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Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes

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Phosphoinositides are both substrates for second messenger-generating enzymes and spatially localized membrane signals that mediate vital steps in signal transduction, cytoskeletal regulation and membrane trafficking. Phosphatidylcholine-specific phospholipase D (PLD) activity is stimulated by phosphoinositides, but the mechanism and physiological requirement for such stimulation to promote PLD-dependent cellular processes is not known. To address these issues, we have identified a site at which phosphoinositides interact with PLD and have assessed the role of this region in PLD function. This interacting motif contains critical basic amino acid residues that are required for stimulation of PLD activity by phosphoinositides. Although PLD alleles mutated at this site fail to bind to phosphoinositides *in vitro*, they are membrane-associated and properly localized within the cell but are inactive against cellular lipid substrates. Analogous mutations of this site in yeast PLD, Spo14p, result in enzymes that localize normally, but with catalytic activity that has dramatically reduced responsiveness to phosphoinositides. The level of responsiveness to phosphoinositides *in vitro* correlated with the ability of PLD to function *in vivo*. Taken together, these results provide the first evidence that phosphoinositide regulation of PLD activity observed *in vitro* is physiologically important in cellular processes *in vivo* including membrane trafficking and secretion.

Keywords: phosphatidylinositol 4,5-bisphosphate/phospholipase D/pleckstrin homology domain/secretion

Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids, resulting in the generation of phosphatidic acid (PtdOH) and the respective head group. Two classes of phosphatidylcholine (PtdCho)-specific PLD enzymes have been identified in mammalian systems. One of these can be stimulated by fatty acids such as oleate, but the identity of the protein responsible for this widespread activity is not known (Okamura and Yamashita, 1994). The second class of PLD enzymes is characterized by a dramatic activation by phosphoinositides when assayed *in vitro* using the most widely employed method (Brown and Sternweis, 1995). Two mammalian isoforms of PLD, PLD1 and PLD2, have been cloned based on sequence homology to previously identified plant and yeast enzymes, and both are activated by phosphoinositides *in vitro* (Hammond *et al*., 1995; Colley *et al*., 1997).

A role for phosphoinositides in the regulation of PLD activity was first suggested by the finding that phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] was required for optimal stimulation of PLD activity partially purified from HL60 cell membranes (Brown *et al*., 1993). This effect of PtdIns(4,5)P_2 on PLD activity is specific because other acidic phospholipids including phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate [PtdIns(4)P], and PtdOH are ineffective (Brown *et al*., 1993; Liscovitch *et al*., 1994). However, *in vitro*, PLD activity becomes much less dependent on phosphoinositides when short-chain PtdCho substrates are used (Vinggaard *et al*., 1996). Consistent with a role of PtdIns(4,5)P_2 in PLD regulation, it was demonstrated that the phosphoinositide-binding aminoglycoside antibiotic, neomycin, inhibited activity of the membrane-bound PLD from rat brain. This inhibition could be overcome by the inclusion of exogenous PtdIns(4,5)P_2 (Liscovitch *et al*., 1994). Subsequent studies showed that phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] can also stimulate PLD activity (Liscovitch *et al*., 1994; Hammond *et al*., 1997).

The structural basis for the activation of PLD by PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 observed *in vitro* and the role of this mode of regulation in the physiological control of PLD activity are not known. PtdIns(4,5)P_2 synthesis was required for the activation of PLD by guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) and phorbol 12-myristate 13-acetate (PMA) in permeabilized U937 cells (Pertile *et al*., 1995). Activation of GTP-binding proteins and protein kinase regulators of mammalian PLD involves a number of steps that are themselves dependent on phosphoinositides (reviewed in Sciorra *et al*., 1999). Consequently, the basis for this requirement for PtdIns(4,5)P_2 synthesis in activation of PLD is unclear. Recently, it was shown that PLD activity localized to detergent-resistant membrane domains (Czarny *et al*., 1999; Kim *et al*., 1999; Sciorra and Morris, 1999), which are plasma membrane domains reported to be enriched in PtdIns(4,5)P_2 (Hope and Pike, 1996). Collectively, these studies support a role for PtdIns(4,5)P_2 in PLD regulation; however, it is not clear if this requirement is direct and whether physiological changes in phosphoinositide levels control PLD *in vivo*.

Although a growing body of evidence supports roles for mammalian PLD in signal transduction and regulation of vesicular transport (reviewed in Exton, 1998), precise definition of the pathways involved remains a challenge because activation of PLD is accompanied by stimulation

of other signaling pathways. The greatest insights into the function of the phosphoinositide-dependent PLD enzymes have come from studies of the yeast PLD, Spo14p. Spo14p activity is essential for meiosis and is strongly activated by PtdIns(4,5)P_2 *in vitro* (Rose *et al.*, 1995). Spo14p is regulated by phosphorylation-mediated redistribution within the cell and appears to be intimately involved with the formation of the prospore membrane (Rudge *et al.*, 1998a). Consistent with this proposed role in membrane movement, Spo14p catalytic activity is also required for Golgi secretory function under the unusual circumstance when the yeast PtdIns-transfer protein, Sec14p, is dysfunctional and a secondary bypass mutation is present (Sreenivas *et al.*, 1998; Z.Xie *et al.*, 1998). However, the role of phosphoinositides in these cellular processes is unclear.

We have examined the structural basis and physiological significance of the activation of mammalian and yeast PLD enzymes by PtdIns(4,5)P_2. Our results show that activation is mediated by direct interaction of this lipid with a selective binding site for PtdIns(4,5)P_2 that is conserved among PtdIns(4,5)P_2-activated PLD enzymes. We demonstrate that binding to PtdIns(4,5)P_2 is not the sole determinant of membrane association for mammalian and yeast PLD proteins, but that interaction with PtdIns(4,5)P_2 is required for activation of these enzymes in a number of physiological settings.

Results

Direct interaction between PLD enzymes and PtdIns(4,5)P_2

Activation of PLD by PtdIns(4,5)P_2 has been proposed to occur through a direct interaction between the enzyme and lipid regulator but this has not been demonstrated. We therefore began our investigation by examining the binding of purified PLD2 to sucrose-loaded phospholipid vesicles with a constant composition containing 5% PtdIns(4,5)P_2, PtdOH or PtdIns. The vesicles were sedimented by ultracentrifugation and >95% of the [^{14}C]PtdCho tracer was recovered in the pellet fractions (data not shown). Measurement of PLD activity (Figure 1A) or protein (Figure 1B) revealed that PLD2 bound strongly and specifically to the vesicles containing PtdIns(4,5)P_2. To determine the affinity of PLD2 for PtdIns(4,5)P_2 in liposomes, we examined the binding of PLD2 to increasing concentrations of liposomes containing 5% PtdIns(4,5)P_2 or PtdIns. Binding of PLD2 to vesicles containing PtdIns was weak with an approximate K_d of 200 μM; however, inclusion of 5% PtdIns(4,5)P_2 dramatically increased the affinity of PLD2 binding to these vesicles to an approximate K_d of 10 μM (Figure 1C and D).

The results shown in Figure 1 demonstrate that PtdIns(4,5)P_2 promotes binding of PLD to bilayer vesicles but do not prove that the interaction between PtdIns(4,5)P_2 and PLD is direct. To address this issue, we used a photo-affinity radiolabeling strategy. This approach has been effective in characterizing phosphoinositide-binding interactions for many other proteins, including phospholipase Cδ1 (Tall *et al.*, 1997) and profilin1 (Chaudhary *et al.*, 1998). PtdIns(4,5)P_2 derivatives, with a photo-reactive benzophenone group linked to the SN_1-substituted acyl chain {BZDC-[^{3}H]acyl-PtdIns(4,5)P_2} or to the P1 phosphate group forming a triester linkage {BZDC-[^{3}H]triester-PtdIns(4,5)P_2}, were employed for photo-affinity labeling. Both photo-reactive PtdIns(4,5)P_2 analogs were effective activators of PLD2 (Figure 2A) and PLD1 (data not shown), producing increases in PLD activity that were 70–80% of those observed with bovine brain PtdIns(4,5)P_2. We next determined whether the photo-reactive probe interacted with PLD directly. Purified PLD2 was incubated

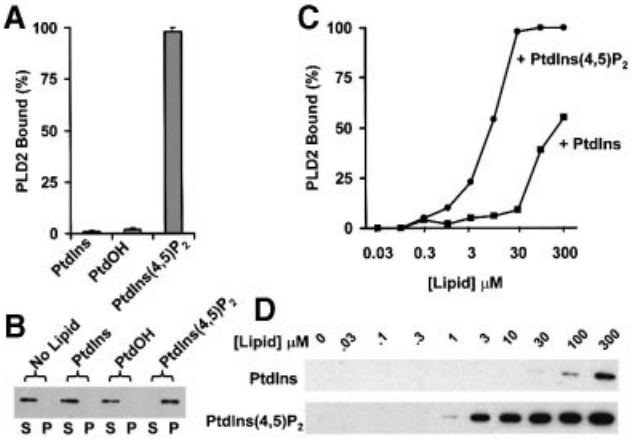

Fig. 1. PtdIns(4,5)P_2-dependent binding of PLD2 to sucrose-loaded phospholipid vesicles. (**A**) The binding of purified PLD2 to phospholipid vesicles (PtdCho:PtdSer:PtdEtn, 1:1:1) containing 5 mol% PtdIns, PtdOH or PtdIns(4,5)P_2 was determined at total lipid concentrations of 23.1, 24 and 24.9 μM, respectively. The data shown are means ± SD of triplicate determinations from a single representative experiment. (**B**) Supernatant (S) and pellet (P) fractions from the incubations described in (A) were recovered and analyzed for PLD2 by SDS–PAGE and Western blotting. (**C**) The binding of purified PLD2 to increasing concentrations of phospholipid vesicles containing either 5 mol% PtdIns(4,5)P_2 (●) or PtdIns (■) was determined. The data shown are means of triplicate determinations from a single representative experiment. (**D**) Pellet fractions from the incubations described in (C) were recovered and analyzed by SDS–PAGE and Western blotting.

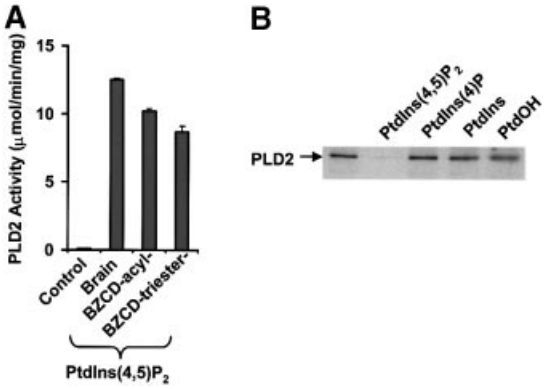

Fig. 2. Activation and labeling of PLD2 by photo-reactive PtdIns(4,5)P_2 derivatives. (**A**) Activity of purified PLD2 was determined as described in Materials and methods using sonicated phospholipid dispersions containing a 5 μM concentration of either bovine brain PtdIns(4,5)P_2, the unlabeled BZDC-acyl-PtdIns(4,5)P_2 or the BZDC-triester-PtdIns(4,5)P_2. The data shown are means ± SD of triplicate determinations from a single representative experiment. (**B**) Purified PLD2 (2 μg) was incubated with an equimolar concentration of the BZDC-[^{3}H]triester-PtdIns(4,5)P_2 probe alone or in the presence of a 1000-fold molar excess of PtdIns(4,5)P_2, PtdIns(4)P or PtdIns. The incubations were exposed to a 360 nM UV light source, proteins separated by SDS–PAGE and radiolabeled proteins visualized by fluorography after impregnation of the gels with EN^{3}HANCE.

with an equimolar concentration of BZDC-[^{3}H]triester-PtdIns(4,5)P_2 alone or in the presence of a 1000-fold molar excess of PtdIns(4,5)P_2, PtdIns(4)P, PtdIns or PtdOH. The radiolabeled probe was incorporated into the PLD2 protein with an estimated efficiency of 3% and this incorporation could be competed by excess unlabeled PtdIns(4,5)P_2 but not by PtdIns(4)P or PtdIns (Figure 2B). Taken together, the results suggest that PLD contains a site that promotes direct, selective and high-affinity interaction with PtdIns(4,5)P_2.

The N-terminal pleckstrin homology domain is not involved in the activation of PLD by PtdIns(4,5)P_2

PLD1, PLD2 and Spo14p contain an N-terminal pleckstrin homology (PH) domain (Holbrook *et al.*, 1999), which is a conserved structural motif present in many signal transduction proteins (Lemmon *et al*., 1996). PH domains are thought to be involved in mediating intermolecular interactions, and several PH domains (e.g. PLC δ1) have been shown to bind phosphoinositides with high affinity (Tall *et al.*, 1997). To determine the effect of the PH domain, PLD N-terminally truncated forms (which lack the PH domain) of PLD1 (ΔN325) and PLD2 (ΔN308) were expressed in Sf9 cells, and their sensitivity to activation by PtdIns(4,5)P_2 was examined. The concentration dependence of activation of truncated PLD2 (Figure 3A) and PLD1 (data not shown) by PtdIns(4,5)P_2 was very similar to that of the wild-type proteins, indicating that the N-terminal PH domain plays no significant role in activation of the enzymes by PtdIns(4,5)P_2. Consistent with these results, Sung *et al.* (1999a,b) demonstrated that truncated PLD1 or PLD2 mutants lacking the N-terminal PH domains are catalytically active when ectopically expressed in COS-7 cells.

Activation of PLD enzymes by PtdIns(4,5)P_2 requires a unique conserved region of basic amino acids

Selective interactions with phosphoinositides and inositol phosphates that do not involve PH domains have been demonstrated for a number of proteins including synaptotagmin, villin, gelsolin and phospholipase C-β (reviewed in Martin, 1998). Although a firm consensus sequence has not emerged, these interactions all involve stretches of sequence (10–20 residues) loosely denoted as 'KR' motifs, which are rich in basic amino acids and often interspersed with aromatic and aliphatic amino acids. We hypothesized that similar sequences were involved in the interaction of the PLD with PtdIns(4,5)P_2.

Close examination of the sequences of the phosphoinositide-dependent and -independent PLDs, and observations based on previous work (Sung *et al*., 1997, 1999a,b; Rudge *et al.*, 1998a), allowed us to exclude many regions (Figure 3B). Regions of amino acids (regions I–IV) are involved in catalysis and were excluded because of the observation that these sequences are highly conserved among plant and bacterial PLD enzymes that are not activated by phosphoinositides (Morris *et al.*, 1996; Ponting and Kerr, 1996). Other regions of the phosphoinositide-activated PLD enzymes including the C-termini are considerably divergent in primary sequence. However, inspection of the PLD1, PLD2 and Spo14p sequences did reveal a highly conserved region of 21 amino acids

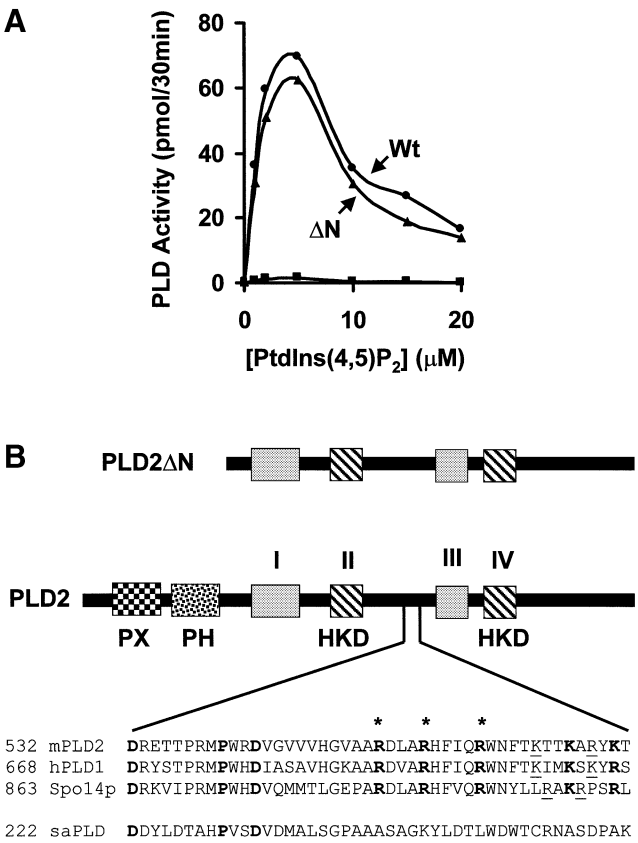

Fig. 3. Activation of PLD2 by PtdIns(4,5)P_2 does not require the N-terminal PH domain. (**A**) Sf9 cells were infected with baculovirus vectors for expression of wild-type PLD2, PLD2 lacking the N-terminal 308 amino acids (PLD2ΔN) and an irrelevant control protein. The cells were harvested and total membrane fractions prepared and adjusted to give equivalent maximal activities (~0.4 μg). PLD activity was determined using phospholipid substrate dispersions containing increasing concentrations of PtdIns(4,5)P_2. Incubations contained 5 μM GTPγS-activated ARF1 (Sung *et al.*, 1999). The data shown for control (■), wild-type PLD2 (●) and PLD2ΔN (▲) are means of triplicate determinations from a single representative experiment. (**B**) Structural representation of PLD2 and PLD2ΔN. Regions I, II, III and IV are conserved among all PLD isoenzymes. Regions II and IV contain the catalytic 'HKD' motif. An alignment of the regions in PLD1, PLD2 and Spo14p that contain the putative phosphoinositide-interacting motif and the corresponding region in *Streptomyces antibioticus* PLD (saPLD), which does not contain this motif, is shown. Conserved basic amino acid residues are indicated in bold, additional lysine and arginine residues are underlined, and asterisks indicate the amino acids targeted in this study.

containing many conserved basic and hydrophobic residues. This sequence (amino acids 554–575 in PLD2, 691–712 in PLD1 and 885–906 in Spo14p) is N-terminal to conserved region III (which contains the putative choline-interaction site; Morris *et al.*, 1996; Ponting and Kerr, 1996; Sung *et al.*, 1997). The region of sequence identified is absent from PLD enzymes that are not activated by PtdIns(4,5)P_2 such as bacterial PLD (Figure 3B).

We used PLD2 as a model enzyme to explore the role of this conserved region in the binding and activation by PtdIns(4,5)P_2 because PLD2 activity is independent of the protein activators described for PLD1 (Colley *et al*., 1997). Because arginine and lysine residues have been reported to be critical for phosphoinositide binding (reviewed in Martin, 1998), and the first three arginine residues of this

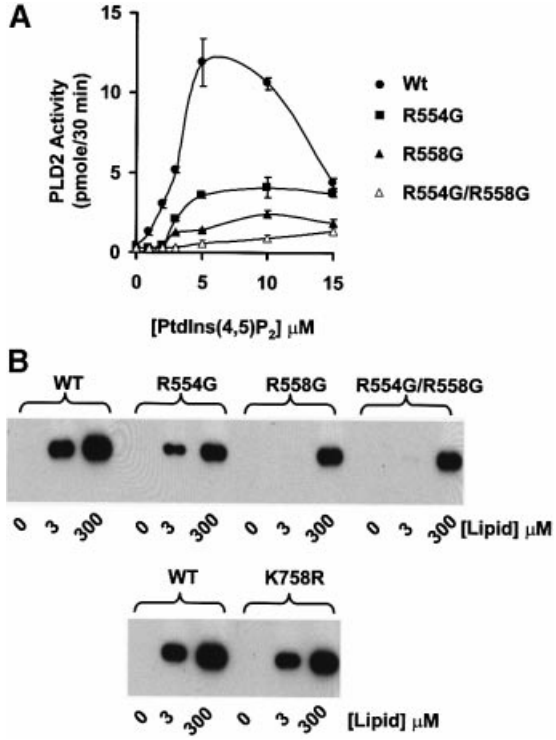

Fig. 4. Characterization of PLD2 mutants with reduced responsiveness to PtdIns(4,5)P_2. (**A**) Wild-type PLD2 and the R545G, R548G and R554G/R558G PLD2 mutants were expressed in Sf9 cells using baculovirus vectors and purified by immunoaffinity chromatography. PLD activity was determined using sonicated phospholipid dispersions containing increasing concentrations of PtdIns(4,5)P_2 as indicated. Incubations contained 5 ng of protein. The data shown are means ± SD of triplicate determinations from a single experiment. (**B**) The wild-type and mutant PLD proteins including a catalytically inactive PLD2 mutant (K758R) were incubated with sucrose-loaded liposomes containing 5 mol% PtdIns(4,5)P_2 at the indicated liposome concentrations. The liposomes were sedimented by centrifugation and the pellets analyzed for PLD2 proteins by SDS–PAGE and Western blotting.

newly identified motif in PLD1, PLD2 and Spo14p are conserved, we constructed site-directed mutants of PLD2 in which the first two conserved arginine residues of the sequence (R554 and 558) were substituted with glycine (G). These proteins were expressed in Sf9 cells and purified by immunoaffinity chromatography. The purity and stability of the mutants were verified by SDS–PAGE and Western blotting (data not shown). PLD activity was determined *in vitro* using substrate-containing vesicles with different amounts of PtdIns(4,5)P_2. As we have reported previously, purified wild-type PLD2 has readily detectable activity in the absence of PtdIns(4,5)P_2, but inclusion of PtdIns(4,5)P_2 produces a dramatic increase in activity of the enzyme (Colley *et al*., 1997; Figure 4A). When assayed in the absence of PtdIns(4,5)P_2, the specific activity of R554G, R558G and R554G/R558G PLD2 mutants was 84 ± 12, 83 ± 10 and 76 ± 6% of that of the wild-type enzyme (mean ± SD of triplicate determinations). However, activation of each of these PLD2 mutants by PtdIns(4,5)P_2 was significantly attenuated [at 5 mol% PtdIns(4,5)P_2, R554G, R558G and R554G/R558G exhibited activities that were 30, 12 and 5% of the wild type; Figure 4A]. We also note that these mutants appear less responsive than the wild-type PLD2 to activation by lower concentrations of PtdIns(4,5)P_2.

We next compared the binding of wild-type PLD2 and mutants to sucrose-loaded phospholipid vesicles containing 5% PtdIns(4,5)P_2 at a total lipid concentration of 3 or 300 μM. As shown previously (Figure 1), the binding of PLD2 to vesicles at low total lipid concentrations is dependent on the presence of PtdIns(4,5)P_2. In comparison with the wild-type enzyme, binding of the R554G mutant to vesicles at a total lipid concentration of 3 μM was significantly attenuated, and binding of the R558G and R554G/R558G mutants to this concentration of vesicles was not detected (Figure 4B). However, at the higher total lipid concentration (300 μM), the mutated PLD proteins were capable of binding to the lipid vesicles in a similar manner to the wild-type enzyme (Figure 4B) but, at this concentration, the binding was not dependent on PtdIns(4,5)P_2 (Figure 1C and D). Finally, the catalytically inactive mutant, PLD2 K758R (Sung *et al*., 1997), bound to lipid vesicles similarly to the wild-type enzyme (Figure 4B). This demonstrates that binding and catalysis are independent. These results also suggest that the altered binding patterns of the PLD2 mutants (Figure 4B) are not caused by their aberrant enzyme activities. Although wild-type PLD2 could be effectively labeled with BZDC-[^{3}H]triester-PtdIns(4,5)P_2 (Figure 2B), labeling of the R554G, R558G and R554G/R558G PLD2 mutants with this compound was reduced dramatically or not detected (data not shown). Collectively, these results suggest that the sequence motif identified is a site of interaction of the PLD enzymes with PtdIns(4,5)P_2.

PtdIns(4,5)P_2 binding in vitro is required for catalytic activity in vivo

The experiments presented above demonstrate that PLD enzymes are strongly activated by and require PtdIns(4,5)P_2 for efficient binding to lipid vesicles *in vitro*. We next explored whether the reduced responsiveness to PtdIns(4,5)P_2 of the PLD2 mutants *in vitro* indicated that they would display decreased activity *in vivo*. We compared the activity of wild-type PLD2, R554G and R554G/R558G PLD2 mutants in transfected COS-7 cells using endogenously labeled phospholipid substrates. In comparison with cells transfected with empty vector (pCGN) alone, cells expressing PLD2 exhibited a high basal activity that could be stimulated ~2-fold by phorbol ester treatment (Colley *et al*., 1997; Figure 5A). Cells expressing R554G or R554G/R558G PLD2 mutants exhibited basal and phorbol ester-stimulated levels of PLD activity that were comparable to that of vector-transfected cells (Figure 5A). Although catalytically competent *in vitro* (Figure 4A), hydrolysis of endogenously labeled phospholipid substrates by these mutated PLD2 proteins was ablated (Figure 5A). These results suggest that activation of PLD by PtdIns(4,5)P_2 is important *in vivo*.

PtdIns(4,5)P_2 binding in vitro is not critical for membrane association in vivo

We next investigated whether the ability to bind PtdIns(4,5)P_2 *in vitro* was necessary for membrane association *in vivo*. Wild-type PLD2 and the R554G and R554/R558G PLD2 mutants were expressed by transient transfection of COS-7 cells using pCGN vectors, which append an N-terminal hemagglutinin (HA)-epitope tag to the proteins. Wild-type PLD2 was almost exclusively mem-

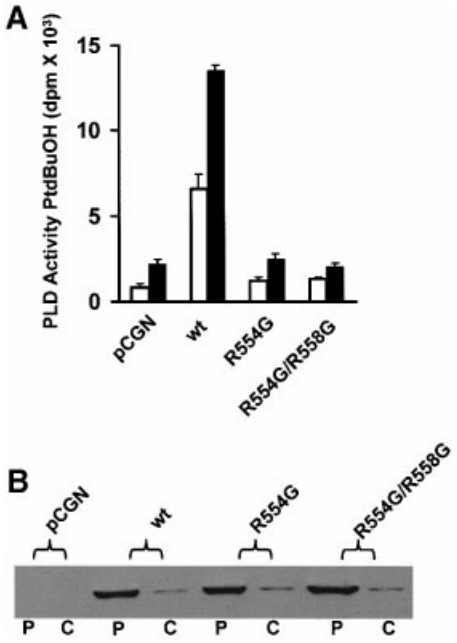

Fig. 5. Activity and subcellular localization of wild-type and mutant PLD2. (**A**) COS-7 cells were transfected with pCGN vector or with pCGN constructs harboring wild-type PLD2 (wt) or the R554G and R554G/R558G PLD2 mutants. The cells were labeled with [^{3}H]palmitate and PLD activity determined by measuring the formation of [^{3}H]phosphatidylbutanol (PtdBuOH). Incubations contained vehicle (white bars) or 100 nM PMA (black bars). The data shown are means ± SD of triplicate determinations. (**B**) Immunoblot of paticulate (P) and cytosolic (C) fractions prepared from COS-7 cells transfected with pCGN vector or with pCGN constructs harboring wild-type D2 (wt), and the R554G and R554G/R558G PLD2 mutants. Under the centrifugation conditions used for this experiment, cellular membranes are largely pelleted.

brane-associated in these cells (in the particulate fraction, Figure 5B). Distribution of the R554G and R554/R558G PLD2 mutants between the particulate and cytosolic fractions was not appreciably different from that of the wild-type protein (Figure 5B). In addition, the mutated PLD2 proteins were expressed at levels comparable to wild-type PLD2 protein, indicating that the mutated proteins are stable *in vivo*. The distribution of wild-type PLD2 and R554G/R558G PLD2 mutant in transfected COS-7 cells was also examined by indirect immunofluorescence using anti-HA primary antibody and rhodamine-conjugated secondary antibody (Figure 6A). As reported in fibroblasts (Colley *et al*., 1997), wild-type PLD2 localizes to submembranous vesicles near or at the plasma membrane (Figure 6A). We occasionally saw perinuclear staining, which may be the result of overexpression in COS-7 cells. Nevertheless, the localization of the R554G/R558G PLD2 mutant was similar to that of the wild-type enzyme (Figure 6A). Consistent with the biochemical fractionation (Figure 5B), these data suggest that the localization of PLD to cellular membranes does not require direct association with PtdIns(4,5)P_2.

Several groups have reported that both endogenous and transiently expressed PLD proteins are partially resistant to extraction with Triton X-100 in a number of cell types (Czarny *et al*., 1999; Hodgkin *et al*., 1999; Kim *et al*., 1999; Sciorra and Morris, 1999). Many cells contain cholesterol and sphingomyelin-enriched membrane domains or rafts, which have been implicated as sites of interactions between proteins involved in cell signaling (Simons and Ikonen, 1997). These membrane domains have been reported to contain high concentrations of PtdIns(4,5)P_2 (Hope and Pike, 1996). We have characterized these insoluble membrane domains in HEK 293 cells (Sciorra and Morris, 1999), and used this cell line to determine whether PtdIns(4,5)P_2 binding *in vitro* was required for localization to these specialized membrane domains. Cells expressing Gbx2, PLD2 and R554G/R558G PLD2 mutant were extracted with Triton X-100 and the extracts fractionated by sucrose-density gradient centrifugation (Brown and Rose, 1992). The fractions obtained were analyzed by Western blotting using anti-HA primary antibody. PLD2 was found in both the Triton X-100 soluble (Heavy) and insoluble (DRM) fractions (Figure 6B). As expected, Gbx2, a homeodomain-encoding protein that localizes to the nucleus (Sung *et al*., 1999a; Figure 6A), is present solely in the Triton X-100-soluble fractions. The inability of the R554G/R558G PLD2 mutant to bind PtdIns(4,5)P_2 *in vitro* (Figure 4B) did not interfere with its ability to associate with these specialized insoluble domains (Figure 6B). Collectively, these data suggest that direct interaction with PtdIns(4,5)P_2 is not required for membrane association of PLD2 in cells, or for localization of the protein to Triton X-100-insoluble membrane domains, but that phosphoinositides are required for catalytic activity of the enzymes in intact cells.

Requirement of PtdIns(4,5)P_2 activation for the cellular function of Spo14p

The *Saccharomyces cerevisiae SPO14* gene encodes a PtdCho-specific PLD (Rose *et al*., 1995) whose catalytic activity is essential for both mediating the synthesis of the prospore membrane during meiosis (Rudge *et al*., 1998a), a prerequisite event for spore formation, and Sec14p-independent Golgi secretion (Sreenivas *et al*., 1998; Z.Xie *et al*., 1998). Similarly to mammalian PLD1 and PLD2, the catalytic activity of Spo14p *in vitro* can be elevated by the inclusion of PtdIns(4,5)P_2 in the substrate vesicles (Rose *et al*., 1995; Waksman *et al*., 1996; Figure 7A). However, the requirement of PtdIns(4,5)P_2 activation for the cellular function of Spo14p remains unknown. We therefore investigated the role of PtdIns(4,5)P_2 activation of Spo14p in these events (sporulation and Sec14p-independent Golgi secretion) by substituting one of the two arginine residues (R885 and 889), corresponding to the analogous PtdIns(4,5)P_2 binding domain of Spo14p, with glycine (G).

In contrast to mammalian PLD2, mutation of the first conserved arginine residue in the PtdIns(4,5)P_2 binding motif of Spo14p [spo14(R885G)p] resulted in only slightly reduced PtdIns(4,5)P_2-stimulated activity compared with the wild type (data not shown). Not surprisingly, this mutant [spo14(R885G)p] retained the capacity of the wild-type protein to allow Sec14p-independent Golgi secretion and mediate the formation of the prospore membrane during sporulation (data not shown). Consistent with our findings made with the mammalian PLD enzymes, mutation of the second arginine residue in this motif of Spo14p [spo14(R889G)p] exhibited a dramatically attenuated response to PtdIns(4,5)P_2 compared with Spo14p (9% of the wild type; Figure 7A). This mutant [spo14(R889G)p] failed to allow homozygous *spo14* dip-

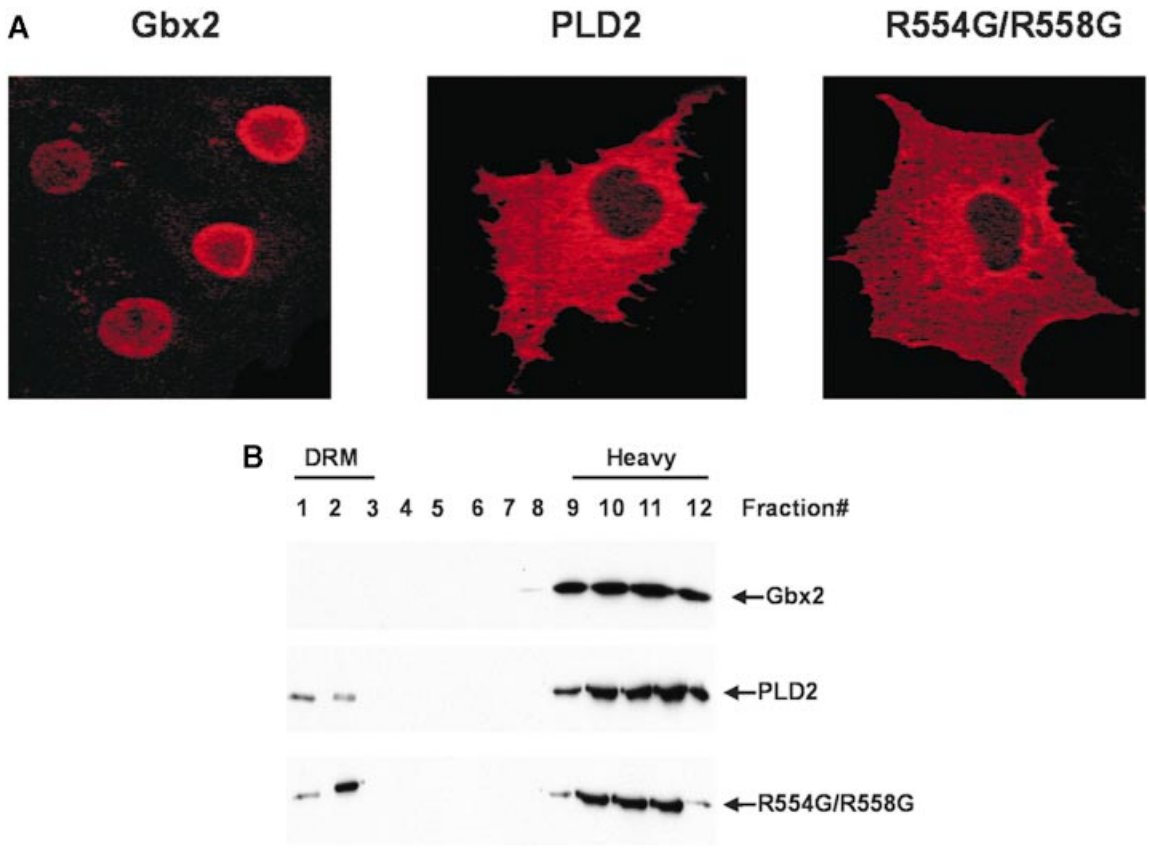

Fig. 6. Subcellular localization of PLD2 does not require PtdIns(4,5)P_2 binding *in vitro*. (**A**) COS-7 cells were grown on coverslips and transiently transfected with pCGN-Gbx2, pCGN-PLD2 or pCGN-PLD2R554G/R558G, each appending an HA-epitope tag at their N-termini. The distribution of the expressed proteins was examined by indirect immunofluorescence staining using anti-HA primary antibody and rhodamine-conjugated goat anti-mouse IgG secondary antibody. The fluorescence staining pattern was analyzed by confocal microscopy. (**B**) HEK 293 cells expressing Gbx2, PLD2 or R554G/R558G PLD2 mutant were lysed in 1% Triton X-100 and fractionated on a sucrose-density gradient (6 × 10-mm dishes per gradient) as described in Materials and methods. Aliquots (1 ml) were collected with fraction 1 representing the top of the gradient, and fraction 12 the bottom. Protein patterns in 1/30 of each fraction were analyzed for the protein indicated by Western blotting using anti-HA antibody.

loids to sporulate (Figure 7A) or *spo14 sec14*-bypass mutants to grow in the absence of functional Sec14p (no growth at 37°C; Figure 7C).

Because spo14(R885G)p gave only a modest reduction in PtdIns(4,5)P_2-responsive activation, and was functional, we determined whether the third conserved arginine (R894) in this domain was required for PtdIns(4,5)P_2 activation and *SPO14* function (corresponding to PLD2 R563). Although spo14(R889G)p and spo14(R894G) retained similar basal activity (Figure 7A), only spo14(R894G) was able to partially rescue the sporulation defect of homozygous *spo14* diploids (Figure 7A) and the growth defect of *spo14 sec14*-bypass mutants at 37°C (Figure 7C). These data are consistent with the result that spo14(R894G)p (25% of the wild type; Figure7A) was more responsive to PtdIns(4,5)P_2 than spo14(R889G)p (9% of the wild type; Figure 7A). In addition, the reduced responsiveness of spo14(R889G)p and spo14(R894G)p to PtdIns(4,5)P_2 was not a result of protein instability, because the mutated proteins were expressed at levels comparable to that observed for the wild type (Figure 7C). These results demonstrate that activation of Spo14p by PtdIns(4,5)P_2 is necessary for *SPO14* function.

Green-fluorescent-protein-tagged Spo14p (GFP–Spo14p) is fully functional, allowing both *spo14* homozygous diploids to sporulate as efficiently as wild-type cells (Rudge *et al.*, 1998a) and *spo14 sec14*-bypass strains to suppress *sec14*^{ts} defects (data not shown). Furthermore, localization of GFP–Spo14p can readily be monitored within living cells. Since both the catalytic activity and the correct subcellular localization are essential for Spo14p function (Rudge *et al*., 1998a; Z.Xie *et al*., 1998), we investigated the subcellular localization of GFP-Spo14(R889G)p. Examination of GFP-Spo14(R889G)p in wild-type cells (cells that have a functional PtdCho-PLD) revealed that the mutant protein localized to the developing prospore membrane (Figure 7D). These data suggest that direct binding to PtdIns(4,5)P_2 is not a primary determinant for Spo14p localization during meiosis. In support of this idea, a deletion of 150 amino acids in the extreme N-terminus of Spo14p retains PtdIns(4,5)P_2-responsive PLD catalytic activity *in vitro*, but fails to localize properly (Rudge *et al*., 1998a). Collectively, these data suggest an absolute requirement of phosphoinositide activation for PLD function.

Discussion

We have identified a conserved region in mammalian and yeast PLDs that is required for both PtdIns(4,5)P_2 binding and stimulation of catalysis. This region is not located within the N-terminal PH domain found in these phosphoinositide-activated PLD enzymes. PH domains mediate interactions of many proteins with inositol lipids and

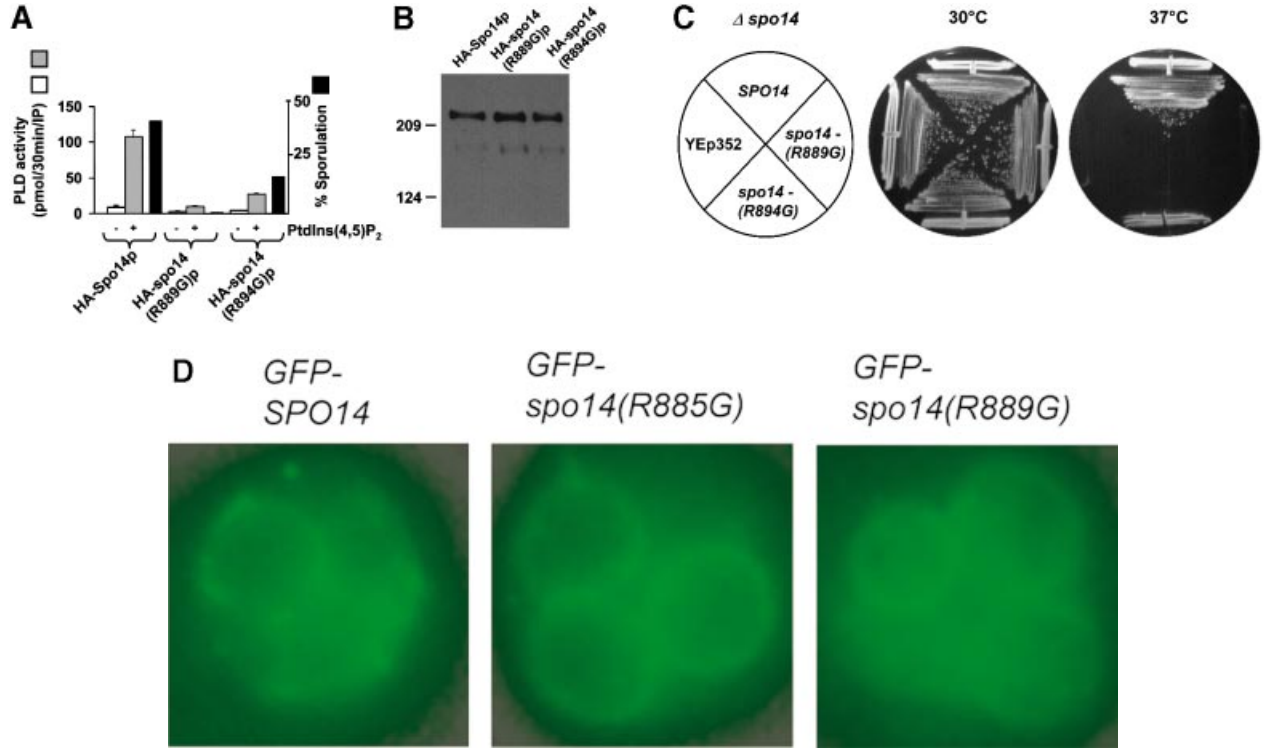

Fig. 7. Requirement of PtdIns(4,5)P_2 activation for the cellular function of Spo14p. (**A**) Sporulation (black bars) and immunoprecipitated PLD activity of the indicated proteins were determined as described (Rudge *et al*., 1998a,b). PLD activity was measured using 50 μM BODIPY-PtdCho in the presence (gray bars) or absence (white bars) of 5 μM PtdIns(4,5)P_2. (**B**) Immunoblot analysis of immunoprecipitated HA-Spo14p, HA-spo14(R889G)p and HA-spo14(R894G)p. The number on the left of the immunoblot indicates the position of the molecular mass standard in kDa. (**C**) Complementation analysis of *spo14* deletion, *sec14*-bypass mutant phenotype (no growth at 37°C) with spo14(R889G) and spo14(R894G). *spo14* deletion mutants harboring wild type (*SPO14*) and empty vector (YEp352) represent *sec14*-bypass-competent (growth at 37°C) and -incompetent (no growth at 37°C) cells. (**D**) Live cells expressing GFP–Spo14p, GFP–Spo14(R885G)p and Spo14(R889G)p were visualized during meiosis.

phosphates. PH domains that interact with phosphoinositol headgroups containing different positional substituents with a selectivity for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 which is very similar to that of the PLD enzymes have been described (Martin, 1998). Accordingly, it is surprising that the PH domain in PLD plays no significant role in activation of the enzymes by phosphoinositides. It is also unclear why PLD2 mutants lacking both the phox consensus sequence (PX) and PH domains require ADP-ribosylation factor (Arf) for PtdIns(4,5)P_2-stimulated activity (Sung *et al.*, 1999a,b). Interestingly, it has been shown that specific deletions of the N-terminal region of rat PLD1 (Park *et al.*, 1998) and Spo14p (Rudge *et al*., 1998a) are cytosolic. However, the precise function of the PH domain in PLD is not currently known.

An increasing number of non-PH-domain-containing proteins that interact with phosphoinositides with high affinity and selectivity have been identified. In general, these interactions involve regions of basic amino acids, often interspersed with aliphatic amino acids (Lu and Chen, 1997; Chaudhary *et al.*, 1998; Martin, 1998). Inspection of the PLD1, PLD2 and Spo14p sequences, coupled with results from our published and ongoing mutagenesis studies with these enzymes, focused attention on a highly conserved region adjacent to but not overlapping with region III of the conserved sequence common to all PLD enzymes. This sequence contains three conserved arginine residues, and our results obtained with PLD2 demonstrate that the first two of these play an essential role in activation of the enzyme by PtdIns(4,5)P_2. In the absence of any further structural information it is tempting to speculate that these residues form electrostatic interactions with the phosphate substituents of the inositol lipid headgroup.

One notable difference between the PLD2 and Spo14p mutants described in this study is that whereas mutation of the first conserved arginine residue in the phosphoinositide-binding motif of PLD2 produced a dramatic decrease in activation by PtdIns(4,5)P_2, mutation of the cognate residue in Spo14p produced only a very modest decrease in PtdIns(4,5)P_2-stimulated PLD activity and, unlike mutation of the second conserved arginine residue, did not ablate Spo14p function *in vivo*. Although the corresponding mutant was not examined in mammalian PLD2, mutation of the next arginine residue to glycine [Spo14(R894)p] significantly attenuated Spo14p function *in vivo*, and responsiveness to PtdIns(4,5)P_2 was decreased. Because mammalian PLD enzymes can also be activated by PtdIns(3,4,5)P_3 (Liscovitch *et al.*, 1994; Hammond *et al*., 1997), there may be subtle differences between the phosphoinositide-interacting sites in the yeast and mammalian PLD enzymes. Unlike mammalian PLD enzymes, part of the PtdIns(4,5)P_2-binding motif in Spo14p contains the consensus, (R/K)X_n(R/K)X(R/K)(R/K), which has been shown to be critical for phosphoinositide binding in other proteins (reviewed in Martin, 1998). In addition, sequences of basic amino acid residues that interact with phosphoinositides also contain aromatic amino acids (Lu and Chen, 1997; Martin, 1998), which have been demonstrated to play an essential role in defining the selectivity

of the interaction with phosphoinositides (Lu and Chen, 1997). The PLD phosphoinositide-binding motif also contains conserved tyrosine, phenylalanine and tryptophan that may serve this function.

Although PLD enzymes from peanut and cabbage are not activated by PtdIns(4,5)P_2 (Brown *et al*., 1995), stimulatory effects of this lipid on certain recombinant *Ricinus* PLD isoenzymes have been reported (Pappan *et al*., 1997; Qin *et al*., 1997). Unlike the mammalian and yeast PLDs, activity of the plant enzymes is strongly dependent on Ca^{2+} and is stimulated by PtdIns(4)P (Qin *et al*., 1997). The plant PLD enzymes do not contain the phosphoinositide-binding motif that we have identified, although a different sequence of basic residues, also close to the catalytic domain of the protein, was postulated to be involved in their activation by these lipids (Qin *et al*., 1997). An alternative possibility is suggested by the finding that the plant PLD enzymes contain an N-terminal C2 domain, which presumably mediates Ca^{2+}-dependent activation and, by analogy with studies using protein kinase C (PKC) isoenzymes (Newton and Johnson, 1998), may be capable of binding phosphoinositides and may thereby be responsible for interaction of the plant PLD enzymes with these lipids.

Our results suggest that the dramatic stimulation of PLD activity by phosphoinositides observed *in vitro* results from a direct interaction between the proteins and the phosphoinositol headgroups of the lipids. One mechanism involved in this *in vitro* activation may be the tethering of the enzymes to a substrate-containing phospholipid surface, increasing their catalytic efficiency by allowing them to function in a processive mode of catalysis. Alternatively, PtdIns(4,5)P_2 binding may stabilize a conformational change that brings both HKD motifs of PLD into close proximity (X.Xie *et al.*, 1998). Although we found that activation by phosphoinositides was required for expression of PLD activity *in vivo*, at least in the case of PLD2, simple recruitment to the membrane surface can not be the mechanism involved because both the wild type and phosphoinositide-unresponsive PLD2 mutants were found to be membrane localized. On the other hand, interactions with these lipids may play a more subtle role in lateral organization of the enzymes at the membrane surface or their ability to co-localize or interact with signaling proteins such as tyrosine kinase receptors (Slaaby *et al.*, 1998).

While the phosphoinositide-binding motif is required for PLD activation and function *in vivo*, it is not the sole determinant for proper localization within the cell. Targeting of the PLD enzymes to their appropriate intracellular sites has been postulated to be mediated by protein–protein interactions (Park *et al*., 1998; Rudge *et al*., 1998a; Slaaby *et al.*, 1998). PLD2 localizes to detergent-resistant membrane rafts (Czarny *et al.*, 1999; Sciorra and Morris, 1999), specialized membrane domains that are enriched in phosphoinositides (Hope and Pike, 1996). We found that localization of PLD2 to these domains is not mediated by interaction with PtdIns(4,5)P_2. Czarny *et al*. (1999) suggested that PLD2 is anchored to these domains in a PtdIns(4,5)P_2-independent manner, possibly involving interactions with a resident protein, caveolin-1.

Stimulation of phosphoinositide synthesis or stimulus-dependent re-localization of PLD enzymes to regions of the cell with high levels of these lipids (e.g. caveolae) may constitute a direct mechanism for regulation of PLD activity. Although this possibility has not yet been examined in detail for the mammalian PLD enzymes, Spo14p has been shown to translocate from a detergent-insoluble to a detergent-soluble cell fraction during sporulation (Rudge *et al*., 1998a,b). Translocation of proteins to their lipid substrate and/or activator is a common theme for the activation of proteins involved in signal transduction. However, we found that interaction of Spo14p with PtdIns(4,5)P_2 is not directly involved in this translocation. In this regard, it is notable that although the localization of PLD2 to caveolae has been speculated to result in enzyme activation (Czarny *et al.*, 1999), it is equally possible that sequestration to this fraction maintains the enzyme in an inactive state through inaccessibility to substrates and regulators.

Although the precise function of PLD in mammalian cell regulation is not known, essential roles for yeast PLD in sporulation and vesicular transport have been identified (Rose *et al*., 1995; Sreenivas *et al*., 1998; Z.Xie *et al*., 1998). *myo*-inositol auxotrophic yeast strains require inositol to sporulate (Schroeder and Breitenbach, 1981), but the dependence of this process on phosphoinositides has not been examined. Phosphoinositide levels are perturbed in one occurrence of *sec14*-bypass (Guo *et al*., 1999; Stock *et al*., 1999). Certain mutant alleles (e.g. *sac1-22*) of *SAC1*, a gene that encodes a polyphosphoinositide phosphatase (Guo *et al*., 1999), allow yeast to bypass their essential requirement for Sec14p (Cleves *et al*., 1989). *sec14*-bypass is also dependent on Spo14p (Sreenivas *et al*., 1998; Z.Xie *et al*., 1998), and during *sac1-22*-mediated *sec14*-bypass the levels of PtdIns(4)P are significantly elevated, with modest elevations of PtdIns(3)P and PtdIns(3,5)P_2 (Guo *et al*., 1999; Stock *et al*., 1999). While PtdIns(4)P and PtdIns(3)P do not significantly activate Spo14p *in vitro* (V.A.Sciorra, S.A.Rudge, A.J.Morris and J.Engebrecht, unpublished observations), effects of PtdIns(3,5)P_2 on Spo14p activity have not yet been evaluated. We show that responsiveness to phosphoinositides is required for Spo14p function and, in turn, this finding implies that these lipids have important regulatory roles in meiosis and Sec14p-independent Golgi function.

Dissecting the role of phosphoinositides in PLD regulation in mammalian systems has been complicated, because in addition to activating PLD directly these lipids play critical roles in controlling the activation state of members of the ARF and Rho families of GTP-binding proteins that are upstream of PLD and have significant effects on protein kinase C (PKC) activity (reviewed in Sciorra *et al.*, 1999). Further analysis of the PLD mutants described herein will be of particular value for dissecting the direct and indirect roles of phosphoinositides in PLD regulation. In this respect, we note that mutation of the phosphoinositide-binding site in PLD1 inactivates PLD activity against cellular lipid substrates (V.A.Sciorra, S.Wu, M.A.Frohman and A.J.Morris, unpublished observations). The precise regulatory properties of these PLD1 mutants (notably their sensitivity to activation by ARF, Rho and PKC) and their subcellular localization are currently under investigation.

In conclusion, we identified a region of PLD that is

Table I. Yeast strains

Strain	Genotype	Source
Y594	*HO MAT***a** *leu2 thr1-4 trp1-1 ura3 arg4-8 ade2-1 spo14::URA3*	
	HO MATα leu2 thr1-4trp1-1 ura3 arg4-8 ade2-1 spo14::URA3	Rudge *et al*. (1998a)
Y1684	Y594, plus pME1096 (*GFP–SPO14 LEU2* 2μ)	this study
Y1685	Y594, plus pME1360 [*GFP–spo14(R885G) LEU2* 2μ]	this study
Y1686	Y594, plus pME1361 [*GFP–spo14(R889G) LEU2* 2μ]	this study
Y1687	Y594, plus pME962 (*SPO14 LEU2* 2μ)	Rudge *et al*. (1998a)
Y1799	*MAT***a** *leu2 :: hisG his4-x ho::LYS2 ura3 ARG4 SPO14*	
	MATα leu2-k His4 ho::LYS2 ura3 arg4-Nsp spo14::URA3	
	plus pME1360 [*GFP-spo14(R885G) LEU2* 2μ]	this study
Y1800	*MAT***a** *leu2 :: hisG his4-x ho::LYS2 ura3 ARG4 SPO14*	
	MATα leu2-k His4 ho::LYS2 ura3 arg4-Nsp spo14::URA3	
	plus pME1361 [*GFP-spo14(R889G) LEU2* 2μ]	this study
KR52-3C	*HO MAT***a** *ura3-1 leu2 arg4-8 thr1-4 trp1-1 ade2 spo14::URA3*	
	HO MATα ura3-1 leu2 arg4-8 thr1-4 trp1-1 ade2 spo14::URA3	Rose *et al*. (1995)
Y602	KR52-3C, plus pME957 (*HA-SPO14 LEU2* 2μ)	Rudge *et al*. (1998a)
Y1727	Y594, plus pME1441 [*HA-spo14(R889G) LEU2* 2μ]	this study
CTY1130	*MAT***a** *ura3-52, Δhis3-200, lys2-801, sec14-3^{ts}, kes1-1, spo14::HIS3*	V.Bankaitis
Y1530	CTY1130, plus YEp352 (*URA3* 2μ)	this study
Y1531	CTY1130, plus pME1095 (*GFP–SPO14 URA3* 2μ)	this study
Y1690	CTY1130, plus pME1372 [*GFP–spo14(R885G) URA3* 2μ]	this study
Y1691	CTY1130, plus pME1373 [*GFP–spo14(R889G) URA3* 2μ]	this study
Y522	*HO MAT***a** *ura-1 leu2 arg4-8 thr1-4 trp1-1 ade2 spo14::LEU2*	
	HO MATα ura3-1 leu2 arg4-8 thr1-4 trp1-1 ade2 spo14::LEU2	this study
Y1977	Y522, plus pME1576 [*HA-spo14(R894G)URA3* 2μ]	this study
Y2041	CTY1130, plus pME986 (*HA-SPO14 URA3* 2μ)	this study
Y2048	CTY1130, plus pME1569 [*HA-spo14(R889G) URA3* 2μ]	this study
Y1981	CTY1130, plus pME1576 [*HA-spo14(R894G) URA3* 2μ]	this study

required for stimulation of enzymatic activity by phosphoinositides, and used mammalian and yeast PLD enzymes to probe the role of these lipids in regulation of PLD. Our results demonstrate that the interaction with phosphoinositides is required for expression of PLD activity and is obligatory for two PLD-dependent cellular functions.

Materials and methods

General reagents

Unless otherwise stated, all reagents were of analytical grade. PtdCho, phosphatidylserine (PtdSer), PtdIns and PtdOH were obtained from Avanti Polar Lipids, Alabaster, AL. PtdIns(4)P and PtdIns(4,5)P_2 were purified from bovine brain lipid extract by anion-exchange HPLC and converted to their ammonium salts as described previously (Morris *et al*., 1995). 2-decanoyl-1-(*O*-[11-{4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl}amino]undecyl)-sn-glycero-3-phosphocholine (BODIPY-PtdCho) was from Molecular Probes, Inc. (Eugene, OR). L-dipalmitoyl phosphatidylcholine ([^{3}H]methyl choline), ([^{3}H]PtdCho) and [^{3}H]palmitic acid were obtained from American Radiolabeled Chemicals. Rabbit polyclonal anti-PLD2 antibodies were from Quality Control Biochemicals as described previously (Colley *et al*., 1997). 12CA5 antibody (mouse monoclonal anti-HA tag antibody) was from Boehringer Mannheim. Rhodamine-conjugated anti-mouse IgG was from Jackson ImmunoResearch Labs, Inc. Rabbit polyclonal anti-GFP antibody was from Clontech.

Construction of expression plasmids

PLD2 cDNA was subcloned into the *Eco*RI and *Not*I sites of the pFASTBAC vector (Invitrogen). Mutants were generated via site-directed mutagenesis of expression plasmids (pFASTBAC-PLD2, pCGN-PLD2 and GFP–Spo14), carried out using the Quik-change kit (Stratagene). Plasmids were sequenced to confirm the intended mutation using Sequenase (US Biochemicals).

Expression and purification of wild-type and mutant PLD enzymes

Recombinant bacmids were prepared by transfection of DH10Bac cells, and expression was monitored by Western blotting using anti-PLD2 antibodies. Recombinant baculoviruses were amplified and propagated using standard procedures. Monolayer cultures of exponentially growing Sf9 cells were infected with baculoviruses at a multiplicity of 10 and cultured for 48 h at 27°C. The PLD proteins were purified by immunoaffinity chromatography as described previously (Colley *et al*., 1997; Hammond *et al*., 1997). In some cases, the proteins were eluted from the immunoaffinity resin with glycine buffer omitting the octylglucoside. The purified proteins were generally stored at 4°C and used within 48 h of isolation.

Cell culture and transfections

COS-7 and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin and 0.22% $NaHCO_3$. For transfections, the cells were grown in 35-mm dishes (2×10^5 cells/dish) and then switched into Opti-MEM I media (Gibco-BRL). The cells were transfected with 1 μg of DNA/dish using lipofectamine (Gibco-BRL) as described previously (Sung *et al*., 1997). For *in vivo* PLD assays, 24 h post-transfection the medium was replaced with complete DMEM containing 1 μCi of [^{3}H]palmitic acid and the cells incubated for a further 24 h.

Yeast strains and plasmid constructs

Yeast strains used in this study are shown in Table I. The construction of GFP–Spo14p and HA-Spo14p have been reported previously and have been shown to result in fully functional proteins (Rudge *et al*., 1998a). Plasmids ME1360 and ME1361 contain the arginine to glycine change at amino acid residues 885 and 889 in GFP–Spo14p, respectively, and were generated by site-directed mutagenesis of pME1096 (*GFP–SPO14 LEU2* 2μ; Rudge *et al*., 1998a). GFP–spo14(R885G) and GFP–spo14(R889G) were subcloned into *Xba*I and *Asp*718I sites of the 2μ plasmid YEp352 (Hill *et al*., 1986) on a 6 kb *Xba*I–*Asp*718I fragment, generating plasmids ME1372 [*GFP–spo14(R885G) URA3* 2μ] and ME1373 [*GFP–spo14(R889G) URA3* 2μ].

Three copies of the HA epitope were exchanged with the GFP sequence and inserted into the *Sph*I site of plasmid ME1361 by the introduction of a *Sph*I fragment from pME1113 (*HA-SPO14 URA3* 2μ; Rudge *et al*., 1998a), generating plasmid ME1441 [*HA-spo14(R889G) LEU2* 2μ]. HA-spo14(R889G) was subcloned into *Xba*I and *Asp*718I sites of YEp352 on a 6 kb *Xba*I–*Asp*718I fragment, generating plasmid ME1569 [*HA-spo14(R889G) URA3* 2μ]. Plasmid pME1576 [*HA-spo14(R894G) URA3* 2μ] contains the arginine to glycine change at amino acid residue 894, and was generated by site-directed mutagenesis of plasmid ME1113. Plasmids ME1360, 1361 and 1576 were all sequenced to confirm the site of mutagenesis.

Cell fractionation

Cells were harvested in 20 mM Tris pH 7.5, 1 mM EGTA, 0.1 mM benzamidine, 0.1 mM PMSF, and disrupted by brief sonication on ice. Nuclei and unbroken cells were removed by centrifugation at 500 *g* for 10 min and the supernatant obtained from this step was separated into total membrane and cytosolic fractions by centrifugation at 35 000 *g* for 30 min. Samples from an equivalent number of cells were examined for PLD distribution by Western blotting. Sucrose-density gradient fractionation of Triton X-100 lysates was performed as described (Brown and Rose, 1992). Briefly, cells were lysed in 1% Triton X-100, and the cell lysate was adjusted to 40% sucrose. A sucrose gradient (38 and 5%) was layered over the adjusted lysate in an SW41 ultracentrifuge tube (Beckman). Gradients were centrifuged for 16–18 h at 28 000 r.p.m. at 4°C in a Beckman SW41 rotor. Fractions (1 ml) were collected from the top of the gradient and analyzed for PLD distribution by Western blotting. Purity of the Triton X-100-insoluble domains has been demonstrated elsewhere (Sciorra and Morris, 1999).

PLD assays

PLD activity was determined *in vitro* by measuring release of [^{3}H]choline from [^{3}H]methyl-choline-labeled dipalmitoyl PtdCho using previously published procedures (Hammond *et al.*, 1997). In brief, these assays used sonicated dispersions of phospholipids containing phosphatidylethanolamine (PtdEtn), PtdCho and bovine brain PtdIns(4,5)P_2. In some cases, the BZDZ-[^{3}H]acyl-PtdIns(4,5)P_2 or BZDZ-[^{3}H]triester-PtdIns(4,5)P_2 were substituted for the bovine brain PtdIns(4,5)P_2. GFP-Spo14p or HA-Spo14p (and the corresponding mutant proteins) were immunoprecipitated using anti-GFP antibody or anti-HA antibody, respectively, as described previously (Rudge *et al.*, 1998a). Measurements of immunoprecipitated yeast PLD activity used BODIPY-PtdCho as substrate and were assayed as described (Rudge *et al.*, 1998a,b).

Lipid binding assay

The procedure used is described in detail elsewhere (Buser and McLaughlin, 1998). In brief, sucrose-loaded phospholipid vesicles (PtdCho:PtdSer:PtdEtn, molar ratio 1:1:1) containing 5% of various acidic phospholipids were prepared. A trace amount of [^{14}C]PtdCho was included in the vesicles as a recovery marker. The vesicles were resuspended in buffer containing 100 mM KCl and 1 mM MOPS. PLD proteins were added to siliconized microfuge tubes containing these vesicles and incubated on ice for 30 min. The vesicles were then sedimented by centrifugation at 100 000 *g* for 1 h. Samples of the supernatant were removed for determination of PLD activity and analysis of PLD proteins by Western blotting. The pelleted vesicles were resuspended in 100 µl of MOPS buffer. Samples were analyzed for PLD proteins by Western blotting and for efficiency of sedimentation by liquid scintillation counting.

Photolabeling PLD enzymes

The synthesis and characterization of the benzophenone PLD probes have been reported previously (Chen *et al*., 1996; Gu and Prestwich, 1996), and their use in identification of phosphoinositide binding sites in proteins lacking PH domains has also been reported previously (Chaudhary *et al.*, 1998). The procedures used for photolabeling the purified PLD enzymes were adapted from those described (Chaudhary *et al.*, 1998). Purified PLD proteins (generally 1–5 µg) were incubated with 0.5 µCi of the BZDC-triester-PtdIns(4,5)P_2 probe (molar ratio of probe to protein of ~1:1) in 10 mM phosphate buffer pH 7.4 for 10 min on ice in a UV-transparent 96-well plate. Photolysis was achieved by placing the plate directly under a 360 nm UV source for 45 min and the reactions were quenched by addition of SDS–PAGE sample buffer directly to the wells. Proteins were separated by SDS–PAGE, gels were stained with Coomassie Blue and soaked in EN^{3}HANCE (NEN) for 1 h. After rehydration in glycerol, the gels were dried between cellophane sheets in warm air and analyzed by fluorography using Kodak BioMAX film and an intensifying screen for 14–20 days at –80°C.

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