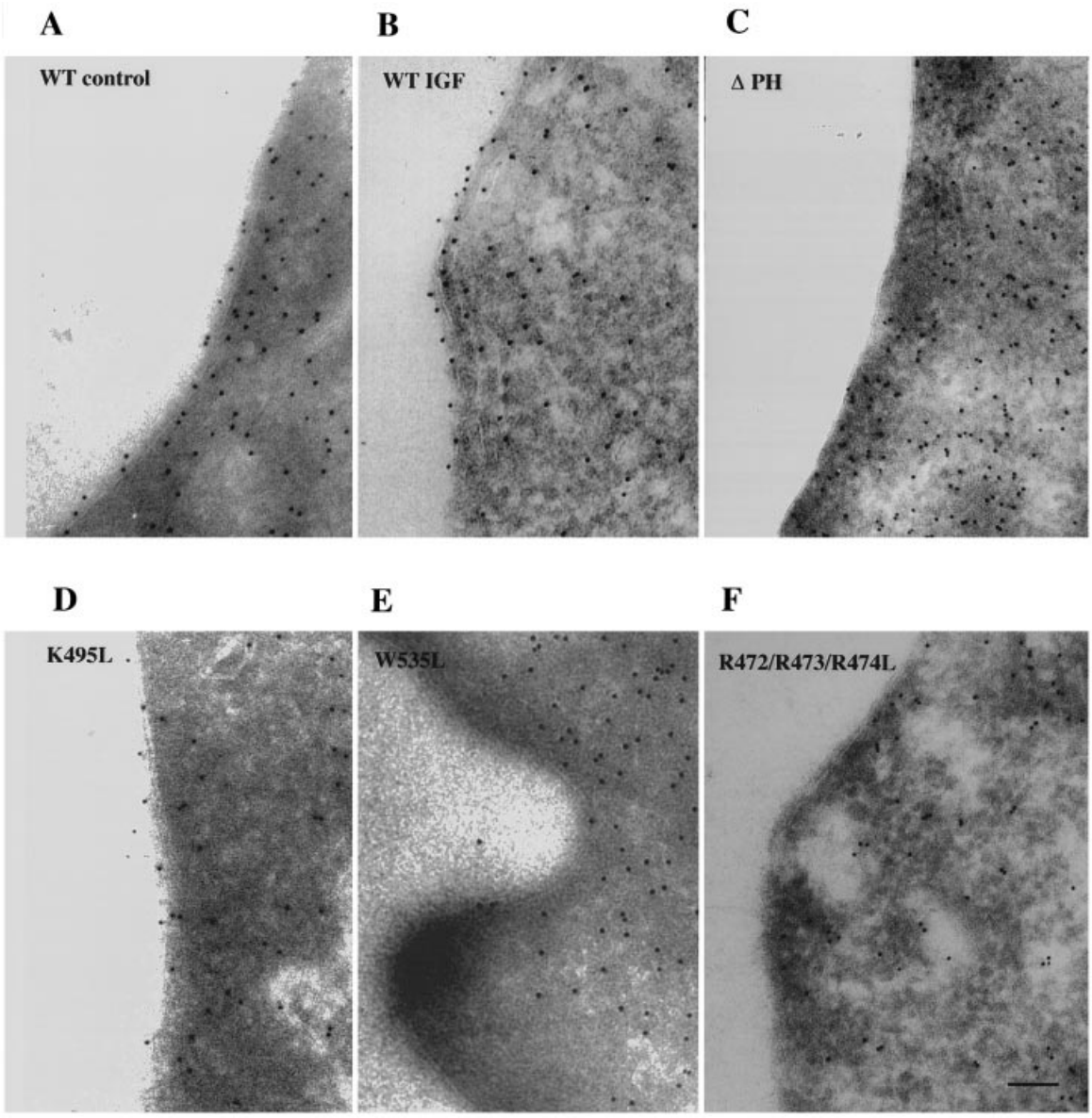


**Figure 3 Localization of wild-type and mutant PDK1 determined by immunoelectron microscopy**

The panels show typical electron micrographs of immunogold labelling at the plasma membrane of 293 cells transfected with wild-type PDK1 (**A**), wild-type PDK1 after stimulation for 5 min with IGF1 (**B**), ΔPH PDK1 (**C**), K495L PDK1 (**D**), W535L PDK1 (**E**) and R472/R473/R474L PDK1 (**F**). Scale bar represents 100 nm.

The activation of wild-type PKBα only occurs in the presence of lipid vesicles containing PtdIns(3,4,5)$P_3$. In the presence of *sn*-1-stearoyl-2-arachidonyl PtdIns(3,4,5)$P_3$, the naturally occurring derivative, K495L-PDK1 activates PKBα at a similar rate to wild-type PDK1. However W535L-PDK1, K495L/W535L-PDK1, R472L/R473L/R474L-PDK1 and ΔPH-PDK1 activate wild-type PKBα much more slowly (Figure 1C). This suggests that the PH domain of PDK1 plays an important role in activating PKBα in lipid vesicles *in vitro* and prompted an analysis of the binding of PtdIns(3,4,5)$P_3$ to wild-type and mutant PDK1.

### Lipid binding of PDK1 PH-domain mutants

We have developed an assay to measure the kinetics of binding of proteins to a supported lipid monolayer containing a low molar fraction of poly-PI using surface plasmon resonance. (A detailed description and characterization of this assay and a comparison with other protein-lipid binding assays is available from R.A.C. or C.P.D. on request). The *sn*-1-stearoyl-2-arachidonyl D-PtdIns(3,4,5)$P_3$ enantiomer of PtdIns(3,4,5)$P_3$ binds to PDK1 with high affinity ($K_D$ = 1.6 nM), whereas PtdIns(3,4)$P_2$ binds 3-fold less strongly, PtdIns(4,5)$P_2$ 15 times less strongly and PtdIns(3)$P$ over 50-fold less strongly (Table 1). PtdIns-(3,4,5)$P_3$ binds to PDK1 about 20-fold more strongly than to PKBα (Table 1), as reported previously using a different assay [21]. It should be noted that PDK1 binds to PtdIns(4,5)$P_2$ (Table 1) at least as strongly as PKB binds to PtdIns(3,4,5)$P_3$, whereas PKB does not bind to PtdIns(4,5)$P_2$ under the assay conditions used (Table 1).

Wild-type PDK1 binds PtdIns(3,4,5)$P_3$ with slightly higher affinity than K495L-PDK1 (Table 2), the decreased affinity of

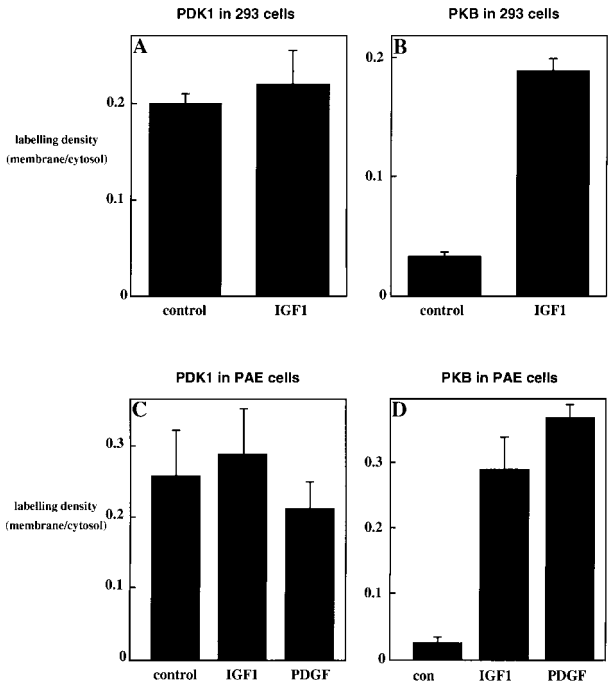


**Figure 4 The amount of PDK1 associated with the plasma membrane is not increased by growth-factor stimulation**

The Figure shows quantification of gold labelling at the plasma membrane of 293 cells and PAE cells relative to the amount in the cytosol. 293 cells (**A**, **B**) or PAE cells (**C**, **D**) were transfected with wild-type PDK1 (**A**, **C**) or wild-type PKB (**B**, **D**). The cells were stimulated for 5 min with 10 ng/ml IGF-1 or 10 ng/ml PDGF as indicated, or left unstimulated. The results are presented as the number of gold particles/$\mu$m of plasma membrane as a fraction of the gold particles/$\mu$m$^2$ of cytosol, and are given as the mean $\pm$ S.E.M. for 24–36 cells (two or three separate experiments).

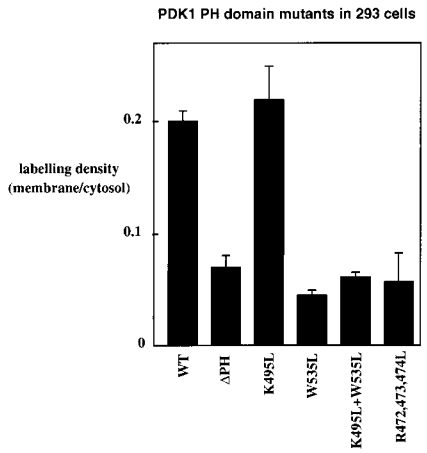


**Figure 5 The association of PDK1 with the plasma membrane is abolished by certain mutations in the PH domain**

The Figure shows quantification of gold labelling at the plasma membrane of 293 cells relative to the amount in the cytosol. The experiments were carried out in unstimulated 293 cells transfected with cDNA constructs encoding wild type or mutant PDK1. Further details are given in the Materials and methods section. The data are presented as the number of gold particles/$\mu$m of plasma membrane as a fraction of the gold particles/$\mu$m$^2$ of cytosol and are given as the mean $\pm$ S.E.M. for 36 cells (three separate experiments).

the mutant being caused by an increase in the dissociation rate constant. W535L-PDK1, K495L/W535L-PDK1 and R472L/R473L/R474L-PDK1, like ΔPH-PDK1, do not bind to PtdIns(3,4,5)$P_3$ at the concentration used (100 nM) (Figure 2 and Table 2). Similar reductions in affinity were observed for the binding of these PDK1 mutants to PtdIns(4,5)$P_2$ (Table 2).

## Localization of PDK1 in 293 and PAE cells by quantitative immunogold electron microscopy

We have previously used quantitative immunogold localization on ultrathin cryosections to demonstrate that transfected PKBα translocates from the cytosol to the plasma membrane of 293 cells in response to IGF-1 [26], presumably by virtue of its interaction with PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$. It was therefore of interest to investigate the subcellular localization of PDK1 in unstimulated and stimulated cells. In unstimulated cells overexpressing PDK1 there is substantial immunogold labelling in both the cytoplasm and at the plasma membrane (Figure 3A), but not in the nucleus (results not shown). No increase in immunogold labelling at the plasma membrane is observed following stimulation with IGF-1 for 5 (Figures 3B and 4A), 30 or 60 min (results not shown). In contrast, PKBα underwent the expected IGF-1-dependent translocation to the plasma membrane in parallel experiments (Figure 4B). Similar results were obtained using PAE cells overexpressing the platelet-derived-growth-factor (PDGF) receptor, where no translocation of PDK1 to the plasma membrane is observed after stimulation with either IGF-1 or PDGF (Figure 4C) under conditions where PKBα is activated 6- and 8-fold respectively (results not shown). Furthermore, PKBα is translocated to the plasma membrane in response to either agonist (Figure 4D).

The K495L-PDK1 mutant that binds PtdIns(3,4,5)$P_3$ showed a similar localization to wild- type PDK1 (Figure 3D). In contrast, W535L-PDK1 (Figure 3E), R472L/R473L/R473L-PDK1 (Figure 3F) and ΔPH-PDK1 (Figure 3C) show greatly decreased binding to the plasma membrane. Quantification of the gold labelling is shown in Figure 5.

Given the high affinity of PDK1 for PtdIns(3,4,5)$P_3$, it is possible that PDK1 binds to the basal levels of PtdIns(3,4,5)$P_3$ in the HEK 293 cells and that this accounts for the constitutive localization at the plasma membrane in unstimulated cells. Therefore, HEK 293 cells were preincubated with the PI 3-kinase inhibitor LY 294002 for up to 16 h before fixing. This treatment reduced basal PtdIns$P_3$ mass (J. van der Kaay and C. P. Downes, unpublished work). However, these cells also showed no difference in the localization of the wild-type PDK1 compared with the untreated cells (results not shown), suggesting that PDK1 does not solely bind to basal PtdIns(3,4,5)$P_3$ at the plasma membrane.

## Localization of a GFP–PDK1 fusion protein

To investigate the localization of PDK1 in live cells, 293 cells were transiently transfected with a construct encoding GFP–PDK1. GFP–PDK1-expressing cells were identified and revealed using confocal microscopy. To control for membrane concentration effects, the plasma membrane was labelled with the lipid-binding dye DiIC$_{16}$(3). A typical result is shown in Figure 6. Figure 6(A) (green) shows GFP–PDK1, Figure 6(B) (red) DiIC$_{16}$(3) from unstimulated cells, and Figures 6(C) and 6(D) the same images from cells stimulated for 15 min with insulin. GFP–PDK1 shows a diffuse cytoplasmic localization in unstimulated cells and is excluded from the nucleus. Membrane

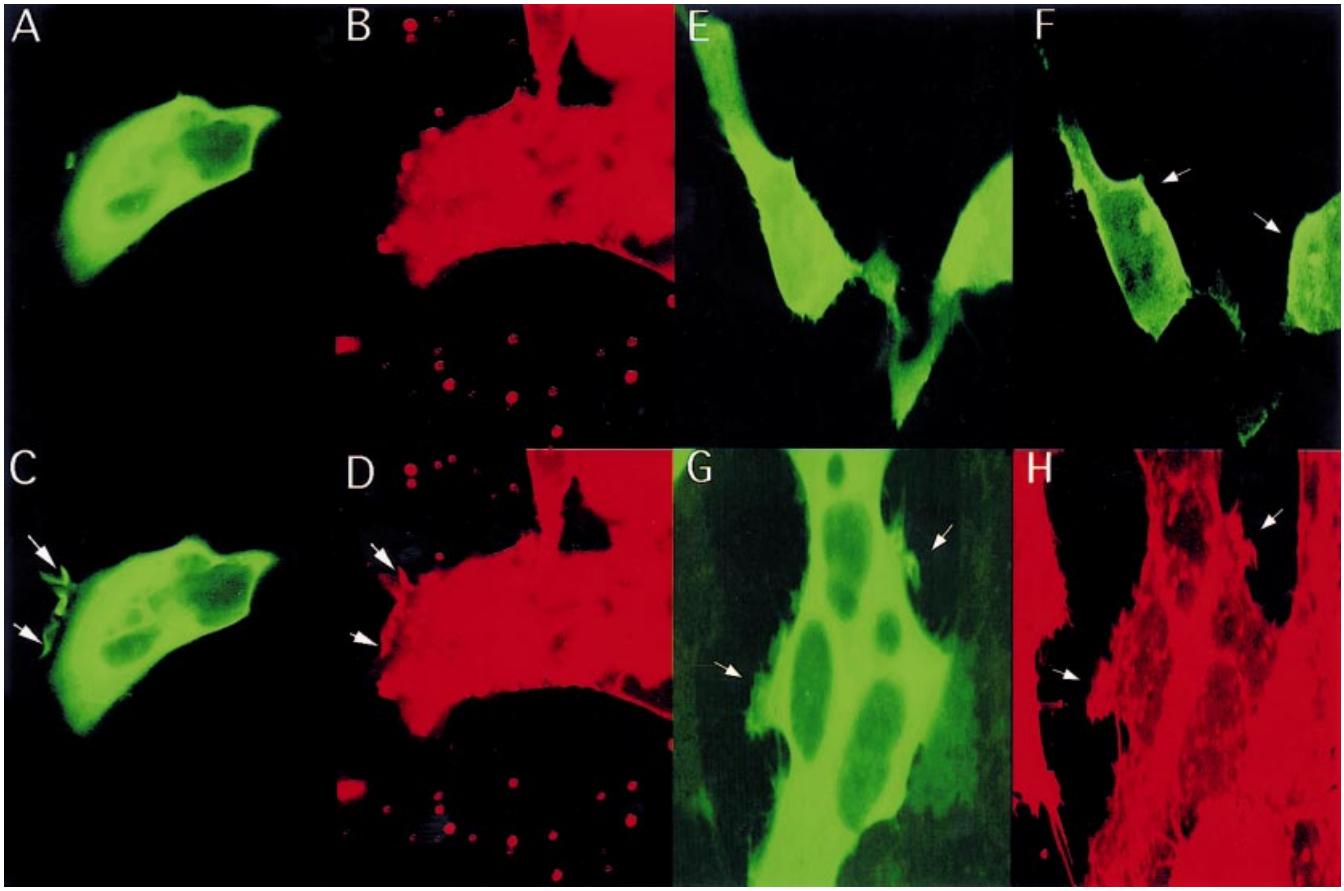


**Figure 6 Confocal localization of GFP–PDK1, GFP–ΔPH-PDK1 and GFP–PKB-PH in control and insulin-stimulated 293 cells**

293 cells were transfected with GFP–PDK1 (**A**, **B**, **C** and **D**), GFP–PKB-PH (**E** and **F**) or GFP–ΔPH-PDK1 (**G** and **H**) and stimulated with insulin (**C**, **D**, **F**, **G** and **H**) or left unstimulated (**A**, **B** and **E**). (**A**) and (**C**), GFP–PDK1; (**B**) and (**D**), the red lipid-staining dye $DiIC_{16}(3)$. The arrows in (**C**) and (**D**) demonstrate that the apparent accumulation of PDK1 at the plasma membrane is caused by membrane ruffling, since the intensity of lipid staining is also increased at these regions. (**E**) and (**F**), GFP–PKB-PH. The arrows in (**F**) demonstrate the plasma-membrane localization of GFP–PKB-PH in insulin-stimulated cells. (**G**) GFP–ΔPH-PDK1; (**H**), $DiIC_{16}(3)$. The arrows in (**G**) and (**H**) demonstrate that although GFP–PH-PDK1 is present in the ruffles, it is not highly concentrated at the membranes within them.

ruffles appear upon stimulation with insulin. GFP–PDK1 fluorescence is concentrated in these ruffles, as indicated by an dentical fluorescence pattern between the GFP–PDK1 and the $DiIC_{16}(3)$ marking of bulk plasma-membrane lipid (arrows in Figures 6C and 6D). Although a fusion protein of GFP followed by PDK1 without its PH domain (GFP–ΔPH-PDK1) also appeared in membrane ruffles (arrows in Figures 6G and 6H), it was not concentrated at the membranes within them, indicating that GFP–PDK1 was bound to the lipid component of the membranes in the ruffles. In contrast, a fusion protein consisting of GFP followed by the PH domain of PKBα (GFP–PKB-PH) shows a striking translocation to the cell periphery in insulin-stimulated cells (Figure 6F) when compared with unstimulated cells (Figure 6E).

## DISCUSSION

In the present study we used a surface-plasmon-resonance technique to confirm that wild-type PDK1 binds to PtdIns-(3,4,5)$P_3$, and with a $K_D$ value an order of magnitude lower than that of PKBα. PtdIns(3,4)$P_2$ binds less strongly to PDK1 than PtdIns(3,4,5)$P_3$, while PtdIns(4,5)$P_2$ and PtdIns(3)$P$ possessed much lower binding affinities for PDK1 under the conditions studied. Nevertheless, since PDK1 exhibits a higher affinity than PKBα for all phosphoinositides tested, its absolute affinity for PtdIns(4,5)$P_2$ (Table 1) is similar to that for PtdIns(3,4,5)$P_3$ binding to PKBα (Table 2). We also generated mutations in the PH domain of PDK1 and studied their binding to PtdIns-(3,4,5)$P_3$, their ability to activate PKBα and their subcellular localization compared with wild-type PDK1. We find that there is a strict correlation between the ability to bind PtdIns(3,4,5)$P_3$, to catalyse the activation of PKBα and to associate with the plasma membrane. Thus the presence of a PH domain in PDK1 that is competent to bind PtdIns(3,4,5)$P_3$ is required for efficient phosphorylation of PKBα *in vitro*, presumably by increasing the concentrations of PKB and PDK1 at the surface of the lipid vesicles. It is also likely that the ability of the PH domain of PDK1 to bind PtdIns(3,4,5)$P_3$ and/or PtdIns(4,5)$P_2$ is critical for the localization of PDK1 to the plasma membrane *in vivo*.

When overexpressed in 293 cells, a small proportion of the PDK1 localizes to the plasma membrane, while most is present in the cytosol. Interestingly, the distribution of PDK1 between the plasma membrane and the cytosol is unaffected by insulin or growth-factor stimulation. This is in contrast with PKBα, which is entirely cytosolic in unstimulated cells and shows a striking translocation from the cytosol to the plasma membrane upon

stimulation with insulin or growth factors (Figure 4). The simplest explanation for these results is that PDK1 is associated with the plasma membrane of unstimulated cells because it binds PtdIns(3,4,5)$P_3$ and/or PtdIns(4,5)$P_2$ much more tightly than PKB$\alpha$ (see above) and is therefore able to interact with the low levels of these lipids that are present in unstimulated cells. In contrast, PKB$\alpha$ is only able to translocate to the plasma membrane when the level of PtdIns(3,4,5)$P_3$ at the membrane is elevated above a particular threshold concentration. However, if the association of PDK1 with the plasma membrane was mediated solely by its interaction with PIs, then one might have expected all the PDK1 to be localized to the plasma membrane, since we estimate that the level of endogenous PtdIns(3,4,5)$P_3$/PtdIns(4,5)$P_2$ greatly exceeds the number of transfected PDK1 molecules. This suggests that, in addition to PtdIns(3,4,5)$P_3$ and/or PtdIns(4,5)$P_2$, another factor/protein may be required for the interaction of PDK1 with the plasma membrane that is limiting when PDK1 is overexpressed. The limiting factor cannot be PtdIns(3,4,5)$P_3$, otherwise PDK1 (like PKB$\alpha$) would have translocated to the membrane in response to stimuli that elevate PtdIns(3,4,5)$P_3$ and would have dissociated from the plasma membrane when basal PtdIns(3,4,5)$P_3$ levels are decreased by treatment with PI 3-kinase inhibitors. PDK1 at the plasma membrane may therefore bind to PtdIns(4,5)$P_2$ under basal conditions. However, the SH2 (Src homology domain 2) domain of PI 3-kinase has been reported to bind to phosphotyrosine as well as PtdIns(3,4,5)$P_3$ [29] and, by analogy, the PH domain of PDK1 may also interact with a protein in the plasma membrane, as well as PtdIns(3,4,5)$P_3$. The PH domain of the protein tyrosine kinase Btk is reported to interact with protein kinase C [30] and G$\alpha$12 [31] as well as PtdIns(3,4,5)$P_3$ and the PH domain of the $\beta$-adrenergic receptor kinase interact with heterotrimeric G-protein $\beta\gamma$ subunits and PtdIns(4,5)$P_2$ [32]. Since the lipid- and protein-binding components of some other PH domains can be synergistic, it is possible that PtdIns(3,4,5)$P_3$/PtdIns(4,5)$P_2$ binding to PDK1 is necessary to produce a subsequent protein–protein interaction which localizes PDK1 at the plasma membrane and that this protein component is limiting.

Our data disagree with those obtained by Anderson et al. [28] who recently reported translocation of some transfected PDK1 molecules to the plasma membrane of PAE cell in response to PDGF. When we examined the localization of PDK1 in PAE and 293 cells by quantitative immunogold electron microscopy, we failed to detect any translocation of PDK1 from the cytosol to the plasma membrane in response to either IGF-1 or PDGF, despite these stimulations causing both translocation and activation of PKB. There are significant methodological differences between our study and that of Anderson et al. [28], which was carried out on fixed, Triton-X-100-permeabilized cells viewed by confocal immunofluorescence microscopy. While quantitative immunogold electron microscopy allows resolution and quantification of reference structures over which gold labelling is located, confocal microscopy cannot do this without the use of compartment specific markers. In the study by Anderson et al. [28] the increased labelling for PDK1 was found in membrane ruffles of the plasma membrane, but no reference marker for that structure was used and the labelling could simply reflect increased amounts of plasma membrane carrying constitutively associated PDK1. We investigated this possibility by labelling the plasma membrane with a fluorescent lipid in cells expressing GFP–PDK1 and stimulated with IGF-1. We found that increases in GFP fluorescence were consistently associated with increased lipid labelling in ruffles of the plasma membrane. Thus, most likely, the results of Anderson et al. [28] reflect movements of plasma membrane and its associated PDK1 to new plasma membrane containing structures that are formed. The constitutive association of PDK1 with the plasma membrane is consistent with the observation that a membrane-targetted form of PKB$\alpha$ becomes fully phosphorylated and activated, even in unstimulated cells (see the Introduction).

It is also possible that PDK1 interacts with a protein that prevents it from binding to PtdIns(3,4,5)$P_3$ and/or PtdIns(4,5)$P_2$ and that this explains why most of the transfected PDK1 is in the cytosol rather than at the plasma membrane of transfected cells. There is increasing evidence that PKB is not the only protein kinase that is activated by PDK1 *in vivo*, and some of its substrates, such as p70 S6 kinase [33,34], may be located in the cytosol. PDK1 may therefore require a dual distribution between membranes and cytosol to be localized to its substrates. The activation of p70 S6 kinase by PDK1 is unaffected by PtdIns(3,4,5)$P_3$ *in vitro* [33,34]. Lipid binding may not be the sole determinant for the localization of PKB either, however, since upon stimulation PKB is first translocated to, and then dissociates from, the plasma membrane to move to the nucleus, despite PtdIns(3,4,5)$P_3$ levels remaining elevated [26,35]. This may require interaction with other protein factors. Confirmation of these hypotheses will require the identification and characterization of these putative PDK1 and PKB-binding proteins.

In summary, our present model of signal transduction immediately downstream of PtdIns(3,4,5)$P_3$ leading to activation of PKB is therefore that PtdIns(3,4,5)$P_3$ and/or PtdIns(4,5)$P_2$ mediates the localization of PDK1 at the plasma membrane under basal conditions and that, upon stimulation with insulin or growth factors, PKB is recruited to the plasma membrane, where it co-localizes with PDK1. This localization, coupled with a PtdIns(3,4,5)$P_3$-mediated enhancement in the ability of PKB to become a substrate for PDK1 [19,20], leads to phosphorylation and activation of PKB.

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