Interaction of the Type I α PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity

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Phosphoinositides are localized in various intracellular compartments and can regulate a number of intracellular functions, such as cytoskeletal dynamics and membrane trafficking. Phospholipase Ds (PLDs) are regulated enzymes that hydrolyse phosphatidylcholine (PtdCho) to generate the putative second messenger phosphatidic acid (PtdOH). *In vitro*, PLDs have an absolute requirement for higher phosphorylated inositides, such as phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂]. Whether this lipid is able to regulate the activity of PLD *in vivo* is contentious. To examine this hypothesis we studied the relationship between PLD and an enzyme critical for the intracellular synthesis of PtdIns(4,5)P₂: phosphatidylinositol 4-phosphate 5-kinase α (Type I α PIPkinase). We find that both PLD1 and PLD2 interact with the Type I α PIPkinase and that PLD2 activity *in vivo* can be regulated solely by the expression of this lipid kinase. Moreover, PLD2 is able to recruit the Type I α PIPkinase to its intracellular location. We show that the physiological requirement of PLD enzymes for PtdIns(4,5)P₂ is critical and that PLD2 activity can be regulated solely by the levels of this key intracellular lipid.

Keywords: phosphatidic acid/phosphatidylinositol 4,5-bisphosphate/phosphatidylinositol 4-phosphate 5-kinase α /phospholipase D/porcine aortic endothelial cells

Introduction

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PtdCho) to generate phosphatidic acid (PtdOH), which remains in the membrane, and choline, a water-soluble head group (Exton, 1998; Liscovitch *et al.*, 2000). Thus far, two isoforms of PLD have been described, PLD1 and PLD2, both of which exist as two splice variants (Colley *et al.*, 1997; Hammond *et al.*, 1997; Park *et al.*, 1997). The 124 kDa PLD1 appears

localized to vesicles derived from the endosomal/lysosomal pathway (Ktistakis *et al.*, 1995; Brown *et al.*, 1998), but has also been detected at the plasma membrane (Brown *et al.*, 1998); it has been suggested that it is involved in the regulation of membrane coating that occurs during vesicle formation (Austin and Shields, 1996; Ktistakis *et al.*, 1996; Chen *et al.*, 1997; Siddhanta *et al.*, 1998) and in the regulation of secretion (Metz and Dunlop, 1990; Stutchfield and Cockcroft, 1993; Cockcroft, 1996; Bi *et al.*, 1997; Morgan *et al.*, 1997; Brown *et al.*, 1998; Caumont *et al.*, 1998). PLD1 activity is regulated by multiple inputs, including phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] (Liscovitch *et al.*, 1991, 1994; Pertile *et al.*, 1995; Schmidt *et al.*, 1996b), protein kinase C (PKC) (Conricode *et al.*, 1992; Eldar *et al.*, 1993; Cockcroft, 1996a,b; Kiss, 1996; Ohguchi *et al.*, 1996; Park *et al.*, 1998; Kim *et al.*, 2000), the Rho family proteins Cdc42, Rac (Hess *et al.*, 1997; Plonk *et al.*, 1998) and Rho (Malcolm *et al.*, 1996; Hess *et al.*, 1997; Karnam *et al.*, 1997; Schmidt *et al.*, 1997; Vinggaard *et al.*, 1997), and Arf proteins (Brown *et al.*, 1993; Cockcroft *et al.*, 1994; Ktistakis *et al.*, 1995; Kuribara *et al.*, 1995; Whatmore *et al.*, 1996) that act in an additive manner, leading to greater PLD1 activity than each alone (Hodgkin *et al.*, 1999).

PLD2 is a 106 kDa protein that shares 50–55% homology with PLD1 but lacks the 116 amino acid loop region following the first HKD motif (Plonk *et al.*, 1998). This isoform has been suggested to be localized to the plasma membrane (Plonk *et al.*, 1998) and to an as yet undefined submembrane vesicle compartment, which translocates to the membrane on stimulation with epidermal growth factor (EGF) (Honda *et al.*, 1999). PLD2 is reported to have a much higher basal activity than PLD1 when expressed in Cos-7 cells or isolated from insect cells and it has been suggested that cellular proteins, such as fodrin and synucleins (Jenco *et al.*, 1998), act to reduce the basal activity. Consequently, growth factor stimulation, rather than activating the enzyme catalytically, may repress the action of these endogenous inhibitors. Phorbol ester (phorbol 12-myristate 13-acetate) and members of the ARF family activate PLD2 2-fold only; however, deletion of the non-core N-terminal amino acids generates a protein with a much lower basal activity, which can be activated by ARF some 13-fold (Sung *et al.*, 1999). Whether PLD2 can be activated in response to growth factors is still unclear.

Both mammalian PLD1 and PLD2 have an absolute requirement for the higher phosphorylated inositol lipids (Berstein *et al.*, 1992; Colley *et al.*, 1997; Hammond *et al.*, 1997; Sciorra *et al.*, 1999; Hodgkin *et al.*, 2000). In keeping with this, we have recently demonstrated that PLD1 has a functional pleckstrin homology (PH) domain, specific for PtdIns(4,5)P₂, that regulates both activity and

localization (Hodgkin *et al.*, 2000). Sequence analysis demonstrates considerable homology between the PH domains of PLD1 and PLD2, thus a similar specificity can be presumed.

Cellular PtdIns(4,5)P₂ levels are, in part, governed by two families of enzymes, namely the Type I and Type II phosphatidylinositol 4-phosphate 5-kinases (PIPkinases) (Hinchliffe *et al.*, 1998; Anderson *et al.*, 1999). The Type II enzymes are composed of three isoforms that synthesize PtdIns(4,5)P₂ by the 4-phosphorylation of PtdIns(5)P (Rameh *et al.*, 1997). The role of these enzymes in the maintenance and synthesis of cellular PtdIns(4,5)P₂ pools is not yet understood. Type I PIPkinases comprise at least three distinct isoforms that phosphorylate the 5-position of PtdIns(4)P (Ishihara *et al.*, 1996; Loijens and Anderson, 1996; Shibasaki *et al.*, 1997). However, *in vitro*, these enzymes will phosphorylate a number of inositol lipids such as PtdIns, PtdIns(3)P, PtdIns(4)P and PtdIns(3,4)P₂ (Zhang *et al.*, 1997; Tolias *et al.*, 1998b). The Type I PIPkinases have been shown to associate with at least two low molecular weight G proteins of the Rho family (Chong *et al.*, 1994; Ren *et al.*, 1996; Tolias *et al.*, 1998a) and recently to be activated by members of the ARF family (Honda *et al.*, 1999; Jones *et al.*, 2000). Previous data demonstrated that the Type I PIPkinases were activated by PtdOH (Jenkins *et al.*, 1994), one product of PLD hydrolysis of PtdCho, and regulation of the Type I PIPkinase by ARF, *in vitro*, is dependent upon this acidic phospholipid (Honda *et al.*, 1999). The Type I PIPkinases have been shown to be required for secretion in permeabilized cells, for Rho-mediated ezrin, radixin and moesin (ERM) phosphorylation (Matsui *et al.*, 1999) and Rac-regulated capping/uncapping of actin in platelets (Tolias *et al.*, 2000). Recent evidence has suggested that PIPkinases may also be involved in ARF-regulated vesicle coating and budding at the Golgi and lysosomal membranes (Arneson *et al.*, 1999). A role in the internalization of activated receptors has also been suggested. Genetic studies in mice lacking a functional synaptojanin, a PtdIns(4,5)P₂ 5-phosphatase, have demonstrated increased PtdIns(4,5)P₂ levels with an accumulation of clathrin-coated vesicles in the cytosol, further supporting a role for PtdIns(4,5)P₂ in vesicular trafficking (Cremona *et al.*, 1999).

In most, if not all of these cellular processes, a role for PLD has also been implicated. A feed-forward cycle has been postulated whereby the generation of PtdIns(4,5)P₂ activates PLD, which leads to enhanced PtdOH formation able to activate further the Type I PIPkinase. This would lead to a rapid local increase in both PtdOH and PtdIns(4,5)P₂, which has been suggested to be important in both the generation of membrane curvature, vesicle budding and the recruitment/activation of proteins involved in coating of vesicles (Liscovitch and Cantley, 1995). Although enticing, there is a paucity of evidence to corroborate this theory. In this report we demonstrate that both PLD1 and PLD2 interact with the murine Type I α PIPkinase and that PLD2 is able to lead to the recruitment of Type I α PIPkinase in porcine aortic endothelial (PAE) cells. Finally, expression of the Type I α PIPkinase leads to the activation of PLD2 activity *in vivo*. These data suggest a molecular mechanism by which PtdIns(4,5)P₂ can be

Fig. 1. Cos-7 cells were transfected either with LacZ construct (1 μ g) or with the Type I α PIPkinase. These were labelled overnight with orthophosphate (10 μ Ci per dish) and stimulated as indicated for 30 min in the presence of 0.3% butan-1-ol. The reactions were quenched, and lipids were extracted and analysed as described in Materials and methods. The closed bars show the levels of ³²P-labelled PtdIns(4,5)P₂, while the open bars are the counts in PtdBut. The data are presented as the mean $\pm$ SD for triplicate samples (arbitrary units from analysis using a phosphoimager) and are typical of two independent experiments.

generated in a localized environment, leading to activation of PLD2.

Results

Expression of a Type I PIPkinase in Cos-7 cells leads to the activation of an endogenous PLD

Cos-7 cells transfected with LacZ (cont) or murine Type I α PIPkinase were treated as controls or stimulated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or lysophosphatidic acid (LPA) and PLD activity was assessed. PLD activity is measured easily by the inclusion of a primary alcohol, such as butanol, which is used as a nucleophile, in place of water, leading to the production of phosphatidylbutanol (PtdBut). Unlike PtdOH, which can be synthesized in a cell by at least three separate mechanisms, the more metabolically stable PtdBut can only be formed by the action of PLD. Transfection of the Type I α PIPkinase leads to an increase in endogenous PLD activity [from 856 $\pm$ 75 to 1432 $\pm$ 86 U (where U are arbitrary phosphoimager units); Figure 1]. As only 50% of the cells become transfected under these conditions, this suggests that the true stimulation would be at least 4-fold. This increase is equivalent to that seen *in vivo* using transfection with constitutively activated RhoA or with ARF-1, known activators of PLD1 (data not shown). TPA induced an increase of 1391 $\pm$ 81 U in non-transfected cells; however, in Type I PIPkinase-transfected cells TPA gave an increase of 2626 $\pm$ 207 U. Expression of Type I alone led to an increase of 575 $\pm$ 52 U, and added to the increase from the TPA treatment this would account for 1966 U of PLD activity. This suggests that Type I α PIPkinase-induced PLD activity is unlikely to occur through increased PtdIns(4,5)P₂ formation and subsequent hydrolysis leading to enhanced PKC activation. The fact that the activity in Type I α PIPkinase-transfected cells stimulated with TPA is greater than the sum of the two activities alone may suggest that TPA may be able to activate the Type I α PIPkinase.

Analysis of the phosphatidylinositol lipids after transfection of the Type I PIPkinase demonstrated a 2-fold increase in the level of $\text{PtdIns}(4,5)\text{P}_2$ (Figure 1). These data are consistent with the hypothesis that transfection of the Type I PIPkinase enhances $\text{PtdIns}(4,5)\text{P}_2$ synthesis, leading to the stimulation of an endogenous PLD activity.

Co-expression of Type I α PIPkinase leads to the activation of PLD2

To investigate which isoform of PLD is activated by the Type I α PIPkinase we studied the effect of its expression together with either PLD1 or PLD2 on PtdBut formation. As the extent of transfection differs between experiments, the counts obtained in the LacZ alone transfection have been subtracted. Thus, the data represent the PLD activity due to the transfection of the various constructs. Transfection of PLD1 into Cos-7 cells led to an increase in the basal activity that was stimulated potently by TPA, but we have been unable to demonstrate enhanced activation by co-transfection with the Type I α PIPkinase (data not shown). This was also shown to be the case when the cells were stimulated with LPA. Although not conclusive, these data suggest that the endogenous PLD that is activated by expression of the Type I α enzyme is not PLD1.

Transfection of PLD2 led to a small increase in both PtdOH (102 U) and PtdBut (327 U) in serum-starved cells. Co-transfection of PLD2 with the Type I α PIPkinase led to a much larger increase in both PtdOH and PtdBut formation (1756 and 2216 U, respectively). Type I PIPkinase alone yielded an increase of 306 and 42 U in PtdOH and PtdBut formation, respectively (Figure 2A). No enhancements of these changes were seen after stimulation with EGF, although MAP-kinase was activated by this agonist (Figure 2B). The gel also shows that there was approximately equal green fluorescent protein (GFP)–PLD2 and Type I PIPkinase expression in the transfected cells (each lane represents a single transfection and is the protein recovered from the interface of the lipid extractions used to generate the PtdBut data) (Figure 2A). These data suggest that co-expression of the Type I PIPkinase is able to lead to activation of PLD2 *in vivo*.

Murine Type I α PIPkinase interacts with PLD

To assess whether PLD2 and Type I α PIPkinase interact, we co-transfected Cos-7 cells with haemagglutinin (HA)-tagged PLD2 and EE-tagged Type I α PIPkinase. Immunoprecipitation was carried out using antibodies directed against the Glu-Glu (EE) tag (Type I PIPkinase), followed by western blot analysis using the anti-HA antibody (PLD2). Analysis of the whole cell lysates showed that HA-PLD2 was expressed equally (Figure 3A, lanes 5 and 7), but was only immunoprecipitated by the EE antibody when co-expressed with the EE-Type I α PIPkinase (Figure 3A, lane 3). These data demonstrate that PLD2 is able to interact with the Type I α PIPkinase.

To define regions of PIPkinase that interact with PLD2, deletion mutants of the Type I α PIPkinase (Figure 3B) were constructed and expressed as recombinant glutathione *S*-transferase (GST)-tagged proteins in *Escherichia coli*. These were purified using glutathione beads and then used to affinity purify PLD2 that had been expressed in Cos-7 cells (Figure 3B, blot). As controls,

Fig. 2. Cos-7 cells were transfected with Type I, PLD2 or a combination and were labelled overnight using orthophosphate. Cells were then left as controls or were stimulated with EGF (10 ng/ml) for 30 min in the presence of butan-1-ol (0.3%). Cells were quenched and the lipids and proteins were recovered and analysed by TLC (A) or western blotting (B) respectively. (A) Analysis of PtdOH (open bars) and PtdBut (closed bars). Lanes 1, 3 and 5 are controls, while lanes 2, 4 and 6 were treated with EGF. Lanes 1 and 2 were transfected with Type I PIPkinase (1 μg), lanes 3 and 4 were transfected with PLD2 (1 μg) and lanes 5 and 6 were transfected with Type I (1 μg) and PLD2 (1 μg). These data are expressed as the mean $\pm$ the range of values and are typical of two independent experiments. (B) Total protein was recovered from the interface of the lipid extraction used to generate the data for (A), redissolved in 8 M urea and separated using SDS–PAGE. Proteins were transferred to nitrocellulose and expression of the constructs determined using specific antibodies (anti-GFP for PLD and an antipeptide antibody for the Type I α PIPkinase). To demonstrate that EGF was active (+), the blot was reprobed using a phospho-specific anti-p42/44 MAP-kinase antibody.

either GST alone or GST–Type II α PIPkinase was used. The full-length Type I α PIPkinase was able to affinity purify PLD2. Deletion of the first 120 amino acids ($\delta 1$ –420) did not prevent affinity purification of PLD2. However, deletion of the first 306 amino acids ($\delta 1$ –912) completely abolished PLD2 binding. Deletions in the other direction demonstrated that even removal of the C-terminal 425 amino acids ($\delta 325$ –1626), leaving only the N-terminal 108 amino acids, still allowed binding of PLD2. It should be noted that there is no overlap between $\delta 325$ –1626 and $\delta 1$ –420, suggesting that either these two regions form distinct PLD2 binding sites or that they are both part of a single binding site. No affinity purification of PLD2 was achieved with either GST alone or GST–Type II PIPkinase.

Although we were unable to show that Type I α PIPkinase could regulate PLD1 activity *in vivo*, we were able to demonstrate that these two proteins interact (Figure 4). After co-transfection of the two proteins, wild-type PLD1 was immunoprecipitated and the Type I α PIPkinase activity associated with this enzyme was assessed. Co-transfection of the two cDNAs led to an enhancement of the PIPkinase activity immunoprecipitated with PLD1 (there was an increase in the PIPkinase activity in Type I-transfected cells alone, which may represent immunoprecipitation of endogenous PLD1) (Figure 4). Immunoprecipitation of the Type I activity, using an anti-myc antibody, demonstrated that its expres-

Fig. 3. (A) Cos-7 cells were transfected as indicated, lysed and immunoprecipitated using the EE antibody directed against the tagged Type I α PIPKinase. The immunoprecipitates and total lysates were separated by SDS-PAGE and immunoprobed using an anti-HA antibody directed against the tagged PLD2. PLD2 was expressed to equal levels either in the presence or absence of the Type I α PIPKinase. PLD2 was immunoprecipitated only when both PLD2 and Type I α PIPKinase were co-transfected (lane 3). (B) Deletions were generated in the Type I PIPKinase as indicated (δ x-y defines the deleted bases in the cDNA, where 1 refers to the start ATG) and cloned into a pGEX-4T expression vector. Proteins were induced, purified and used to affinity purify PLD2 from transfected Cos-7 cell lysates. The proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed using an anti-HA antibody against the tag for PLD2. Only deletion δ 1-912 did not bind PLD2. Interestingly, there is no overlap between δ 325-1626 and δ 1-420, suggesting that either the PLD2 binding site resides across this stretch or that there are two distinct binding sites. The experiment shown was carried out independently on two occasions.

Fig. 4. PLD1 interacts with the Type I α PIPKinase. Cos-7 cells were transfected with the HA protein (control) or with PLD1, myc-tagged Type I α , Type II PIPKinase, or in combinations as shown. The cells were lysed and immunoprecipitated using an anti-PLD1 polyclonal antibody. The immunoprecipitations were then used in a PIPKinase assay. As can be seen, co-transfection leads to enhanced immunoprecipitation of Type I activity. The inset shows the total Type I PIPKinase activity after immunoprecipitation using a myc antibody for co-expression with the HA protein (lane 1) or with PLD1 (lane 2).

Fig. 5. Confluent PAE cells were microinjected with various constructs, left for 3 h, and then fixed and stained. In (A, 1–3), the constructs were injected alone. In (B, 1–3), they were injected with Type Iα PIPkinase together with GFP-PLD2. (B, 1) shows the GFP-PLD2 staining, (B, 2) is the Type Iα PIPkinase expression visualized using a polyclonal antibody and (B, 3) is the merge. (C, 1–3) Cells microinjected with both Type Iα PIPkinase and PLD2, but viewed through a Z plane. (D, 1–3) Co-injection of the GFP-8325–1626 together with HA-tagged PLD2. The lasers were set up such that no bleed through from one channel to the other was detected.

sion, either in the presence or the absence of PLD1, was equivalent (Figure 4, inset). No PIPkinase activity was associated with PLD1 immunoprecipitates when it was co-expressed with the Type IIα PIPkinase. These data demonstrate that both isoforms of PLD are able to interact with the Type Iα PIPkinase, but not with the Type II enzyme. However, $\text{PtdIns}(4,5)\text{P}_2$ generated by this enzyme can only activate PLD2 *in vivo*.

PLD2 recruits the Type Iα PIPkinase when co-expressed in PAE cells

To assess whether Type Iα PIPkinase and PLD2 interact *in vivo*, we co-expressed these proteins in confluent PAE cells by microinjection of the cDNAs and stained for the various proteins 3 h later. This experiment was carried out using both GFP-tagged PLD2 and myc-tagged Type I PIPkinase or GFP-tagged Type Iα PIPkinase and HA-tagged PLD2. In both cases the same result was observed. PLD2 was localized in a submembrane vesicular compartment (Figure 5A, 2), which did not co-localize with markers for either the endoplasmic reticulum or the Golgi (data not shown). Expression of the Type I PIPkinase alone showed a complex staining pattern associated with the plasma membrane, cytosol and in

intracellular structures that resemble microtubuli (Figure 5A, 1). Co-expression of PLD2 and Type Iα PIPkinase resulted in a dramatic relocation of the Type Iα PIPkinase to the submembrane PLD2-positive patches (Figure 5B). This co-localization was also seen when viewing the cells through a Z section (Figure 5C). As the Type Iα PIPkinase is able to associate with a number of different proteins (Rac1, PLD1 and PLD2, PKD and ARFs) and to test the requirement for PIPkinase activity for this recruitment, we also tested the localization of the GFP-8325–1626 deletion mutant, which contains only the first 108 amino acids but is still able to bind to PLD2 (Figure 3C). When injected alone, the GFP-8325–1626 was completely cytosolic (Figure 5A, 3); however, co-injection with PLD2 also led to a complete co-localization at the sub-membrane patches (Figure 5D). Thus, PLD2 is able to recruit the Type Iα PIPkinase to its own intracellular location.

Local generation of $\text{PtdIns}(4,5)\text{P}_2$ is required for TPA-stimulated PtdBut formation

To determine whether the Type I PIPkinase activity was required for the activation of PLD2, we constructed a kinase-dead (KD) version of the Type I PIPkinase by

substituting a single amino acid in the ATP-binding loop as described previously (Ishihara *et al.*, 1998). This mutant was expressed in Cos-7 cells, immunoprecipitated and assayed for PIPkinase activity. The wild-type enzyme yielded 109 146 U, while the KD mutant produced 1435. This mutant was transfected into Cos-7 cells, in the presence or the absence of PLD2, and the activity of the expressed PLD2 was assessed. Overexpression of the KD mutant significantly reduced the basal activity of PLD2 (Figure 6A). The reason for the incomplete inhibition probably resides in the fact that both PLD2 and Type I α KD were co-transfected. Thus, the expression of the KD may not be high enough to completely abrogate the heterologously expressed PLD2 activity (anti-GFP antibodies showed that GFP-PLD2 with GFP-wild-type Type I α PIPkinase were expressed at equimolar levels).

Previous data showed that PtdIns(4,5)P₂ was also required for TPA-stimulated PLD activity *in vivo*. We therefore investigated whether the kinase activity was also required. PLD2 was co-expressed together with the δ 325–1626 construct, which we showed was able to bind PLD-2, but is inactive with respect to kinase activity. Expression of this mutant results in a 50% decrease in the TPA-induced PLD2 activity (Figure 6B). Western analysis showed that there was no significant change in the expression of PLD2 in these transfections. Identical data were obtained using the KD mutant Type I α instead of the δ 325–1626 mutant (Figure 6A, striped bars). These data suggest that interaction with Type I α PIPkinase is required for the generation of PtdIns(4,5)P₂, which is required for the activation of PLD2 by TPA.

Type I α PIPkinase and PLD interact *in vivo*

Although the previous data are consistent with the hypothesis that PLD family members and the Type I α PIPkinase interact, these experiments were carried out with overexpressed proteins. To assess the *in vivo* significance, we employed anti-peptide antibodies specific for the Type I PIPkinase and assessed whether they were able to immunoprecipitate PLD activity assayed *in vitro*. PAE cells were grown to confluency, lysed using a mild detergent buffer and the proteins were immunoprecipitated using specific antibodies to the Type I or Type II PIPkinase as a control. After extensive washing, the beads were assayed for PIPkinase activity by phosphorylation of PtdInsP to PtdIns(4,5)P₂ in the presence of *N*-(*N*-[6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino]caproyl) (NBD)-PtdCho as a substrate for PLD. The reactions were carried out for 20 min and the products were separated by thin layer chromatography (TLC). PIPkinase activity was assessed by phosphorimager analysis of the ³²P incorporated into PtdIns(4,5)P₂, while PLD activity was assessed by the generation of NBD-PtdOH. As a control, HA-tagged PLD2 was expressed in Cos-7 cells and immunoprecipitated using the anti-HA antibody (Figure 7, lane 3). No PLD activity was found when either protein G-Sepharose or protein G-Sepharose coupled to anti-Type II PIPkinase antibodies was used (Figure 7). In contrast, anti-Type I PIPkinase antibodies immunoprecipitated PLD activity. As the assays were carried out in the presence of 10 μ M PtdIns(4,5)P₂, the increased PLD activity is not a reflection of the amount of conversion of PtdIns(4)P to

Fig. 6. (A) Cos-7 cells were transfected with PLD2 alone or in the presence of the KD Type I PIPkinase. The cells were labelled overnight and incubated in the presence of butan-1-ol for 30 min. The lipids were extracted and the formation of PtdBut was analysed (filled bars). In a separate experiment, PLD2 was expressed alone or together with the KD Type I α and then stimulated with TPA (striped bars). The data are expressed as the means from three separate transfections $\pm$ SD. The difference is significant with $P < 0.05$. (B) Cos-7 cells were transfected with PLD2 alone or in the presence of GFP- δ 325–1626 and labelled as above, but were treated with TPA in the presence of butan-1-ol for 30 min. The data are represented as the means of two separate transfections. The panel underneath shows the expression levels of PLD2 in the experiment and was carried out after recovery of the protein from the interface after extraction of the lipids (see Materials and methods).

PtdIns(4,5)P₂. These data suggest that, *in vivo*, PLD can associate with Type I PIPkinase isoforms in PAE cells.

Discussion

A number of studies have demonstrated that PLD activity requires the presence of PtdIns(4,5)P₂. It has been suggested that the inositide interacts with a site within domain IV (Sciorra *et al.*, 1999), but contradicting evidence has now pointed to the effect being mediated

Fig. 7. PAE cells were grown to confluence, lysed and immunoprecipitated using either an anti-Type II (lane 1) or anti-Type I antibody (lane 2). The beads were then assayed for PIPkinase activity in the presence of NBD-labelled PtdCho. The reaction was carried out for 20 min and the lipids were extracted, separated by TLC, and NBD-PtdOH was viewed using an eagle Eye video camera. Radioactivity incorporated into PtdIns(4,5)P₂ was quantitated using a phosphorimager. Lane 3 shows the immunoprecipitation of HA-tagged PLD2 expressed in Cos-7 cells. The production of NBD-PtdOH is shown, which co-migrated with NBD-PtdOH generated by the phosphorylation *in vitro* of NBD-1,2-diacylglycerol.

through a PtdIns(4,5)P₂ selective PH domain (Hodgkin *et al.*, 2000). The PH domain binding site appears to have two functions, as point mutations abolish PLD activity, whilst deletion of the domain prevents membrane association. The demonstration that Rho family proteins are able to regulate PIPkinase activity (Chong *et al.*, 1994; Ren *et al.*, 1996; Tolia *et al.*, 1998a) together with the use of a number of bacterial toxins, such as the *Clostridium difficile* toxin B (which inactivates Rho family proteins) and *Clostridium sordelli* toxin (which inhibits both Ras and Rho family proteins), has been used to implicate the importance *in vivo* of phosphoinositides in the regulation of PLD activity (Schmidt *et al.*, 1996a,b, 1997). These data are, however, limited as the small molecular weight G proteins, which are targets for these toxins, are also potential activators of PLD1. In toxin B-pretreated HEK 293 cells, the reduced GTPγS-mediated PLD activity could be fully restored by the re-addition of PtdIns(4,5)P₂ (Schmidt *et al.*, 1996b). These data, although implying a specific role for PtdIns(4,5)P₂ in the regulation of PLD activity, do not demonstrate that PLD activity can be regulated *in vivo* by the levels of this lipid. In this study we demonstrate that the endogenous PLD in Cos-7 cells can be stimulated solely by the overexpression of a Type I PIPkinase. Furthermore, we suggest that this occurs through the interaction between the two proteins. Recruitment of the Type Iα PIPkinase to the intracellular compartment where PLD2 resides would lead to enhanced synthesis of polyphosphoinositides. Although both PLD1 and PLD2 have an absolute requirement for PtdIns(4,5)P₂ and are able to interact with the Type I PIPkinase, we were only able to show activation of PLD2. As PLD1 activity is regulated by multiple, independent inputs: two separate GTPases, a Rho family member and an ARF family member and PKC, in a phosphorylation-independent manner, it may be that PtdIns(4,5)P₂ levels alone are not enough to regulate this enzyme. PLD2, in contrast, is not regulated by the same multiple inputs.

The mechanism behind the regulation of PLD family members by phosphoinositides is not clear, as it has been demonstrated that PLD2 activity may be negatively regulated in cells by proteins such as fodrin and synucleins (Jenco *et al.*, 1998) and by actinin and amphiphysins (Lee *et al.*, 2000; Park *et al.*, 2000). It is possible that the role of polyphosphoinositides is to alleviate the inhibition by

these proteins. The recent demonstration that ARF family members are able to regulate Type Iα PIPkinase activity (Honda *et al.*, 1999; Jones *et al.*, 2000), together with the data from this study, suggest that ARF-mediated regulation of PLD activity may occur through enhanced local PtdIns(4,5)P₂ synthesis. Alternatively, phosphoinositides may regulate ARF activation. The regulation of ARF by phosphoinositides is complex. PtdIns(4,5)P₂ has been shown to mediate the activation of an ARF GAP and its interaction with ARF (Randazzo, 1997); this would lead to an increase in the GDP loading. However, PtdIns(4,5)P₂ has also been suggested to positively regulate the activity of an ARF-guanine nucleotide exchange factor (ARNO), leading to an increase in the levels of GTP bound ARF (Paris *et al.*, 1997). Further complexity arises through the ability of PtdIns(4,5)P₂ to stimulate guanine nucleotide exchange on ARF (Terui *et al.*, 1994). At present, there are no methods to look specifically for activated ARF *in vivo* and the importance *in vivo* of ARF family members in regulating PLD2 remains controversial.

A number of studies have previously suggested a link between PtdIns(4,5)P₂, PtdOH and vesicle formation (Liscovitch and Cantley, 1995; Pertile *et al.*, 1995; Arneson *et al.*, 1999). The generation of PtdIns(4,5)P₂ by PIPkinase, and the subsequent activation of PLD2 to generate PtdOH, positively regulates PIPkinase, leading to high local concentrations of these lipids in the membrane, which may be important in the induction of membrane curvature. It may also be important in the recruitment of adapter proteins involved in the generation of a coat required to make the vesicle. Whether this feedback occurs is not known. Previous data from Moritz *et al.* (1992) showed that treatment of membranes with bacterial PLD, which leads to increased PtdOH formation, also led to the activation of an endogenous PIPkinase.

The generation of PtdOH at membranes may be a general mechanism for regulating PIPkinase at these intracellular domains. Recently, it has been demonstrated that endophilin-1, which is able to stimulate synaptic vesicle budding, acts as a lysophosphatidic acid acyl-transferase generating PtdOH. The authors suggest that this may be required to activate a PIPkinase able to generate PtdIns(4,5)P₂ and thus leading to the recruitment/activation of dynamin (Schmidt *et al.*, 1999). A role for PtdIns(4,5)P₂ in clathrin-coated vesicle formation

has been genetically established in mice that are homozygous for a deletion of synaptojanin, a PtdIns(4,5)P₂ 5-phosphatase (Cremona *et al.*, 1999).

The data presented in this paper are consistent with the hypothesis that PLD2 is able to lead to the recruitment of the Type I α PIPkinase that leads to enhanced PtdIns(4,5)P₂ production, which is able to regulate PLD2 activity. This study provides the first evidence *in vivo* for activation of PLD by PtdIns(4,5)P₂ and places PLD2 as a downstream effector of the Type I α PIPkinase. This may have important implications as a number of studies have suggested that both PIPkinase and PLD activities are up-regulated in the development of cancer.

Materials and methods

General reagents

Unless otherwise stated all reagents were of analytical grade. PtdCho, phosphatidylserine (PtdSer), PtdIns, PtdInsP and PtdInsP₂ were purified from bovine brain. NBD-PtdCho was purchased from Molecular Probes. All radiochemicals were purchased from Amersham International. Anti-HA tag antibody was generated from clone 12CA5, while the myc epitope was from clone 9e10. All secondary antibodies were purchased from Dako Products.

Plasmids

A cDNA encoding the murine Type I α PIPkinase was generated from a murine brain cDNA library using PCR and cloned into either Pjex 4T (Pharmacia) for expression in bacteria, or pCDNA3 for expression in cos cells. Deletions were generated using PCR and cloned into the above vectors and into pEGFP-C2 for GFP fusion protein expression. PLD1 and HA-PLD2 were subcloned into pCDNA3. GFP-PLD2 was cloned into PEGFP-C2.

Cell culture

Cos-7 cells were routinely maintained at ~30% confluency and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum (FCS) (v/v). The cells were split 1 day before transfection to 30%, and transfected the following day using DEAE-dextran. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and incubated with plasmid DNA [2 μ g per 6 cm dish in 560 μ l of PBS + 30 μ l of DEAE-dextran (10 mg/ml)] for 30 min. Four millilitres of DMEM-8% FCS containing 80 μ M chloroquine were added and left for a further 2.5 h. This medium was replaced with 2 ml of DMEM-8% FCS-10% DMSO, left for 2 min and replaced with fresh DMEM-8% FCS. Transfection was carried out for a further 24 h, after which the cells were labelled and treated as below. PAE cells were maintained in DMEM-8% FCS and routinely passaged to 30% confluency. Microinjection of plasmids (0.1 μ g/ml) into the nucleus of PAE cells was carried out using an automated Eppendorf injector. The cells were allowed to recover for 3 h and then were fixed and processed as described below.

In vivo PLD assay

Cells were transfected as above, left for 24 h and labelled overnight with [³²P]orthophosphate (10 μ Ci) in phosphate-free DMEM. The cells were washed twice with RPMI-salts buffer and incubated in this buffer containing butan-1-ol (0.3% v/v) for 30 min. This medium was aspirated, and 0.45 ml of 2.4 M HCl added. The cells were maintained and scraped on ice, removed to a clean Eppendorf tube and the dishes washed with 0.5 ml of methanol, which was pooled with the HCl. Half a millilitre of chloroform containing 5 μ g of Folsch lipid extract was added, together with 0.25 ml of water. The two phases were mixed vigorously, centrifuged (2 min at 21 000 g in an Eppendorf bench centrifuge) and the lower phase was removed carefully, so as not to disturb the protein interface (see below), and was washed once with theoretical upper phase (chloroform:methanol:1 M HCl 15:245:235, 0.7 ml), with the lower phase being removed to a clean Eppendorf tube. The first upper phase was then back extracted with 0.2 ml of chloroform, which after mixing and centrifugation was removed to the tube containing the theoretical upper phase wash. This was mixed and centrifuged and the lower phase removed to the tube containing the first lower phase. The samples were dried and kept at -20°C. Samples were analysed for PtdBut formation

and for PtdIns/PtdInsP/PtdInsP₂ labelling (see the following section). To assess the expression levels of various constructs, the protein layer at the initial interface was recovered after lipid removal by the addition of 0.5 ml of methanol to the upper phase. The Eppendorf tube was centrifuged (21 000 g for 10 min), and the protein pellets were washed once with 70% acetone. These were allowed to dry and were resuspended overnight in 50 mM Tris pH 8.0, 8 M urea. SDS-PAGE sample buffer was added, the samples were boiled, resolved by SDS-PAGE and transferred to nitrocellulose. The blots were probed with various antibodies (at dilutions indicated in the figure legends) and visualization was carried out using ECL according to the manufacturer's instructions (Amersham International).

Co-immunoprecipitations

Cells were transfected as described and left for 48 h, after which they were lysed [1 ml lysis buffer (50 mM Tris pH 8.0, 50 mM KCl, 10 mM EDTA, 1% NP-40)], scraped, and nuclei and cellular debris removed by centrifugation (14 000 r.p.m., 4°C Eppendorf centrifuge). Immunoprecipitations were carried out using monoclonal antibodies against epitope tags 12CA5 (anti-HA tag), 1E10 (anti-myc tag) and an antibody derived against the EE tag. Immunoprecipitation of the endogenous Type I α PIPkinase from PAE cells was carried out using an anti-peptide antibody. The immunoprecipitates were collected using protein G-Sepharose, and washed five times with IP wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA 0.1% Tween-20), then once with PIPkinase buffer. Immunoprecipitations were either used for western blotting or were used to assess the PIPkinase and/or PLD activities as described below.

Lipid vesicles were prepared using 1 nmol of PtdInsP isolated from pig brain together with 3 nmol of PtdOH. Reactions were carried out at 30°C for 5 min in PIPkinase buffer (50 mM Tris pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 70 mM KCl) containing cold ATP (20 μ M) and 1 μ Ci of [³²P]ATP in a final volume of 100 μ l. Reactions were quenched with 0.5 ml of chloroform:methanol [1:1 (v:v)] and the phases were split by the addition of 125 μ l of 2.4 M HCl. The lower phases were removed to a new tube, dried and separated by TLC either using an acidic (chloroform:methanol:acetone:glacial acetic acid:water 240:78:72:70:42) or an alkaline solvent [chloroform:methanol:ammonia (28%):water 45:35:2:8]. Incorporation into PtdIns(4,5)P₂ was quantitated using a phosphorimager.

To assess PLD and PIPkinase activity in the same reaction the following lipid vesicles were made: 3 nmol PtdOH, 1 nmol PtdInsP, 1 nmol PtdIns(4,5)P₂, 10 nmol PtdSer and 2 nmol NBD-labelled PtdCho. Reactions were carried out for 20 min, after which they were extracted as above and reaction products separated using the acidic solvent system. Incorporation into PtdIns(4,5)P₂ was carried out as above, while the PLD activity was assessed by the production of NBD-PtdOH.

Type I α PIPkinase deletions

Deletions in the 3' direction were carried out using a constant 5' primer tagged with the myc epitope and a unique restriction site, while deletions in the other direction were with a constant 3' primer tagged with the myc epitope. PCR was carried out using Pfu and the reaction products were restricted and cloned into either pCDNA3 for eukaryotic expression, or in a GST vector for bacterial expression and purification. In some cases, the products were cloned into a GFP vector. Site-directed mutagenesis was carried out using the Quick Change kit from Stratagene. All constructs were verified by sequencing.

GST-PIPkinase affinity purification of PLD2

Lysates were prepared from Cos-7 cells expressing HA-PLD2 and were incubated with GST fusion proteins purified from bacterial lysates by incubation with glutathione-Sepharose. Incubations were carried out for 2 h, after which the affinity beads were washed three times with immunoprecipitation buffer (see above). The beads were then placed in SDS loading buffer, separated by SDS-PAGE, transferred to nitrocellulose and probed using antibodies against the HA tag. GST alone, GST-Type II α PIPkinase or glutathione beads were used as controls for this experiment. No binding to any of these was found.

Immunolocalizations in PAE cells

Constructs were microinjected into the nucleus of PAE cells, allowed to recover for 3 h and then fixed with formaldehyde (3.6% in PBS). The cells were permeabilized with Triton X-100 (0.1% in PBS) and blocked with PBS-bovine serum albumin (1%). Coverslips were incubated with primary antibody [PIPkinase (1:50), anti-HA (1:100)] for 1 h, washed in PBS three times and incubated with the corresponding secondary antibody conjugated to either fluorescein isothiocyanate or Texas red.

The coverslips were washed with PBS, after which they were mounted using vectashield. Fluorescence was viewed using a Leica confocal microscope.

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