Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B

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Background: Protein kinase B (PKB) is involved in the regulation of apoptosis, protein synthesis and glycogen metabolism in mammalian cells. Phosphoinositide-dependent protein kinase (PDK-1) activates PKB in a manner dependent on phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which is also needed for the translocation of PKB to the plasma membrane. It has been proposed that the amount of PKB activated is determined exclusively as a result of its translocation, and that a constitutively active pool of membrane-associated PDK-1 simply phosphorylates all the PKB made available. Here, we have investigated the effects of membrane localisation of PDK-1 on PKB activation.

Results: Ectopically expressed PDK-1 translocated to the plasma membrane in response to platelet-derived growth factor (PDGF) and translocation was sensitive to wortmannin, an inhibitor of phosphoinositide 3-kinase. Translocation of PDK-1 also occurred upon its co-expression with constitutively active phosphoinositide 3-kinase, but not with an inactive form. Overexpression of PDK-1 enhanced the ability of PDGF to activate PKB. PDK-1 disrupted in the pleckstrin homology (PH) domain which did not translocate to the membrane did not increase PKB activity in response to PDGF, whereas membrane-targeted PDK-1 activated PKB to the extent that it could not be activated further by PDGF.

Conclusions: In response to PDGF, binding of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 to the PH domain of PDK-1 causes its translocation to the plasma membrane where it co-localises with PKB, significantly contributing to the scale of PKB activation.

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Background

Protein kinase B (PKB) [1–3] is a key protein serine/threonine kinase that can be activated by cell-surface receptors and phosphorylates cellular targets involved in processes such as apoptosis [4,5], protein synthesis [6] and glycogen metabolism [7,8]. The activation of PKB appears to be mediated by type-1 phosphoinositide (PI) 3-kinases and their lipid products, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) and PtdIns(3,4)P_2, as inhibitors of PI 3-kinases and dominant-negative PI 3-kinase constructs abolish PKB stimulation [6,9], whereas constitutively active PI 3-kinases directly stimulate PKB activity [10,11].

Several distinct events are associated with the activation of PKB. As a result of binding of the amino-terminal pleckstrin-homology (PH) domain of PKB to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 produced upon appropriate receptor activation, PKB translocates to the plasma membrane [12–14]. PKB is also phosphorylated on Thr308 and Ser473, and this phosphorylation is dependent on PI 3-kinase [15]. We have previously used point mutational analysis to show that phosphorylation of Thr308 is necessary and sufficient for activation of PKB by platelet-derived growth factor (PDGF) in porcine aortic endothelial (PAE) cells, as assessed by kinase activity *in vitro*, whereas phosphorylation of Ser473 is not sufficient but is necessary for maximal activation [13]. Protein kinases dependent on 3-phosphoinositides and capable of phosphorylating Thr308 of PKB — PDK-1s — have been purified from muscle [16] and brain [17], peptide sequenced and cloned [18,19]. Thus far a single PDK-1 gene has been identified; the various activities that have been purified might result from differential splicing of this gene. PDK-1 has been shown to phosphorylate Thr308 of PKB *in vitro* but only in the presence of PtdIns(3,4,5)P_3-containing lipid vesicles or detergent micelles into which PKB can translocate [17,16].

A key issue that remains to be resolved is whether membrane localisation of PKB is sufficient to enable PDK-1 to phosphorylate Thr308, or whether the 3-phosphorylated lipids also 'activate' PDK-1 (in that they either increase its intrinsic catalytic activity or its access to substrates). The fact that PDK-1 contains a carboxy-terminal PH domain and can bind lipid vesicles containing PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 [18] appears to suggest that 3-phosphorylated lipids can 'activate' PDK-1 in some way. This

remains to be clarified, however. Insulin, which increases the levels of 3-phosphorylated lipids, has been reported to have no apparent effect on the kinase activity of PDK-1 *in vitro* [19]. Furthermore, the concentrations of 3-phosphorylated inositol lipids required to translocate PDK-1 to lipid vesicles are very small [18], such that the concentrations of these lipids in unstimulated cells might be sufficient to localise significant quantities of PDK-1 to the membrane. Finally, PDK-1 has been shown to phosphorylate Thr252 of soluble $p70^{S6K}$, a site analagous to Thr308 of PKB, in a PtdIns(3,4,5)P_3-independent manner [20,21]. Here, we have studied the subcellular localisation of PDK-1 in both unstimulated and PDGF-stimulated PAE cells, and investigated the effect of the membrane localisation of PDK-1 on the ability of PDGF to activate PKB.

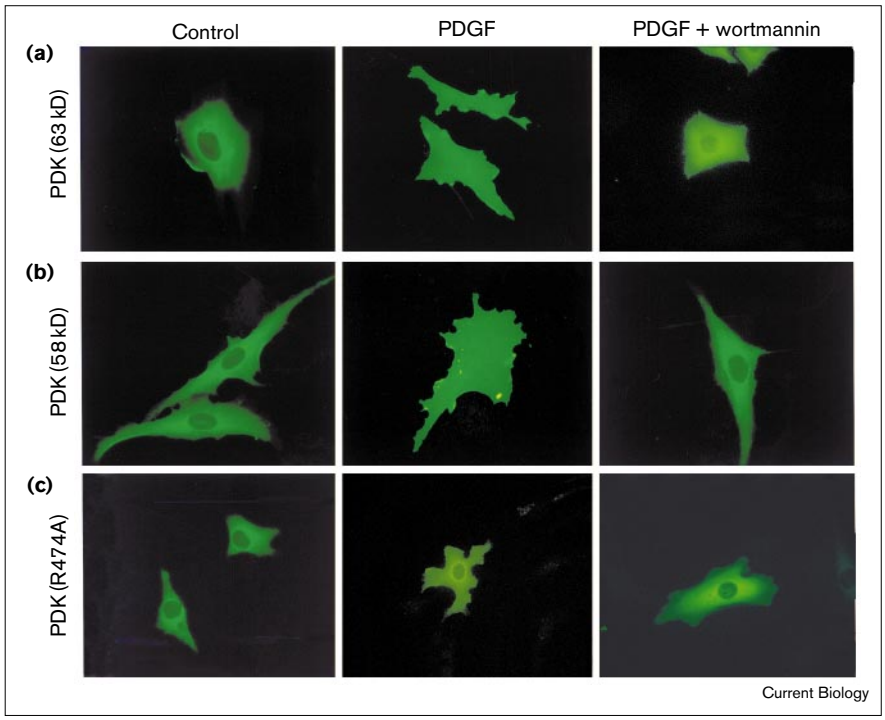
Results

We have previously cloned the PDK-1 gene from a U937 cDNA library as a cDNA with a potential open reading frame (ORF) encoding a 556 amino acid protein (predicted size 63 kDa). It is clear from peptide sequencing of purified protein that this gene locus also gives rise to some smaller proteins [18]. We do not yet understand the mechanism for this, but in the only alternatively spliced transcripts we have discovered so far, the initiation of protein translation would have to be from internal methionine codons of the defined sequence (unpublished data). For the purposes of this study, therefore, as well as using 63 kDa PDK-1, an additional construct was made encoding a 507 amino acid protein (predicted size 58 kDa) that commences 50 amino acids into the sequence of the 63 kDa protein at a candidate internal methionine residue. We also previously identified another ORF in the PDK-1 gene which is missing an exon encoding part of the kinase domain; it encodes an apparently catalytically inactive protein [18]. Mammalian expression vectors encoding tagged versions of these PDK-1 variants were constructed and used to investigate the regulation of PDK-1 and PKB in response to PDGF stimulation.

PDGF induces PDK-1 translocation to the plasma membrane in a manner dependent on PI 3-kinase

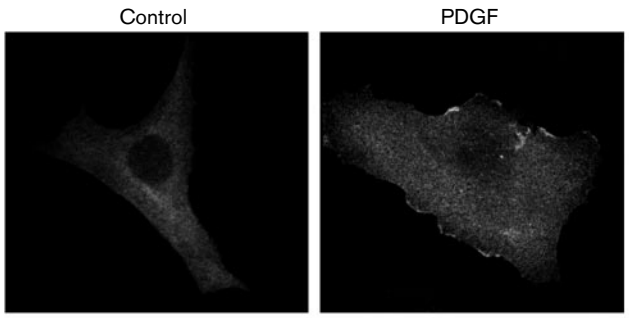
PAE cells were transiently transfected with expression vectors for 63 kDa PDK-1 tagged at its amino terminus with the Myc epitope (PDK(63kD)), plated onto coverslips for 30 hours and then serum starved for an additional 15 hours. Immunohistochemical analysis of unstimulated cells revealed a pattern of staining indicative of a cytosolic distribution for ectopically expressed PDK-1. No nuclear staining was evident, while only minor levels of staining were observed on the periphery of the cell (Figure 1a). Stimulation of cells with the B chain of PDGF (PDGF-BB) produced a marked alteration in the cellular distribution of PDK(63kD). Homogenous staining across the entire cell surface was observed, with a sharply defined edge to the cells, indicative of plasma membrane localisation (Figure 1a). This interpretation was confirmed by confocal microscopy (Figure 2). Thus PDK-1 translocates

Figure 1



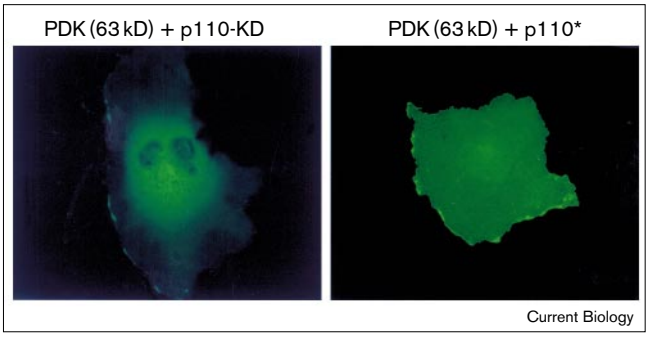
Immunolocalisation of PDK-1 in unstimulated and PDGF-stimulated PAE cells. PAE cells were transiently transfected with 20 μg of the vector for either **(a)** PDK(63kD) **(b)** myc-tagged 58 kDa PDK-1 (PDK(58kD)) or **(c)** a version of PDK(63kD) containing a point mutation in its PH domain (PDK(R474A)), described in Materials and methods. Transfected cells (2×10^6) were plated onto glass coverslips for 30 h then serum-starved for 15 h (control). Where indicated, cells were stimulated with PDGF-BB (10 ng/ml, 5 min) following a 10 min 100 nM wortmannin pretreatment as appropriate. Cells were processed for immunohistochemistry as described in Materials and methods using anti-Myc 9E10 monoclonal antibody as a primary antibody and FITC-conjugated goat anti-mouse as a secondary antibody.

Figure 2



Confocal images of PDK-1 immunolocalisation in unstimulated and PDGF-stimulated PAE cells. PAE cells were transiently transfected with 20 μg of the vector for PDK(63kD) as described in Materials and methods. Cells were grown, stimulated with PDGF, and processed for immunohistochemistry as described for Figure 1, followed by confocal imaging.

Figure 3



PDK-1 translocation in the presence of active PI 3-kinase. Cos-7 cells were transiently cotransfected with 10 μg of the vector for PDK(63kD) and 10 μg of plasmid encoding either a constitutively active p110γ PI 3-kinase catalytic subunit (p110*) or a 'kinase-dead' version (p110-KD), as described in Materials and methods. Cells were incubated for 20 h, replated onto glass coverslips for 12 h, then serum starved for 12 h. Cells were processed for immunohistochemistry as described for Figure 1.

to the plasma membrane in response to PDGF stimulation. This PDGF-induced translocation was dependent on PI 3-kinase, as it was inhibited by pretreatment of cells with wortmannin (100 nM) for 10 minutes (Figure 1a). PAE cells were also transfected with a vector encoding a Myc-tagged 58 kDa form of PDK-1 (PDK(58kD)), which showed a similar PI-3-kinase-dependent, PDGF-induced translocation to the plasma membrane (Figure 1b).

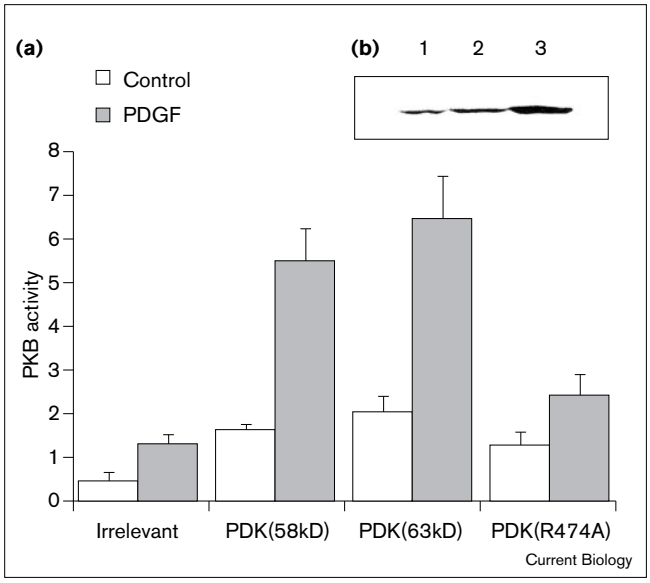
To further investigate the involvement of PI 3-kinase and its lipid products in the observed translocation of PDK-1, Cos-7 cells were transiently co-transfected with the vector encoding PDK(63kD) and vectors for either a constitutively active p110γ PI 3-kinase catalytic subunit (p110*) or a 'kinase-dead' version (p110-KD). Plasma membrane translocation of PDK(63kD) was observed upon co-expression with the active PI 3-kinase, but not the kinase-dead version (Figure 3), indicating direct involvement of the lipid products of PI 3-kinase in the translocation process. Parallel experiments using a catalytically inactive version of PDK-1 demonstrated that it would also translocate to the plasma membrane when co-transfected with p110* but not p110-KD (data not shown). Thus, PDK-1 translocates to the plasma membrane in response to activated PI 3-kinase and this response does not depend on the catalytic activity of PDK-1. Studies were performed to determine the effects of this observed translocation on the ability of PDK-1 to enhance PDGF-induced PKB activity.

PDK-1 potentiates PDGF-stimulated PKB activity

PAE cells were transfected with plasmids encoding PDK(63kD) or PDK(58kD) and a glutamine (EE)-tagged phosphorylation site mutant of PKB (PKB(S473D)), in which the amino acid substitution mimics phosphorylation at residue Ser473 of PKB [15]. As phosphorylation of both Thr308 and Ser473 of PKB is critical for maximal PKB activity [13,15] and, furthermore, PDK-1 is known to phosphorylate Thr308 exclusively [16,17], we used PKB(S473D) to investigate the effects of Thr308 phosphorylation of PKB by PDK-1 in isolation. PKB(S473D) was found to have the same immunolocalisation as wild-type PKB and to respond similarly to co-expression with PDK-1, under both control and PDGF-stimulated conditions (data not shown). Transfected PAE cells were incubated for 12 hours, then serum starved for 11 hours. Cells were briefly stimulated with a submaximal dose of PDGF (2 ng/ml for 45 seconds), to ensure that the scale of the response we were analysing was not limited by these parameters.

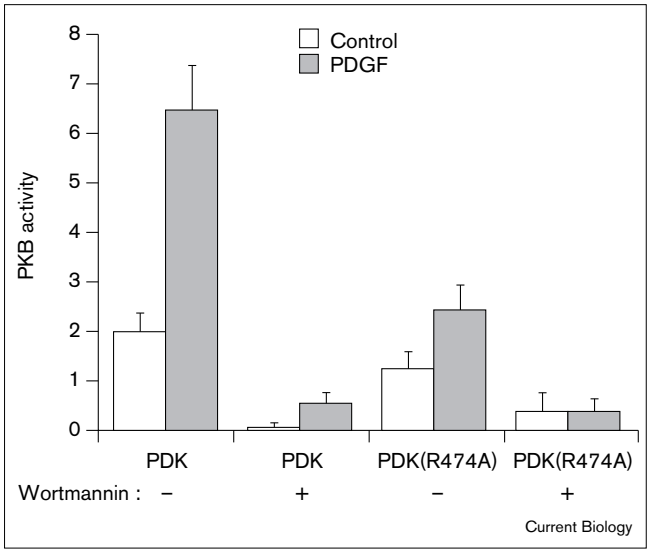
The anti-EE-immunoprecipitated PKB activity in PAE cells expressing PKB(S473D) in the absence of PDK-1 was low and increased with submaximal PDGF stimulation (Figure 4a). Co-expression of PDK(63kD) and PKB(S473D) resulted in a rise in basal PKB activity, as well as a dramatic increase in PDGF-stimulated PKB activity (Figure 4a). Expression of PDK-1 resulted in PDGF eliciting a fivefold greater increase in EE-associated PKB activity than in cells transfected with an irrelevant cDNA. Similar potentiation of PDGF-stimulated PKB activity occurred with co-transfection of PDK(58kD) (Figure 4a). The potentiation of PKB activity by PDK-1 was dependent on PI 3-kinase, being abolished by pretreatment of the cells with wortmannin (Figure 5). Expression of either PDK(58kD) or PDK(63kD) did not affect the level of co-expressed PKB(S473D) in PAE cells (data not shown). The observed PDGF-stimulated translocation of either 63 kDa or 58 kDa PDK-1 to the plasma membrane therefore appears to be associated with the ability of PDK-1 to enhance PKB activity. The two variants of PDK-1 used in this study, PDK(58kD) and

Figure 4



Effects of PDK-1 co-expression on PKB activity in PAE cells. **(a)** PAE cells were cotransfected with 20 μg of the PKB(S473D) vector and either 3 μg of the PDK(58kD), PDK(63kD) or PDK(R474A) vector, or an irrelevant DNA. Transfected cells were incubated for 12 h, then serum starved for 11 h. Where appropriate, cells were stimulated for 45 sec with 2 ng/ml PDGF-BB (grey bars). The activity of PKB (arbitrary units) in anti-EE immunoprecipitates prepared from lysates of the transfected cells was assessed using myelin basic protein as a substrate. The non-specific kinase activity of the immunoprecipitates was subtracted as background. The data presented are the mean ± SEM for $n \geq 8$ from at least four independent experiments. **(b)** Immunoblot results illustrating expression levels of PDK(58kD) (lane 1), PDK(63kD) (lane 2) and PDK(R474A) (lane 3) in transfected cells. Equal numbers of cells from each transfection were solubilised in SDS sample buffer, subjected to SDS–PAGE, transferred to nitrocellulose and probed with an anti-Myc monoclonal antibody (9E10) as described in Materials and methods.

Figure 5



Wortmannin inhibits the effects of PDK(63kD) and PDK(R424A) expression on PKB activity. PAE cells were cotransfected with PKB(S473D) and either PDK(63kD) or PDK(R474A) plasmids and treated as described for Figure 4. Where indicated, cells were pretreated with 100 nM wortmannin for 90 min prior to a 45 sec stimulation with 2 ng/ml PDGF-BB (grey bars). The activity of ectopically expressed EE-tagged PKB was assessed as described for Figure 4. The data presented are the mean ± SEM for $n \geq 4$ from at least two independent experiments.

PDK(63kD), appear to behave in a similar manner, translocating to the plasma membrane and potentiating PKB activity in response to PDGF stimulation.

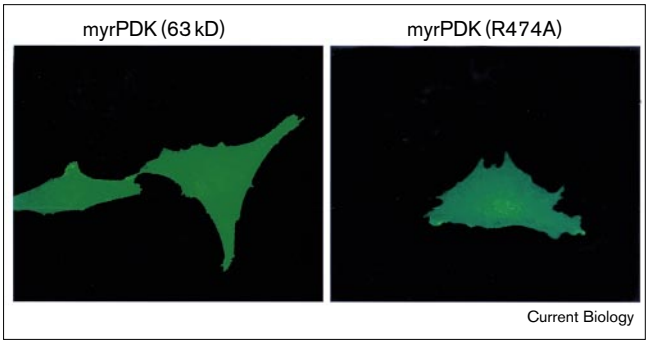
The PH domain of PDK-1 mediates its PI-3-kinase-dependent translocation and activation of PKB

Given the observed PI 3-kinase dependence of the translocation of PDK-1 in response to PDGF, the ability of PDK-1 to bind PtdIns(3,4,5)P_3 with high affinity *in vitro* [18], and its possession of a potentially lipid-binding carboxy-terminal PH domain, experiments were performed to investigate the involvement of the PH domain of PDK-1 in its PDGF-stimulated translocation. For these purposes, point mutation of the Arg474 of PDK(63kD) to alanine (R474A) was performed. This arginine residue in the PH domain of PDK(63kD), is also found in an analogous position in the PH domains of both phospholipase C-δ (PLC-δ) and Bruton's tyrosine kinase (Btk), and has been shown to be critical in both PLC-δ [22] and Btk [23] for ligand recognition, and in PLC-δ for membrane association [22]. Furthermore, this residue is found to be mutated in Btk in patients suffering from agammaglobulinaemia and in mice carrying an X-linked immunodeficiency [23].

Immunohistochemical analysis of PAE cells transiently transfected with Myc-tagged R474A PDK-1 (PDK(R474A)) demonstrated similar cytosolic staining under basal conditions as seen for wild-type PDK(63kD) (Figure 1). In marked contrast to wild-type PDK(63kD), however, PDGF stimulation of the cells expressing PDK(R474A), while producing significant cellular ruffling, did not induce a significant change in the localisation of PDK(R474A) (Figure 1c).

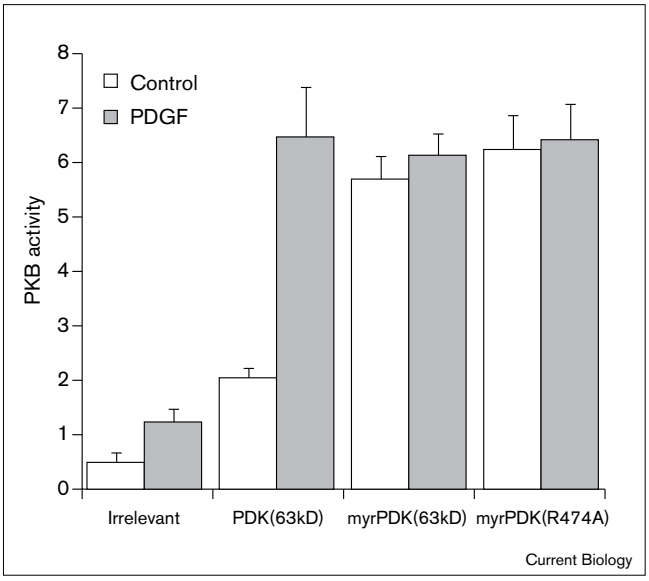
The ability of PDK(R474A) to stimulate the PDGF-induced activity of co-expressed PKB(S473D) in PAE cells was low in comparison to that of PDK(63kD) (Figure 4a). Western blot analysis of transfected cell lysates revealed comparable expression levels of the Myc-tagged proteins, PKD(63kD), PDK(58kD) and PDK(R474A), in these experiments (Figure 4b; if anything, PDK(R474A) was expressed at a higher level), suggesting that the inhibition of PDK-1's ability to potentiate PDGF-stimulated PKB activity by the PH-domain mutation R474A was not a result of reduced

Figure 6



Effect of myristoylation on cellular localisation of 63 kDa PDK-1 and 63 kDa R424A PDK-1. PAE cells were transiently transfected with 20 μg of the plasmids encoding the indicated constructs, grown and processed for immunohistochemistry as described for Figure 1, using the anti-Myc monoclonal antibody 9E10 as a primary antibody and FITC-conjugated goat anti-mouse as a secondary antibody.

Figure 7



Effect of membrane targeting of 63 kDa PDK-1 and 63 kDa R474A PDK-1 on PKB activity. PAE cells were cotransfected with the vector for PKB(S473D) and either plasmids encoding the indicated constructs or an irrelevant DNA. Cells were treated, and the EE-associated PKB activity quantitated as described for Figure 4. The data presented are the mean ± SEM for $n \geq 8$ from at least two independent experiments.

expression levels. The fact that PDK(R474A) failed both to translocate to the plasma membrane and to support activation of PKB in response to PDGF indicates the importance of plasma membrane localisation of PDK-1 for its activation of PKB.

Membrane targeting of PDK-1 produces a constitutively active protein

To further investigate the relationship between plasma membrane localisation of PDK-1 and its ability to amplify the PDGF-stimulated PKB activity, both 63 kDa PDK-1 and its R474A variant were modified by the addition of a myristoylation/palmitoylation sequence to the amino-terminal end and a Myc tag to the carboxy-terminal end (giving myrPDK(63kD) and myrPDK(R474A), respectively). Both constructs expressed well and showed clear plasma membrane localisation in unstimulated cells, with some cells displaying additional nuclear staining (Figure 6). Expression of myrPDK(63kD) led to the constitutive activation of co-expressed PKB(S473D) in cell lysates prepared from unstimulated cells and, under the conditions studied, this PKB activation did not increase further when cells were stimulated with PDGF (Figure 7). The effect of myristoylation on PDK-1 was sensitive to inhibition by wortmannin (data not shown), suggesting that at some level PtdIns(3,4,5)P_3 must bind to PKB to promote its phosphorylation by PDK-1. In marked contrast to non-membrane-targeted PDK(R474A), myristoylation of the PH-domain mutant of PDK-1 induced a high level of PKB activation, comparable to that induced by wild-type myrPDK(63kD), under both unstimulated and stimulated conditions (Figure 7). This ability of myrPDK(R474A) to activate co-expressed PKB indicated that the point mutation of Arg474 in the PH domain of PDK-1 did not affect the ability of the enzyme to phosphorylate and activate PKB. Western blot analysis showed that both myrPDK(63kD) and myrPDK(R474A) were expressed at lower levels than the non-myristoylated constructs (although differential detection of carboxy-terminal and amino-terminal Myc tags cannot be excluded). Expression levels of myrPDK(63kD) and myrPDK(R474A) were comparable, however, (data not shown).

To confirm the role of the PH domain in PDK-1's translocation, a plasmid was generated that would drive production of a Myc-tagged form of the PH domain of PDK-1 (residues 411–556 of the 63 kDa form) in PAE cells. Immunolocalisation of this construct in unstimulated cells revealed nuclear staining — suggesting that a nuclear localisation sequence might have been unmasked with removal of the first 410 amino acids or that, given the small size of the isolated PH domain, it might accumulate in the nucleus by diffusion — and significantly less cytosolic staining than for the 63 kDa form of PDK-1 (data not shown). PDGF stimulation of the cells resulted in reduced nuclear staining and additional homogenous staining across the cell (data not shown), suggesting that the PH domain of PDK-1 could, in isolation, translocate to the plasma membrane in response to PDGF. Furthermore, translocation of the isolated PH domain was inhibited by wortmannin (data not shown) and, hence, is likely to be dependent on PI 3-kinase.

Discussion

Our results clearly show that PDGF stimulates a substantial translocation of ectopically expressed PDK-1 from the cytosol to the plasma membrane in PAE cells. This translocation is dependent upon PDGF-stimulated PI 3-kinase activity and requires an intact PDK-1 PH domain. These findings are consistent with our previous data describing the translocation of purified PDK-1 to artificial lipid vesicles containing low molar ratios of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 *in vitro* [18], and thus support a model in which the lipid products of PI 3-kinase affect the 'activity' of PDK-1 in some way.

Co-expression of PDK-1 with PKB(S473D) significantly elevates the activity of ectopically expressed PKB in unstimulated cells. It is possible that resting cells contain sufficient levels of the lipid products of PI 3-kinase in the membrane to provide a certain degree of membrane targeting of PDK-1. Co-expression of PDK-1 also substantially elevates PKB(S473D) activity during a 45 second stimulation with PDGF. We have recently used similar results to argue that PDK-1 is used by growth factor receptors as a primary mechanism for activating PKB [18]. Our results here show that PDK-1 with a defective PH domain, which cannot translocate to the plasma membrane in response to PDGF, cannot support this increased activation of PKB. This indicates that translocation to the plasma membrane is important for the ability of PDK-1 to phosphorylate and activate PKB. This conclusion is supported by our results showing that PDK-1 artificially targeted to the plasma membrane is constitutively active with respect to its ability to activate PKB.

How might translocation of PDK-1 and its substrate PKB to the plasma membrane lead to increased phosphorylation and activation of PKB? In principle, the mechanism could involve either a substrate 'concentrating' effect, some change to PKB that facilitates phosphorylation of Thr308, or an increase in the intrinsic catalytic activity of PDK-1. The latter mechanism appears unlikely in view of the recent evidence suggesting that PDK-1's ability to phosphorylate a soluble target, $p70^{S6K}$, is not regulated by PtdIns(3,4,5)P_3 [20,21]. The observation that the ability of PDK(R474A) to activate PKB in unstimulated cells is sensitive to wortmannin is evidence in favour of an effect of PtdIns(3,4,5)P_3 on PKB beyond membrane targeting. This issue requires further investigation in some form of defined *in vitro* system before it can be fully resolved, however, using mutants of PKB and PDK-1 that are membrane localised independently of PtdIns(3,4,5)P_3 binding. It must be appreciated that ectopic expression of PDK-1 and PKB might distort the relative requirements for translocation of these proteins from the situation that exists in normal cells; for example, given the high affinity of PDK-1 for PtdIns(3,4,5)P_3 *in vitro*, it is possible that in some circumstances the basal levels of PtdIns(3,4,5)P_3 are sufficient to localise significant quantities of PDK-1 to make its translocation essentially 'constitutive'.

The observation that translocation of PDK-1 to the plasma membrane is important for its ability to phosphorylate Thr308 in PKB has some interesting implications for the role of PDK-1 in phosphorylating other potential downstream targets. It has been suggested that PDK-1 might act to phosphorylate conserved sites in the kinase activation loop of a subfamily of protein kinases [24], and this idea has received support from the observation that PDK-1 can phosphorylate the analogous Thr252 residue in $p70^{S6K}$ [20,21]. It has been shown that $p70^{S6K}$, which is found in the nucleus and cytosol of resting cells, can translocate into the nucleus in response to some stimuli [25], but there is no evidence that it can translocate to the plasma membrane upon growth factor stimulation. It will be of great interest, therefore, to investigate what effect targeting of PDK-1 to the plasma membrane has on its ability to phosphorylate $p70^{S6K}$.

This study strengthens the emerging concept that certain PH domains can act as specific membrane recruitment modules controlling the translocation of soluble protein to the part of the membrane in which PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are synthesised. It seems likely, however, that this translocation, for any particular protein, is not the sole factor determining its regulation but that other factors are important — for example, PKB activation requires that at least one additional protein kinase, PDK-1, is also translocated to the membrane.

Materials and methods

PDK-1 constructs

The cDNA encoding either 58 kDa or 63 kDa PDK-1 (which have the amino acid sequences MDGTAAE... and MARTTSQ..., respectively, in single-letter code, at their amino termini) or a catalytically inactive splice variant [18] were ligated in frame with an amino-terminal Myc (MEQKLISEEDLEF) tag into the pCMV3 transient expression vector [26]. Point mutation (R474A) of the PH domain of 63 kDa PDK-1 was generated by a PCR-based mutagenic strategy using Pfu polymerase (Stratagene). For membrane-targeting experiments, carboxy-terminal Myc tags were generated on 63 kDa PDK-1 and R474A PDK-1 and the constructs were ligated into a pCMV3 expression vector containing an amino-terminal myristoylation/palmitoylation sequence from the Yes Src-type kinase (MGLCIKSKEDKSM) [27]. Residues 411–556 of PDK(63kD) were placed in frame with an amino-terminal Myc tag in the pCMV3 transient expression vector to construct an isolated PDK-1 PH domain. For construction of an active PI 3-kinase allele, and a kinase-dead R947P derivative, the cDNA encoding pig 110γ [26] was ligated into the same amino-terminal myristoylation/palmitoylation vector (a gift from Alicia Eguinoa). All constructs were verified by sequencing.

Cell culture

PAE cells expressing the PDGF receptor were grown in F12 nutrient mixture (Ham F12; Gibco BRL) containing 10% heat-inactivated fetal bovine serum (HI-FBS) at 37°C in a 6% CO_2 humidified atmosphere. Cos-7 cells were maintained in Dulbecco's modified Eagle Medium (DMEM; Gibco BRL) containing 10% HI-FBS at 37°C in a 6% CO_2 humidified atmosphere.

Transient transfection

PAE cells (1×10^7) were transfected by electroporation as described previously [13,18]. Transfected cells were replated into 6 cm tissue culture dishes (2×10^6 cells per dish) for PKB kinase assays, or onto glass coverslips (8×10^4 cells per coverslip) for immunofluorescence experiments, in F12 containing 10% HI-FBS for 12 h (PKB assays) or 30 h (immunofluorescence) and then serum starved for 11 h (PKB assays) or 15 h (immunofluorescence) in F12 containing 0.1% fatty-acid-free BSA, in the presence of 1 U/ml penicillin and 0.1 mg/ml streptomycin (see the figure legends). Confluent monolayers of Cos-7 cells were transfected with 20 μg DNA by the DEAE–dextran method as previously described [28]. Cells were grown in DMEM containing 10% HI-FCS, penicillin and streptomycin for 20 h, trypsinised, diluted 1:50 and replated onto glass coverslips for a further 12 h, then serum starved for 12 h in DMEM containing 0.1% fatty-acid-free BSA, penicillin and streptomycin.

PKB assay

PKB assays were performed on transfected PAE cells essentially as described previously [13] with the following modifications. When required, cells were pretreated with 100 nM wortmannin for 90 min (2×45 min) in F12 containing 0.1% fatty-acid-free BSA, penicillin and streptomycin, prior to experiments. Cells were stimulated, when appropriate, with 2 ng/ml PDGF-BB for 45 sec. In a number of experiments, expression levels of transfected PKB and PDK-1 constructs were compared in parallel by western blotting. For this, the transfected cells were grown and serum starved under the same conditions as those for the PKB assay. Equal numbers of cells from each transfection were solubilised in Laemmli SDS sample buffer and the proteins were separated by 8% SDS–PAGE, transferred to nitrocellulose and blotted with an anti-EE monoclonal antibody (to detect PKB) or anti-Myc monoclonal antibody 9E10 (to detect PDK-1). Expression was visualised by the standard enhanced chemiluminescence procedure. Autofluorograms were densitometrically scanned with a BioRad Gel Doc 1000 system and quantified with the BioRad Molecular Analyst program to estimate the differences in expression levels.

Immunofluorescence microscopy

Cells for immunofluorescence microscopy were grown on glass coverslips and serum starved as described above. Where appropriate, cells were pretreated with 100 nM wortmannin for 10 min then stimulated with PDGF (10 ng/ml) for 5 min at 37°C. Activation was terminated by aspirating the medium and fixing cells with the immediate addition of 4% paraformaldehyde in 100 mM Pipes (pH 7.2), 2 mM EGTA, 2 mM $MgCl_2$ for 15 min at room temperature (RT). The cells were washed three times in 150 mM Tris, pH 7.2, permeabilised for 10 min at RT in PBS, 0.1% Triton X-100 and then washed twice in PBS. Coverslips were blocked with PBS, 0.5% BSA for 20 min at RT and then incubated with an anti-Myc monoclonal antibody (9E10; 1:2) for 1 h at RT. The coverslips were washed 3×5 min in PBS, 0.5% BSA and then incubated with the secondary FITC-conjugated goat anti-mouse antibody (1:100; Sigma). Coverslips were washed for 3×5 min in PBS, 0.5% BSA, 5 min in PBS and then in H_2O before being mounted onto microscope slides with Aqua-polymount anti-fading solution (Polysciences Inc.). Confocal imaging was performed using a NORAN Oz confocal microscope (NORAN Instruments) and a confocal z-section depth of < 1 μm.

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