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Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes

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Phospholipase D (PLD) generates lipid signals that coordinate membrane trafficking with cellular signaling. PLD activity in vitro and in vivo is dependent on phosphoinositides with a vicinal 4,5-phosphate pair. Yeast and mammalian PLDs contain an NH_2-terminal pleckstrin homology (PH) domain that has been speculated to specify both subcellular localization and regulation of PLD activity through interaction with phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2). We report that mutation of the PH domains of yeast and mammalian PLD enzymes generates catalytically active PI(4,5)P_2-regulated enzymes with impaired biological functions. Disruption of the PH domain of mammalian PLD2 results in relocalization of the protein from the PI(4,5)P_2-containing plasma membrane to endosomes. As a result of this mislocalization, mutations within the PH domain render the protein unresponsive to activation in vivo. Furthermore, the integrity of the PH domain is vital for yeast PLD function in both meiosis and secretion. Binding of PLD2 to model membranes is enhanced by acidic phospholipids. Studies with PLD2-derived peptides suggest that this binding involves a previously identified polybasic motif that mediates activation of the enzyme by PI(4,5)P_2. By comparison, the PLD2 PH domain binds PI(4,5)P_2 with lower affinity but sufficient selectivity to function in concert with the polybasic motif to target the protein to PI(4,5)P_2-rich membranes. Phosphoinositides therefore have a dual role in PLD regulation: membrane targeting mediated by the PH domain and stimulation of catalysis mediated by the polybasic motif.

Introduction

Phospholipase D (PLD)* hydrolyzes phosphatidylcholine (PC) to form phosphatidic acid. In mammalian cells, PLD activity is under tight control by cell surface receptors acting through pathways involving GTP-binding proteins and protein kinases. Recent studies identify multiple roles for PLD at the interface between cell signaling and membrane trafficking (Liscovitch et al., 2000). In budding yeast, genetic approaches have identified a conditional role for PLD (the product of the *SPO14* gene) in secretion and an obligatory role in sporulation, wherein PLD activity is required for formation of the prospore membrane (Rudge and Engebrecht, 1999).

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*Abbreviations used in this paper: LUV, large unilamellar phospholipid vesicle; PC, phosphatidylcholine; PH, pleckstrin homology; PI3P, phosphatidylinositol 3-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; PS, phosphatidylserine; PX, Phox.

Key words: phospholipases; signal transduction; phosphatidylinositol phosphates; GTP-binding proteins; membrane lipids

Targeting PLDs to different sites within the cell is vital for their functions. The mammalian PLD enzymes, PLD1 and PLD2, exhibit cell-specific differences in subcellular localization. PLD2 is generally found in the plasma membrane and, in some cases, endosomes (Colley et al., 1997; Honda et al., 1999), whereas PLD1 has been reported to localize to the Golgi apparatus, endosomes, lysosomes, and plasma membrane as well as more specialized vesicles such as histamine granules in mast cells and glut4-containing vesicles in adipocytes (Brown et al., 1998; Emoto et al., 2000; Freyberg et al., 2001). In budding yeast, Spo14p moves from a detergent-insoluble membrane compartment in vegetatively growing cells to the prospore membrane during meiosis (Rudge et al., 1998a).

The mechanisms responsible for targeting PLD enzymes to different cellular membranes are poorly understood. PLD enzymes contain domains with proven or potential roles in protein–protein and protein–lipid interactions that are likely involved in this process. All PLD enzymes share a common catalytic core composed of a duplicated "HKD" motif, which constitutes the essential enzymatic machinery flanked

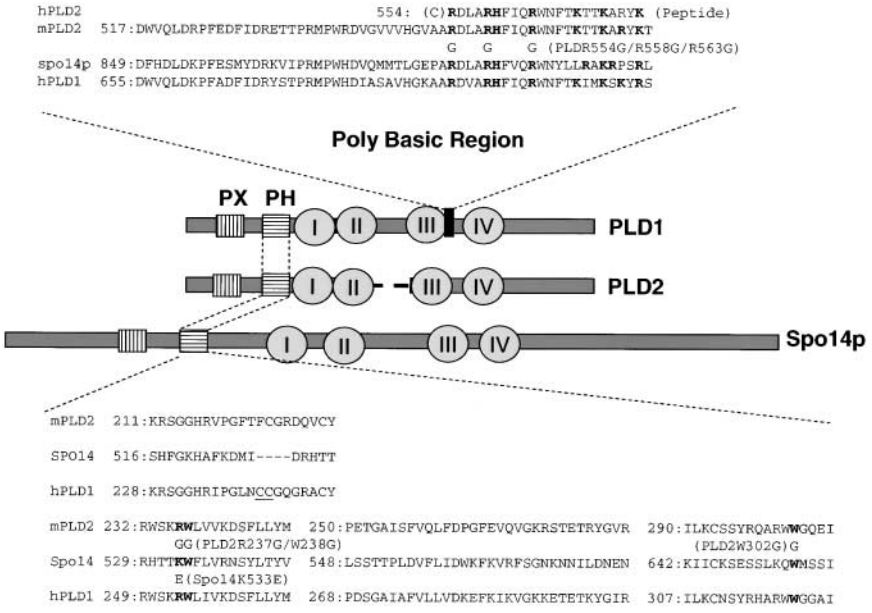

Figure 1. **Domain structure of yeast and mammalian PLD enzymes.** Linear representations of the primary sequences of PLD1, PLD2, and Spo14p are shown noting the four conserved catalytic domains (regions I through IV), the PX and PH domains, and the polybasic region. Sequence comparisons of these latter two domains are shown in detail highlighting conserved amino acid residues and sequences targeted for mutation.

by two additional PLD-specific domains (Hammond et al., 1995). Yeast and mammalian PLDs are potently and specifically activated by phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2) (Brown et al., 1993; Rose et al., 1995). This stimulation is mediated by a polybasic amino acid sequence within the catalytic core. Although mutation of this region does not affect the subcellular localization of PLD2 or Spo14p, mutant alleles of these enzymes with reduced responsiveness to PI(4,5)P_2 are nonfunctional in a number of settings (Sciorra et al., 1999, 2001; Vitale et al., 2001). The NH_2 terminus of the yeast and mammalian PLDs contains Phox and pleckstrin homology (PX and PH) domains. These domains are found in a wide variety of proteins and bind phosphoinositides with varying selectivities and affinities. Although clearly dispensable for stimulation of catalysis by PI(4,5)P_2, this NH_2-terminal region plays a role in both regulation of PLD activity and, in the case of PLD1, localization to the plasma membrane (Sugars et al., 1999; Sung et al., 1999a,b; Hodgkin et al., 2000; Sciorra et al., 2001).

Here we identify potential phosphoinositide-interacting amino acid residues within the PH domains of PLD2 and Spo14p. Mutation of these residues does not affect the intrinsic catalytic activity of either enzyme but renders them nonfunctional in a number of settings, which arises from changes in the distribution of the enzymes between different intracellular membrane compartments. In this study, we show that weak, but highly selective, binding of the PLD2 PH domain to PI(4,5)P_2 underlies the requirement of this domain for PLD localization and function and suggest that the PH domain works in combination with the polybasic motif to target these proteins to PI(4,5)P_2-rich membrane structures. This targeting is vital for PLD function in yeast and mammalian cells.

Results

Sequence comparisons of PLD PH domains

Fig. 1 shows the primary structure of the yeast and mammalian PLD enzymes highlighting the catalytic core (regions I, II, III, and IV), the NH_2-terminal PX and PH domains, and the polybasic motif that encompasses conserved residues that have been previously shown to be vital for binding to phosphoinositides and activation of the enzymes by these lipids.

Structural and biophysical studies reveal that PH domains vary widely in their affinities for phosphoinositides (for review see Lemmon and Ferguson, 2000). Although the overall three-dimensional structure of PH domains is highly conserved, there is strikingly little homology at the primary sequence level. Sequence comparisons of the PH domains of the PLD enzymes with regions of PH domains that bind PI(4,5)P_2, such as PLC-δ_1, spectrin, and Btk, determined in complex with their ligands, identified tandem basic amino acids within the variable "loop" connecting β-strands 3 and 4 that could participate in electrostatic interactions with the phosphate groups of phosphoinositides. In PLD2, these correspond to residues K236 and R237 and in PLD1 to residues K252 and K253. Spo14p has a single lysine (K533) in the cognate sequence at a position that corresponds to the second basic residue in the mammalian PLD sequence. We also identified a conserved tryptophan residue adjacent to R237, W238, that, again by comparison with structures of other phosphoinositide-binding PH domains, could participate in hydrogen bonding interactions with the inositol ring.

An intact PH domain is required for activation of PLD2

To evaluate the effect of PH domain disruption on PLD2 function and localization in cells, we generated a PLD2 mutant in which potential phosphoinositide-interacting residues R237 and W238 were substituted with glycine in a vector for expression of PLD2 with an NH_2-terminal GFP tag. GFP–PLD2 R237G/W238G and GFP-tagged variants of wild-type PLD2 and a catalytically inactive PLD2 mutant (PLD2 K578R) were expressed in HEK 293 cells by transient transfection. The recombinant proteins were immunoprecipitated using an anti-GFP antibody and probed by Western blotting. Each construct produced a protein of the expected size and expression levels were comparable for all

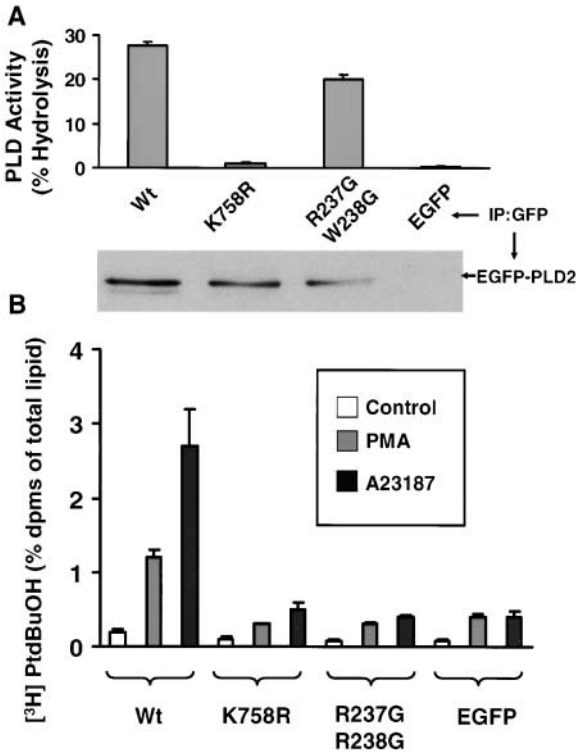

Figure 2. **An intact PH domain is required for activation of PLD in HEK 293 cells.** (A) PLD was immunoprecipitated from HEK 293 cells transiently transfected with pEGFP vector or with pEGFP constructs harboring wild-type PLD2, PLD2 K758R, or PLD2 R237G/W238G using anti-GFP antibody. Activity in the immunoprecipitates (IP) was determined and normalized to equivalent protein amounts determined from corresponding IP by Western blotting. (B) HEK 293 cells were transfected with pEGFP vector or with pEGFP constructs harboring wild-type PLD2, PLD2 K758R, or PLD2 R237G/W238G. The cells were labeled with [^{3}H]palmitic acid and PLD activity was determined by measuring the formation of [^{3}H]phosphatidylbutanol. Incubations contained vehicle (white bars), 100 nM PMA (gray bars), or 19 μM A23187 (black bars) for 30 min at 37°C. The data shown are means ± SD of triplicate determinations. Similar results were obtained in two additional experiments.

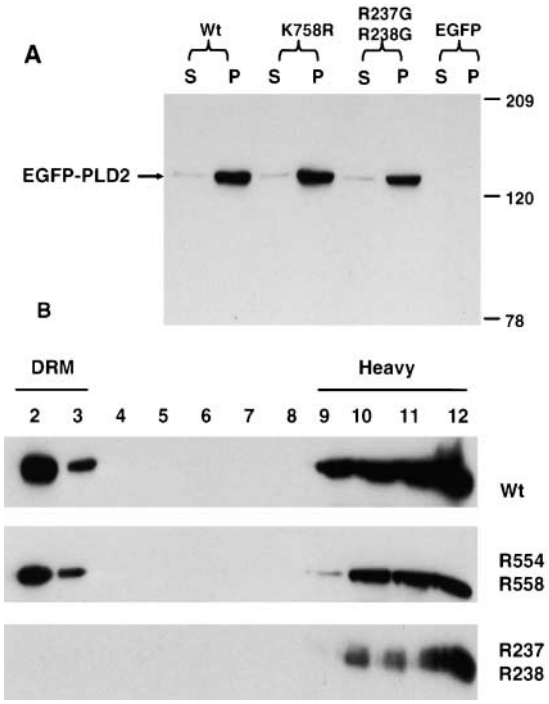

Figure 3. **Localization of PLD to detergent-resistant membrane microdomains requires an intact PH domain.** (A) Immunoblot of particulate (P) and supernatant (S) fractions prepared from HEK 293 cells transfected with pEGFP vector or with pEGFP constructs harboring wild-type PLD2, PLD2 K758R, or PLD2 R237G/W238G. (B) HEK 293 cells expressing HA-tagged wild-type PLD2, PLD2 R554G/R558G, or PLD2 R237G/W238G were lysed in 1% Triton X-100 and fractionated on a sucrose density gradient. 1-ml aliquots 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 indicated protein by Western blotting using anti-HA antibody.

three proteins. The PLD activity associated with these immune complexes was determined in vitro using sonicated phospholipid dispersions. Although PLD2K578R was inactive in these assays, the PH domain PLD2 mutant had an in vitro activity that was comparable to that of wild-type PLD2 (Fig. 2 A). Activity of both wild-type PLD2 and PLD2 R237G/W238G was strongly dependent on inclusion of $PI(4,5)P_2$ in these substrate preparations (unpublished data), underscoring our previous finding that the PH domain plays no role in the stimulation of catalysis by phosphoinositides (Sciorra et al., 1999). In contrast to these in vitro assay results, when PLD activity was measured in cells using a transphosphatidylation assay against radiolabeled cellular substrates, only wild-type PLD2 showed significant increases in activity in response to two known pharmacological activators, PMA and A23187 (Fig. 2 B). Activity of the PH domain mutant was indistinguishable from that of the catalytically inactive PLD mutant. Although the PH domain mutant is expressed at slightly lower levels than wild type (unpublished data; Fig. 3 A), it maintains in vitro activity similar to wild type, so modestly lower expression levels cannot account for these findings. As discussed below, we have expressed PLD2 R237G/W238G in insect cells using a baculovirus vector and found that the activity of this PLD2 mutant and its response to stimulation by $PI(4,5)P_2$ are identical to what we previously reported for wild-type PLD2. These findings show that the PH domain is dispensable for PLD activity against substrates presented in vitro but is critical for the expression of enzyme activity in a cellular setting, at least under conditions where the cells were treated with two pharmacological agents that increase PLD activity.

Localization of PLD2 to detergent-resistant membrane microdomains requires an intact PH domain

One explanation for the inactivity of PLD2 R237G/W238G in vivo is that the mutant enzyme may not be localized correctly to interact with appropriate activators and/or respond to external stimuli. We therefore first examined the distribution of wild-type and PH domain mutant PLD2 between soluble and total membrane fractions in transiently transfected HEK 293 cells. Both the PH domain mutant and the catalytically inactive mutant distributed almost exclusively to the membrane fraction, similar to the wild-type protein (Fig. 3 A). PLD2 partially localizes to detergent-resistant

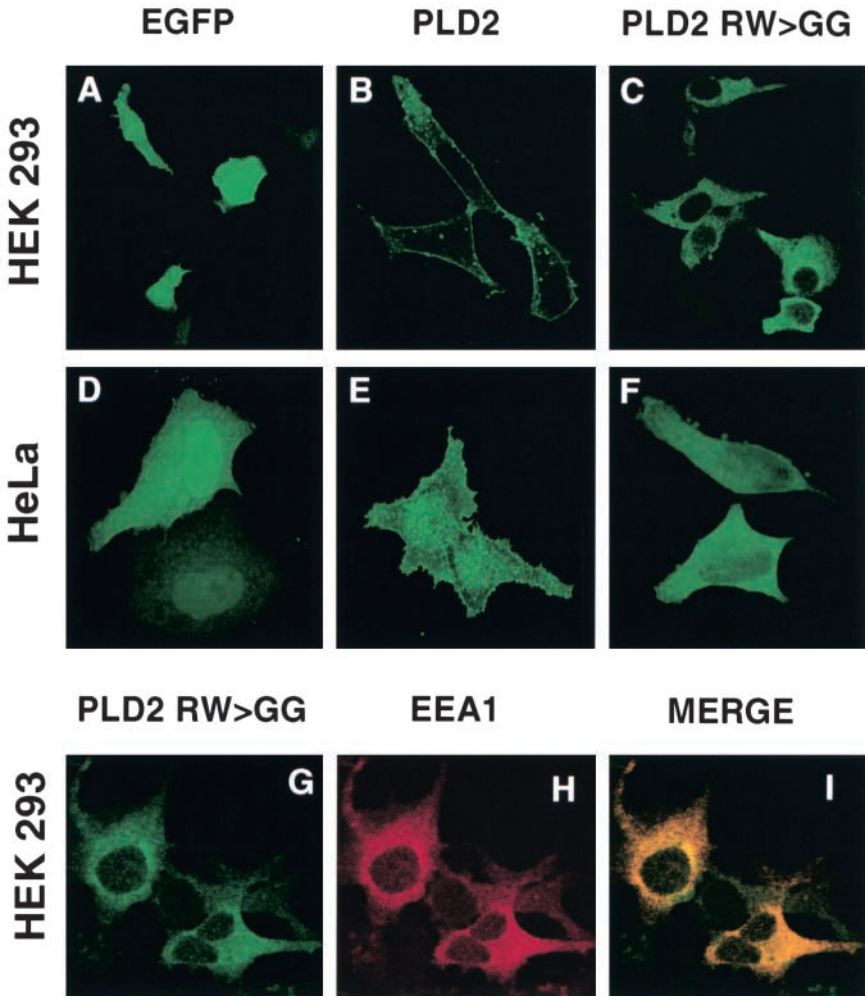

Figure 4. **Effect of PH domain mutations on subcellular localization of PLD2.** HEK 293 cells (A–C and G–I) or HeLa cells (D–F) were grown on coverslips and transiently transfected with pEGFP (A and D), pEGFP-PLD2 (B and E), or pEGFP-PLD2 R237G/W238G (C and F–I). The distribution of the expressed GFP-tagged proteins and markers was examined by laser scanning confocal microscopy. The series of images (G–I) are from cells that were stained with antibody against the early endosome marker EEA1 detected using a rhodamine-conjugated secondary antibody.

membrane microdomains, a specialized domain of the plasma membrane (Sciorra and Morris, 1999). Because these putative domains are speculated to localize many types of signaling molecules, it has been suggested that they provide a proper physiological environment for the activation of PLD2 in a cellular setting (Sciorra and Morris, 1999; Fiucci et al., 2000). We examined the localization of wild-type PLD2 and PLD2 R237G/W238G to detergent-resistant membrane domains by fractionating detergent lysates from HEK293 cells that had been transfected with constructs for expression of these proteins. Wild-type PLD2 was recovered in both detergent-soluble and detergent-resistant fractions, whereas PLD2 R237G/W238G was only detected in the detergent-soluble fraction (Fig. 3 B). These results suggest that a functional PH domain is required for the localization of PLD2 to detergent-resistant membrane microdomains, and that localization to these domains may be critical for PLD2 activation in cells under certain settings.

The PH domain determines localization of PLD2 to the plasma membrane

We used laser scanning confocal microscopy to examine the subcellular localization of wild type and the PH domain mutant PLD2 R237G/W238G in HEK293 and HeLa cells. Whereas GFP alone shows a typically cytosolic and partially nuclear distribution in both cells, in HEK293 cells, wild-type GFP–PLD2 was almost exclusively plasma membrane localized with some punctate staining throughout the cytoplasm (Fig. 4, A, B, and D). The localization of wild-type GFP–PLD2 (Fig. 4 E) or of a catalytically inactive PLD2 mutant in HeLa cells (unpublished data) was overtly similar to that in HEK293 cells, except that in HeLa cells, the protein was more evenly distributed between the plasma membrane and small punctate structures within the cytoplasm. By contrast, in both HEK293 cells and HeLa cells, the PLD2 PH domain mutant PLD2 R237G/W238G was not localized to the plasma membrane and was exclusively found within small punctate structures within the cytoplasm (Fig. 4, C and F). These punctate structures most likely are early endosomes, because we see considerable colocalization of the PH domain mutant with an early endosome marker, EEA1, in HEK293 cells (Fig. 4, G–I). The failure of GFP–PLD2 R237G/W238G to localize to detergent-resistant microdomains is likely due to this redistribution to an intracellular membrane compartment. Integrity of the PH domain of PLD2 is therefore required for localization to the plasma membrane, where the enzyme is responsive to stimulation.

The PH domain of Spo14p is dispensable for stimulation of PLD activity by PI(4,5)P_2 but is required for cellular function in *Saccharomyces cerevisiae*

Budding yeast contain a single PLD enzyme encoded by the *SPO14* gene (Rose et al., 1995). Proper localization and acti-

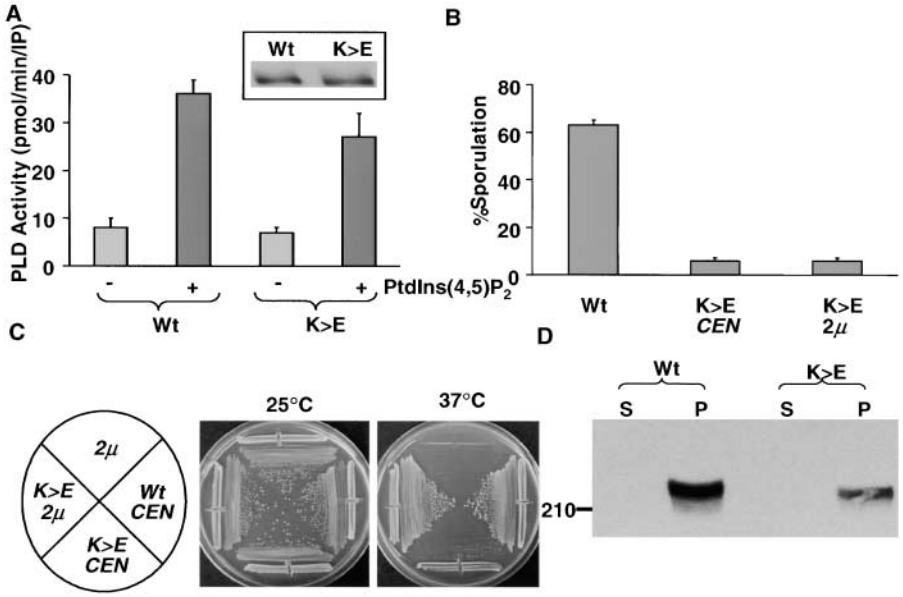

Figure 5. **Analysis of PH domain function in Spo14p.** (A) PLD activity of immunoprecipitates of yeast harboring *HA-Spo14 CEN* (Wt) and *HA-Spo14-K533E CEN* (K>E) as described in Rudge et al. (1998a) and Sciorra et al. (1999). PLD activity was measured using 50 μM BODIPY-PC in the presence (dark gray) or absence (light gray) of $PI(4,5)P_2$. Inset, Western blot of the immunoprecipitated proteins. (B) Sporulation efficiencies of yeast harboring *HA–Spo14 CEN* (Wt), *HA–Spo14-K533E CEN* (K>E *CEN*), and *HA–Spo14-K533E 2μ* (K>E *2μ*). (C) Complementation analysis of *spo14* deletion, *sec14* bypass mutant phenotype (no growth at 37°C) with *HA–spo14-K533E CEN* (K>E *CEN*), and *HA–spo14-K533E 2μ* (K>E *2μ*). (D) Immunoblot of particulate (P) and supernatant (S) fractions (equal volumes not equal protein) prepared from yeast harboring *HA–Spo14 CEN* (Wt) and *HA–Spo14-K533E CEN* as described in Rudge et al. (1998b).

vation of Spo14p is required for sporulation, where it functions in a developmentally regulated secretory pathway that controls the formation of the prospore membrane (Rudge et al., 1998a,b). Spo14p also plays a conditional role in the maintenance of a functional secretory pathway required for permissive actions of a set of genes under conditions where function of the Sec14 lipid transfer protein has been ablated (Sreenivas et al., 1998; Xie et al., 1998; Rudge et al., 2001). We therefore examined effects of mutating the Spo14 PH domain on activity and function of the PLD enzyme under these two physiological settings. The first conserved K residue at position 533 in Spo14p, which is analogous to the R residue in the PLD2 R237G/W238G mutant, was mutated to E. HA–Spo14p and HA–$spo14^{K533E}$p were expressed under the control of the endogenous promoter in homozygous *spo14* diploids and induced to sporulate. Immunoprecipitated proteins were then assayed for PLD activity in response to $PI(4,5)P_2$. As shown in Fig. 5 A, the activity of $spo14^{K533E}$p was stimulated by $PI(4,5)P_2$ to a similar degree as Spo14p. These results are in agreement with those obtained with the mammalian PLD enzymes and indicate that the integrity of the PH domain is not required for $PI(4,5)P_2$-stimulated activity of yeast PLD.

We next evaluated the ability of *spo14-K533E* to complement the sporulation defect of homozygous *spo14* diploids. Whereas *SPO14* efficiently restored sporulation when expressed from a CEN plasmid (~65% spores), *spo14-K533E* only poorly rescued the sporulation defect (~5% spores; Fig. 5 B). Sporulation was not improved when the mutant was expressed at higher levels by using a 2 micron plasmid, indicating that the failure to complement was not due to expression levels (Fig. 5 B). $spo14^{K533E}$p behaved similarly to the wild-type protein with respect to its fractionation properties, both being particulate in nature (Fig. 5 D), indicating that the protein was not grossly altered by this mutation. Thus, an intact PH domain is required for the in vivo function of yeast PLD in meiosis.

We also tested the ability of $spo14^{K533E}$p to restore cell growth in a *sec14* bypass mutant deleted for *SPO14* (*sec14-1*, *kes1*, *spo14*). HA–Spo14 effectively supported growth at a temperature that was nonpermissive for *sec14-1* function (Fig. 5 C). However, *spo14-K533E* only partially complemented the *sec14* bypass phenotype when expressed from a centromere plasmid. In contrast to what was observed during sporulation, when expressed from a 2 micron plasmid, $spo14^{K533E}$ supported wild-type growth (Fig. 5 C). The ability of *spo14-K533E* to support *sec14* bypass when expressed from a high copy number plasmid indicates that the PH domain does not play as critical a role during vegetative Sec14-independent growth as it does during sporulation. Nevertheless mutation of the PH domain of Spo14 results in a catalytically competent $PI(4,5)P_2$-responsive PLD, which is not fully functional in vivo.

The PLD2 PH domain binds to $PI(4,5)P_2$ with low affinity but high selectivity

The studies described above identify a critical role of the PH domain in the localization and function of PLD2 and Spo14p and suggest that this involves interactions with $PI(4,5)P_2$. To test this idea directly, the PLD2 PH domain was expressed in insect cells using a baculovirus vector and purified by immunoaffinity chromatography. The protein was incubated with varying concentrations of sucrose-loaded large unilamellar phospholipid vesicles (LUVs) composed of PC or PC plus a 5% molar fraction of $PI(4,5)P_2$. The vesicles were sedimented by ultracentrifugation and vesicle-bound protein was detected by Western blotting. The PLD2 PH domain did not bind appreciably to PC vesicles at lower lipid concentrations and bound weakly at the highest concentration examined (10^{-3} M total lipid). By contrast, inclusion of $PI(4,5)P_2$ in these vesicles resulted in significant binding of the PLD2 PH domain that could be detected at a lower total lipid concentration of 3×10^{-5} M. Quantitation of immunoreactive protein on these Western blots by scanning densitometry revealed that the recovery of PLD2 PH domain protein in the vesicle-bound fraction at the highest concentration examined was ~80% of protein input and

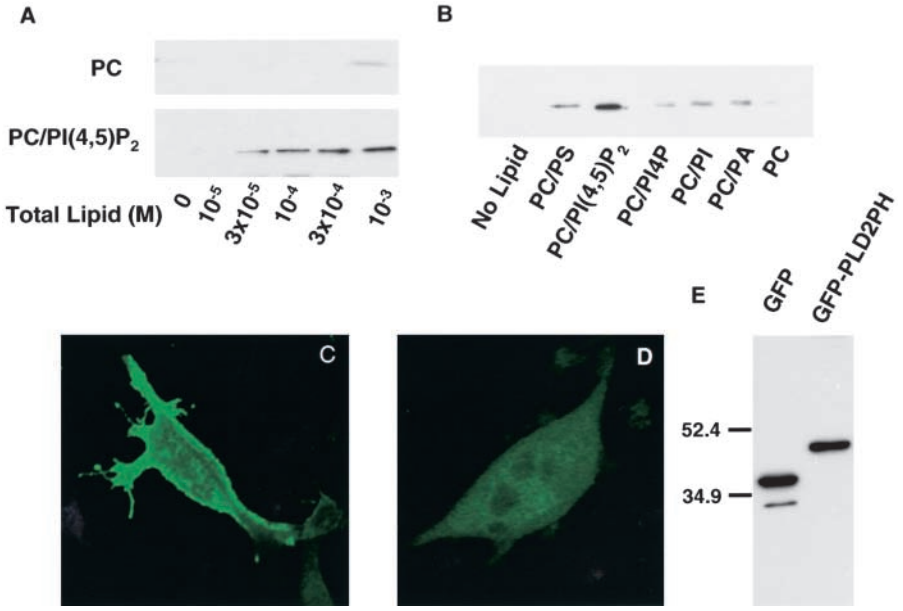

Figure 6. **The PLD2 PH domain binds to PI(4,5)P2 with weak affinity but high selectivity.** (A) Purified PLD2 PH domain was incubated with sucrose-loaded LUVs composed of PC or PC containing 5% PI(4,5)P_2. Incubations contained the indicated total lipid concentrations and the LUVs were sedimented by ultracentrifugation. Vesicle-bound protein was detected by Western blotting using an antibody against the NH_2-terminal glu-glu tag. (B) Purified PLD2 PH domain was incubated with sucrose-loaded LUVs with the indicated phospholipid compositions at a total lipid concentration of 3×10^{-4} M. The LUVs were sedimented by ultracentrifugation and vesicle-bound protein was detected by Western blotting using an antibody against the NH_2-terminal glu-glu tag. These are representative experiments from several that gave similar results. (C) GFP–PLC-δ PH domain was expressed in HEK293 cells by transient transfection and its localization examined by laser scanning confocal microscopy. (D) GFP–PLD2 PH domain was expressed in HEK293 cells by transient transfection and its localization examined by laser scanning confocal microscopy. (E) Proteins from HEK293 cells expressing GFP or GFP–PLD2 PH domain were separated by SDS-PAGE and probed by Western blotting using an anti-GFP antibody.

suggested that binding of the PLD2 PH domain was half maximal at a concentration in the range of 1–3 $\times$ 10^{-4} M total lipid (Fig. 6 A). To examine the phospholipid selectivity of this association, we determined binding of the PLD2 PH domain to LUVs composed of PC or PC containing a 5% molar fraction of PI(4,5)P_2, phosphatidylinositol 4-phosphate (PI4P), PI, phosphatidic acid, or phosphatidylserine (PS). At a fixed total lipid concentration of 3 $\times$ 10^{-4} M, association of the PLD2 PH domain with PI(4,5)P_2-containing LUVs was dramatically greater than that seen with PC vesicles. In comparison to PC LUVs, binding could be detected when each of the acidic lipids was included in the vesicles. This nonspecific binding enhancement has been observed for several different PH domains and likely reflects the characteristic electrostatic polarization of this motif, which produces a net positive charge on the entire membrane binding surface. Using scanning densitometry to quantitate immunoreactive vesicle-bound proteins revealed that binding of the PLD2 PH domain to PI(4,5)P_2-containing vesicles was ~10-fold greater than that seen with any of the other lipids tested (Fig. 6 B). These results show that binding of the PLD2 PH domain to model membranes is significantly and selectively enhanced by PI(4,5)P_2. Although the PLD2 PH domain binds to PI(4,5)P_2 in these membranes with high selectivity, the affinity of this interaction is 10–30-fold lower than that shown by the PH domain of PLC-δ when measured under similar conditions (Lemmon and Ferguson, 2000). The high affinity and selectivity of the PLC-δ PH domain for PI(4,5)P_2 results in the localization of the protein to the plasma membrane in many cell types. We used laser scanning confocal microscopy to compare the localization of the PLC-δ PH domain to that of the PLD2 PH domain when both proteins were expressed in HEK293 cells with GFP fused to the NH_2 terminus. The GFP-tagged PLC-δ PH domain showed a characteristic plasma membrane localization. The GFP-tagged PLD2 PH domain was efficiently expressed and produced an immunoreactive protein of the expected molecular weight. In contrast to what was observed with the GFP–PLC-δ PH domain but consistent with results obtained with GFP–PLD2 R237G/W238G (Fig. 4, C and F), the GFP–PLD2 PH domain was diffusely localized within the cell (Fig. 6, C–E).

Role of the polybasic domain in membrane binding of PLD2

The data presented above show that, when attached to the rest of the PLD protein, the PLD2 PH domain specifies plasma membrane localization and that this function most likely involves association with PI(4,5)P_2. Because the intrinsic affinity of the PLD2 PH domain for PI(4,5)P_2 is insufficient to target the protein to the plasma membrane, we speculated that other determinants within the PLD2 sequence must contribute significantly to the overall affinity of the protein for cellular membranes. We previously identified a sequence rich in basic and aromatic amino acid residues within the catalytic core that is required for activation of PLD2 and Spo14p by PI(4,5)P_2 (Sciorra et al., 1999). This polybasic domain contains six arginine, lysine, or histidine residues that are conserved between PLD1, PLD2, and Spo14p. Substitution of charged residues throughout this domain that include R554, R558, and R563 produces PLD2 enzymes that display significantly attenuated stimulation by PI(4,5)P_2 (Sciorra et al., 1999; unpublished results). We found that binding of wild-type PLD2 to LUVs composed of PC/PS/PE, molar ratio 1:1:1 containing a 5% molar fraction of PI(4,5)P_2, was half maximal at a total lipid concentration of ~10^{-5} M (Sciorra et al., 1999). We repeated these measurements using purified PLD2 R237G/

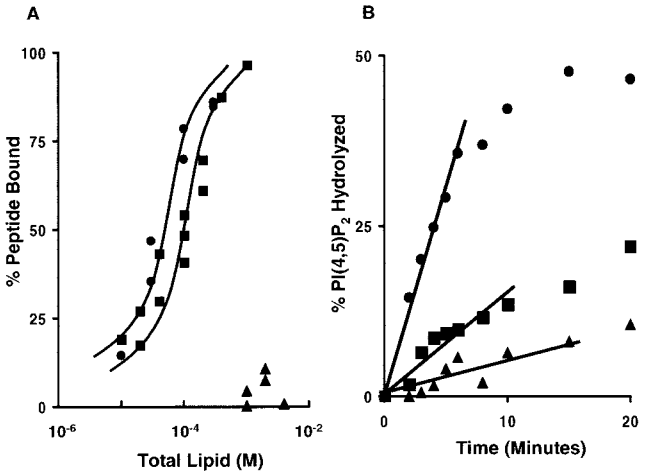

Figure 7. **Binding and sequestration of PI(4,5)P_2 by PLD2 554–575 peptide.** (A) Binding of ^{3}H-PLD2(554–575) to 99:1 PC/PIP$_2$ (●), 5:1 PC/PS (■), and PC (▲) LUVs was measured by centrifugation. The curves are the least square fits of Equation 1 to the data and are from three independent experiments. (B) A monolayer containing 66:33:1 PC/PS/[^{3}H]PI(4,5)P_2 was formed on the surface of 100 mM KCl buffer containing PLC-δ and peptides. After the addition of PLD peptide and PLC-δ to the subphase, PLC activity was determined by measuring PI(4,5)P_2 hydrolysis in the absence of peptide (●) or in the pesence of 5 μM (■) or 10 μM (▲) PLD2 554–575 peptide. The data are representative of two sets of experiments. The lines show linear fits to the data obtained in the first 5 min.

W238G and another PLD2 mutant in which the invariant tryptophan residue that lies in the center of the COOH-terminal α-helix of all PH domains was substituted with G (PLD2 R308G). Both of these PLD2 mutants were stimulated by PI(4,5)P_2 to the same extent and with the same concentration dependence as wild-type PLD2 (unpublished data). Both of these mutants also bound to LUVs composed of PC/PS/PE, molar ratio 1:1:1 containing a 5% molar fraction of PI(4,5)P_2, with approximately the same affinity shown by wild-type PLD2 (unpublished data). These results are consistent with the relatively weak PI(4,5)P_2 binding affinity shown by the isolated PH domain.

Substitution of basic amino acids within the polybasic domain reduced binding of PLD2 to acidic phospholipids, including PI(4,5)P_2, suggesting a general role in membrane association (Sciorra et al., 1999). We have been unable to express this domain independently, most likely because of its proximity to the catalytic core of the enzymes. In an attempt to determine the contribution of this polybasic region to membrane binding by PLD2, we synthesized a peptide corresponding to the entire sequence. This PLD2-derived peptide has a net charge of +6 and has four aromatic residues (Fig. 1). We measured the binding of [^{3}H]PLD2(554–575) to PC, PC/PS, and PC/PI(4,5)P_2 vesicles using the centrifugation technique. Fig. 7 A shows that the PLD peptide binds weakly to PC vesicles, but with significant affinity to both PC/PI(4,5)P_2 (99:1) and PC/PS (5:1) vesicles. Its binding to PC/PI(4,5)P_2 vesicles (molar partition coefficient $K = 10^4$ M^{-1}) is similar to the binding of Lys10 ($K = 3 \times 10^4$ M^{-1}), but is significantly stronger than that of Lys7 ($K = 8 \times 10^2$ M^{-1}), which suggests that both the basic and aromatic residues contribute to the binding (Wang et al., 2002).

We next asked if the peptide could electrostatically sequester PI(4,5)P_2 in a membrane that contained a physiological 30-fold excess of monovalent acidic lipid. We monitored the interaction of PLD2(554–575) with PI(4,5)P_2 by using a PLC assay in a monolayer containing 66:33:1 PC, PS, and PI(4,5)P_2. As shown in Fig. 7 B, PLC hydrolyzes PI(4,5)P_2 at a moderate rate in the absence of PLD peptide. With the addition of 10 μM PLD2(554–575), the initial rate of PI(4,5)P_2 hydrolysis decreased ~10-fold. Similar inhibitions were also seen in a previous study with other peptides containing basic and aromatic residues. The simplest explanation is that basic peptides sequester PI(4,5)P_2 by means of nonspecific local electrostatic interactions (McLaughlin et al., 2002).

Discussion

The broad goal of the work described here was to evaluate the significance of the NH_2-terminal PH domain in the function of yeast and mammalian PLD enzymes. We were interested in testing the hypothesis that this region of the proteins is involved in specific interactions with phosphoinositides and that the interplay between the PH domain and a previously described polybasic motif could be critical for regulating the association of these proteins with cellular membranes and in the control of PLD activity. Our results demonstrate that the integrity of the PH domain is required for proper function of mammalian and yeast PLD. We show that the PLD2 PH domain binds PI(4,5)P_2 with high selectivity but relatively low affinity. Although this affinity is by itself insufficient to target the protein to the plasma membrane, we identify a role for the internal polybasic motif as a higher affinity but less selective membrane anchor for these enzymes. Our data suggest a model in which the polybasic motif serves as a general membrane anchor for the enzymes, allowing the PH domain to specify a targeting function that selectively localizes the enzymes to sites enriched in its ligand PI(4,5)P_2. Because the polybasic motif also serves a critical function in regulation of the catalytic activity of these enzymes by PI(4,5)P_2, our results identify a dual role for phosphoinositides in the regulation of PLD function. The use of combinatorial mechanisms for membrane tethering and localization is a common theme in proteins involved in many aspects of cell regulation. Our proposal for the PLD enzymes is conceptually similar to the situation where, for example, both fatty acylation and electrostatic interactions with phospholipid head groups work together to drive the membrane association observed with the Src tyrosine kinase and a number of extrinsic membrane proteins (for review see McLaughlin et al., 2002). A similar "regulated avidity" mechanism has also been proposed for members of a broad class of PH domains that bind phosphoinositides with weak affinity but that are capable of oligomerization (Lemmon and Ferguson, 2000). This mechanism may be involved in the regulation of the PH domain–containing protein dynamin during receptor-mediated endocytosis. In general, PH domains that bind phosphoinositides with affinities that are low in comparison to those displayed by the PH domains from PLC-δ_1 or spectrin have been reported to show broad specificity for their ligands (Lemmon and Fer-

guson, 2000). Our results suggest that the PLD2 PH domain belongs to a unique class of PH domains with relatively weak, but selective, affinity for $PI(4,5)P_2$. Structural characterization of this domain will be required to understand the basis of this interaction.

Clearly, protein–lipid interactions are not the only determinants of the subcellular localization of the PLD enzymes. In the case of PLD2, we found that in comparison to the wild-type enzyme, mutation of the PH domain resulted in redistribution from the plasma membrane to an intracellular membrane compartment, most likely early endosomes. Because the PH domain mutants of PLD2 are catalytically competent but are uncoupled from activation by stimuli, we suggest that this redistribution results in the movement of PLD2 from a cellular compartment where it is active (the plasma membrane) to a site where it becomes refractory to stimulation. The physiological relevance of this observation is not yet clear, but it is possible that modulation of the $PI(4,5)P_2$ binding affinity of the PH domain through, for example, protein phosphorylation could control sorting decisions that dictate retention of the protein in the plasma membrane or release to the endosomal pathway. The PH domain–independent mechanism responsible for localizing PLD2 to endosomes is presently unclear. PX domains control protein targeting to endosomes through interactions with phosphatidylinositol 3-phosphate (PI3P) (Sato et al., 2001), so the PLD PX domain would be a likely candidate to mediate this interaction. However, we find that several mutations of the PX domain do not affect the endosomal localization of PLD2 (unpublished data).

Although we do not know if the PX domain of PLD2 binds PI3P, this domain may be functionally relevant in other PLD enzymes. PLD1 can apparently bind PI3P in vitro (Hodgkin et al., 2000), and the PX domain of Spo14p can bind PI3P in a dot blot assay with low affinity (Yu and Lemmon, 2001). Substitution of residues within the PX domain of Spo14p, which would be expected to ablate PI3P binding, produces a protein that is nonfunctional in meiosis and *sec14* bypass when expressed from a centromere plasmid, and although increased gene dosage of this mutant fails to improve *sec14* bypass, it does improve sporulation (unpublished data). This suggests that *sec14* bypass and sporulation are differentially sensitive to the loss of functional PX and PH domains, which may in turn relate to their likely roles in controlling subcellular localization of the protein. During *sec14* bypass, Spo14 is found in endosomes (Li et al., 2000), which are rich in PI3P. In contrast, during sporulation, Spo14 is relocalized to the developing prospore membrane, which is rich in $PI(4,5)P_2$ (unpublished data). Although a distinct NH_2-terminal domain is essential for Spo14p localization (Rudge et al., 2002) and mutation of the PH and PX domains does not grossly alter the localization of the enzyme to the prospore membrane (unpublished data), it is likely that these domains both contribute to association of Spo14p to different cellular membranes, which may include endosomes, which are both rich in PI3P and contain an active pool of Spo14p (Li et al., 2000; Rudge et al., 2001).

Although our results suggest that the PH domains of PLD2 and Spo14 p are functionally analogous, the role of the PH domain in the regulation of PLD1 is likely to be different. As with PLD2 and Spo14p, the PH domain of PLD1 is dispensable for stimulation of catalysis by $PI(4,5)P_2$ (Sung et al., 1999a,b; Sciorra et al., 2001). The PH domain of PLD1 also contains amino acid residues in analogous positions to those we suggest are important for $PI(4,5)P_2$ binding. However, other work has identified tandem cysteine residues within this domain that are palmitoylated and would, based solely on energetic considerations, be sufficient to anchor the protein to membranes independently from interactions with $PI(4,5)P_2$ (Sugars et al., 1999). Murine PLD2 used in our studies contains a single cysteine residue at this position, and whereas we can readily detect incorporation of palmitate into human PLD1 protein after radiolabeling of transfected HEK293 cells, murine PLD2 is not labeled (unpublished data). Spo14p does not contain cysteine residues in this region, so palmitoylation is unlikely to be an important posttranslational modification of this protein. Furthermore, PLD1 is regulated by ARF and Rho family GTP-binding proteins and certain conventional protein kinase C enzymes, which are themselves membrane associated, and it is likely that interactions with these proteins also contribute to the membrane targeting of this PLD enzyme. Genetic, biochemical, and biophysical evidence indicates that these families of proteins are not direct regulators of Spo14p or PLD2 (Rudge et al., 1998b; Liscovitch et al., 2000; unpublished data). The role of the PH domain in membrane localization of PLD1 therefore clearly differs from what we report here for PLD2 and Spo14p.

Our previous work identified a region proximal to catalytic region III of all phosphoinositide-dependent PLD enzymes that contains a conserved region of basic and aromatic amino acid residues. Integrity of this polybasic is required for interaction with, and activation by, $PI(4,5)P_2$. This region encompasses seven basic lysine and arginine residues and mutational studies suggest involvement of the entire region in phosphoinositide binding and activation of the enzymes (Sciorra et al., 1999). Here we show that a peptide corresponding to the polybasic motif of PLD2 binds to LUVs containing $PI(4,5)P_2$ with a similar affinity to the intact protein. Detailed studies with this and other small unstructured basic/aromatic peptides suggest a model for the peptide–membrane interaction in which the basic amino acid residues interact through nonspecific, but local, electrostatic interactions with polyvalent acidic lipids such as $PI(4,5)P_2$, and the aromatic residues penetrate the membrane bilayer to contribute additional hydrophobic binding energy (for review see McLaughlin et al., 2002). The observation that this PLD peptide and other basic and basic/aromatic peptides exhibit similar binding to both $PC/PI(4,5)P_2$ (99:1) and PC/PS (5:1) vesicles is consistent with a model in which the local electrostatic potential produced by one multivalent $PI(4,5)P_2$ (or more than one $PI[4,5]P_2$ in the case of peptides with 10 or 13 basic residues) is similar to that produced by 17% PS in the PC/PS bilayer (Wang et al., 2002). The stoichiometry of the interaction between $PI(4,5)P_2$ and the polybasic region has not been determined directly, but clearly at least one and possibly two molecules can bind. The polybasic peptide can also produce a lateral sequestration of $PI(4,5)P_2$ in a model membrane that contains an excess of a monovalent acidic lipid. Although stimulation of catalysis

that is mediated by the polybasic domain is highly specific for $PI(4,5)P_2$, our results suggest a more general role for this motif in anchoring the protein to membrane surfaces containing acidic lipids. Future work on the three-dimensional structure of the catalytic core of PLD that includes this polybasic domain should give insight into how this domain interacts with acidic phospholipids yet confers selectivity for stimulation of catalysis by $PI(4,5)P_2$.

PLD activity has been associated with a very wide range of cellular functions, and this complexity has, for the most part, been ascribed to the complex and isoenzyme-specific regulation of the enzymes by both posttranslational modifications and actions of protein and lipid regulators. Our studies suggest that differential targeting of PLD to intracellular membrane compartments is an equally important determinant of PLD function in yeast and mammalian cells. The work presented here identifies the NH_2-terminal PH domain as an important component of this process and shows that this domain confers selective targeting to $PI(4,5)P_2$-rich membranes through a weak, but selective, interaction with this lipid that operates in conjunction with a higher affinity, but less selective, membrane interaction mediated by the polybasic motif.

Materials and methods

General reagents

Unless otherwise stated, reagents used in this study were of analytical grade. The sources of general reagents used in this study can be found in prior publications (Sciorra and Morris, 1999; Sciorra et al., 1999, 2001). Phospholipids used in this study were purchased from Avanti Polar Lipids, Inc. The ammonium salt of $PI(4,5)P_2$ was either purchased from Avanti Polar Lipids, Inc. or Roche Molecular Biochemicals or purified from bovine brain extract (Sigma-Aldrich) as described elsewhere (Sciorra et al., 1999). (dioleoyl-1-^{14}C)-L-α-dioleoyl-PC and (ethyl-1,2-^{3}H)-*N*-ethyl-maleimide were from PerkinElmer. Non-radioactive *N*-ethyl-maleimide (NEM) was from Sigma-Aldrich. [^{3}H]choline-labeled 1-palmitoyl-2-oleoyl-sn-glycero-3-PC and [^{3}H]oleic and palmitic acids were from American Radiolabeled Chemicals.

Protein expression and purification

Wild-type and mutant PLD1 and PLD2 were expressed in insect cells using recombinant baculoviruses, purified to homogeneity by immunoaffinity chromatography, and quantitated as described previously (Sciorra et al., 1999). The PLD2 mutants were generated using the Quick Change protocol (Stratagene) and confirmed by sequencing and/or restriction digestion. The PH domain from PLD2, which encompassed F196–R315, was amplified by PCR using primer adaptors and used to construct a baculovirus vector for expression of this protein with an NH_2-terminal glu-glu tag to facilitate purification by affinity chromatography (Sciorra et al., 1999). The purified proteins were stored at 4°C and used within 2 d of preparation. PLD activators including ADP ribosylation factor and Rho family GTP-binding proteins were expressed and purified as described in Hammond et al. (1997). Recombinant human PLC-δ1 was purified from *Escherichia coli* as described in Tall et al. (1997).

Peptide synthesis and labeling

The sequence of murine PLD2(554–575) is RDLARHFIQRWNFTKTTKARYK. Two peptides corresponding to this region were obtained from American Peptide Company, Inc. One has an extra Cys at its NH_2 terminus for labeling with [^{3}H]NEM. The other lacks the Cys and was used in the PLC assay. Both peptides are blocked with acetyl at their NH_2-terminal and amide at their COOH-terminal regions. The PLD2(554–575) peptide was covalently labeled with a radioactive [^{3}H]NEM to the NH_2-terminal Cys and purified by HPLC (Wang et al., 2002).

Vesicle preparations

Sucrose-loaded LUVs were prepared as described in Buser and McLaughlin (1998). In brief, the desired lipid mixture was dried on a rotary evaporator and then hydrated in a solution containing 176 mM sucrose, 1 mM MOPS, pH 7.0. The lipid mixture underwent five cycles of freezing and thawing and then was passed through a stack of two polycarbonate filters (0.1-mm diameter pore size) using a Lipex Biomembranes extruder or, in some cases, using a syringe mini extruder from Avanti Polar Lipids, Inc. for 10 cycles. The buffer was then exchanged with 100 mM KCl, 1 mM MOPS, pH 7.0.

Centrifugation binding measurements

The binding of [^{3}H]NEM-labeled PLD2(554–575) peptide to sucrose-loaded PC/$PI(4,5)P_2$ (99:1) LUVs or PC/PS (5:1) LUVs was measured using a centrifugation technique described in Buser and McLaughlin (1998). In brief, sucrose-loaded LUVs were mixed with [^{3}H]NEM-labeled PLD peptide or PLD protein and centrifuged at 100,000 *g* for 1 h. The radioactivity of the supernatant and the pellet was measured to determine the percentage of peptide bound. In experiments using wild type and mutant PLD proteins of the PLD2 PH domain, the above procedure was adapted as described in Sciorra et al. (1999) and PLD binding was quantitated by making measurements of PLD activity remaining in the supernatant or by analyzing supernatant and lipid pellet fractions by SDS-PAGE and Western blotting. In the case of the PLD2 PH domain, we used a monoclonal antibody (1H10) that recognizes the appended glu-glu epitope (Sciorra et al., 2001).

Peptide binding calculations

We use a molar partition coefficient *K* to describe the binding of the peptide to lipid vesicles without making assumptions about the absorption mechanism. The molar partition coefficient *K* is defined in the equation $[P]m/[L] = K[P]$, where $[P]m$ is the molar concentration of peptide partitioned to the membrane, $[P]$ is the molar concentration of free peptide in the bulk aqueous phase, and $[L]$ is the molar concentration of lipid accessible to the peptide. Under our conditions, $[L] >> [P]m$. Thus $[L]$ does not change significantly after the peptide binds and is >>0.5 of the total lipid concentration for these LUVs because the peptide interacts only with the outer leaflet of the bilayer; the vesicles are not permeable to the peptide, which is added to a solution of preformed vesicles. Combining the definition of *K* with the equation $[P]tot = [P]m + [P]$, we get Equation 1:

$$\frac{[P]m}{[P]tot} = \frac{K[L]}{1 + K[L]}. \quad (1)$$

PLC assay in a monolayer system

The effect of the PLD2(554–575) peptide on the PLC-catalyzed hydrolysis of $PI(4,5)P_2$ in a monolayer system as described elsewhere (Wang et al., 2002). In brief, we deposited ~60 μl of a lipid stock in chloroform, which contains 55 μM PC/[^{3}H]$PI(4,5)P_2$ (66:33:1), onto the surface of a 15-ml solution in a 5-cm-diameter Teflon trough. The subphase solution contained 100 mM KCl, 25 mM Hepes, 0.1 mM EGTA, 1 mM DTT, pH 7.0. Once the chloroform had evaporated (10 min), we measured the surface pressure of the monolayer (typically 25 mN/m) using a square piece of filter paper and a balance as described previously (Boguslavsky et al., 1994). We then added PLD peptide to the subphase. After 3 min, we added PLC-δ1 (final concentration ~0.1 nM). After another 3 min, we added 100 mM $CaCl_2$ to the subphase to produce a free concentration of Ca^{2+} (~2 mM) that produces significant activation of PLC-δ. We collected 200-μl aliquots of the subphase at different times after the addition of Ca^{2+} and measured the radioactivity due to [^{3}H]inositol 1,4,5-trisphosphate (Boguslavsky et al., 1994).

PLD activity assays

Activity of wild-type and mutant PLD isoenzymes was measured in vitro using [^{3}H]PC presented in sonicated lipid dispersions containing PE and, in some cases, varying concentrations of $PI(4,5)P_2$. Where indicated, activity was also determined using BODIPY-PC as substrate, as described in Rudge et al. (1998a) and Sciorra and Morris (1999). Measurements of PLD activity in intact cells were performed by prelabeling the cells with [^{3}H]palmitic acid overnight, treating the cells with 0.3% butanol, and measuring PLD-catalyzed formation of phosphatidylbutanol by extraction and TLC analysis of total cellular lipids as described in Sciorra et al. (1999).

Mammalian expression plasmids

Vectors for expression of PLD1 and PLD2 as NH_2-terminal fusions with EGFP have been described previously (Sung et al., 1999b), and these were used as templates for site-directed mutagenesis as described above. The vector for expression of the GFP-tagged PLD2 PH domain was made using the PCR strategy described above for insect cell expression of the protein

Table I. **Yeast strains**

Strain	Genotype
CTY1130	*MAT**a** ura3-52, his3-200, lys2-801, sec14-3ts, kes1-1, spo14::HIS3*
Y1961	CTY1130 + Yep352 *2μ URA3*
Y2041	CTY1130 + pME986 *HA–SPO14 CEN URA3*
Y3853	CTY1130 + pME1765 *HA–spo14-K533E CEN URA3*
Y3854	CTY1130 + pME1767 *GFP–spo14-K533E 2μ URA3*
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
Y2367	Y522 + pME986 *HA–SPO14 CEN URA3*
Y2856	Y522 + pME1765 *HA–spo14-K533E CEN URA3*
Y2858	Y522 + pME1767 *GFP–spo14-K533E 2μ URA3*

to generate a cDNA fragment that was inserted into the pEGFP vector. Mammalian cell transfections were done using lipofectamine (Invitrogen).

Confocal fluorescence microscopy

Cells were cultured on poly-L-lysine–coated glass coverslips and transfected with corresponding plasmids as described in Sciorra and Morris, 1999. Cells were fixed with 4% paraformaldehyde in PBS and then mounted in prolong antifade reagent (Molecular Probes) before analysis by confocal fluorescence microscopy using a Nikon Eclipse PCM system or a ZEISS 410 confocal laser scanning microscope. Images were processed using software provided by the manufacturers and edited with Adobe Photoshop®.

Cell fractionation

HEK293 cells transfected with vectors for expression of wild-type and mutant PLD2 were harvested and fractionated into soluble and total particulate fractions by ultracentrifugation at 100,000 *g* as described in Sciorra et al. (1999). Detergent-resistant membrane fractions were isolated from HEK293 cells that had been transfected with vectors for expression of PLD2 by extraction in 1% Triton X-100 followed by sucrose density gradient fractionation. Fractions were collected from the top of the gradient and analyzed for PLD2 protein by SDS-PAGE and Western blotting (Sciorra and Morris, 1999).

Yeast strains

Genotypes of yeast strains are listed in Table I. Yeast manipulations were performed and media were prepared using standard procedures. Yeast transformations were performed by the lithium acetate procedure. Cells were initiated to sporulate in 2% potassium acetate after transfer from synthetic complete medium without uracil (SC-ura). Sporulation frequencies were determined after 48 h by examining a minimum of 600 cells (Rudge et al., 1998a,b, 2001).

Yeast plasmids

GFP–Spo14p and HA–Spo14p have been reported previously (Rudge et al., 1998a,b). The K533E mutation was generated by site-directed mutagenesis as described using plasmids ME986 and ME1095 as templates for the mutagenesis (Sciorra et al., 1999) to generate pME1765 (HA–spo14K533E URA3 CEN4) and pME1767 (GFP–spo14 K533E URA3 2μ).

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