

KINETIC ANALYSIS OF A MAMMALIAN PHOSPHOLIPASE D:
ALLOSTERIC MODULATION BY MONOMERIC GTPASES,
PROTEIN KINASE C, AND POLYPHOSPHOINOSITIDES

By

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To my son, Teague
and
To my wife, Heather

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ABBREVIATIONS

| | |
|----------------|--|
| Arf | ADP ribosylation factor |
| β -OG | n-octyl- β -D-glucopyranoside |
| DTT | dithiothreitol |
| GTP γ S | guanosine 5'-3-O-(thio)triphosphate |
| n_H | Hill coefficient |
| PA | phosphatidic acid (GP1001) |
| PC | phosphatidylcholine (GP0101) |
| PE | phosphatidylethanolamine (GP0201) |
| PIP2 | phosphatidylinositol 4,5-bisphosphate (GP0801) |
| PLD | phospholipase D |
| PLD1.d311 | phospholipase D1 truncation mutant, amino acids 312 - 1036 |
| PMA | 4 β -phorbol 12-myristate 13-acetate |
| PMSF | phenylmethylsulfonyl fluoride |
| PKC | protein kinase C |

CHAPTER I

INTRODUCTION

Haud ignota loquor – “I say things which are not unknown.”
Vergil (*Aeneid* II, 91)

Many cellular functions and biochemical mechanisms involved in signaling by phosphoinositide breakdown are now well understood (Exton, 1996). Phospholipase C enzymes that catalyze phosphoinositide (PI) hydrolysis have been characterized structurally in complex with substrate, cofactors, and activators (Essen et al., 1996; Rhee, 2001; Singer et al., 2002; Deng et al., 2005). Signal-transduction pathways that connect extracellular stimuli to PI hydrolysis and PI metabolites to cellular functions are known. By contrast, knowledge of cellular signaling by phosphatidylcholine breakdown is less defined. Phosphatidylcholine (PC), the most abundant membrane phospholipid, is hydrolyzed by a number of phospholipases to generate lipid second messengers in response to cellular stimuli. Arachidonic acid is liberated from PC by phospholipase A₂, phosphatidic acid by phospholipase D, lysophosphatidic acid by sequential actions of phospholipases A₂ and D, and diacylglycerol by sequential actions of phospholipase D and lipid phosphate phosphatase. Phospholipase D appears to have an important role in signal transduction by PC catabolism (Augert et al., 1989; Hodgkin et al., 1998). Explicit pathways from extracellular stimuli to this signal-activated phospholipase have not been fully defined.

Recent evidence suggests multiple roles for PLD in rapid responses to cellular stimuli. These roles include secretion, endocytosis, cytoskeletal rearrangements, and

respiratory burst (superoxide production). PLD also mediates long-term responses to cellular signals. PLD signaling is involved in proliferation, apoptosis, and cell survival. Because PLD performs critical cellular functions, it is ubiquitously expressed and its activity is tightly regulated. Disregulated PLD activity leads to pathogenic roles in cancer, tumor invasion, diabetes, and inflammation.

Identification and characterization

Phospholipase D (PLD) enzymes are widely distributed in eukaryotes, prokaryotes, and viruses. In animals, its principle substrate is PC, which it hydrolyzes to phosphatidic acid (PA) and free choline. The production of PA has broad physiological impact and choline release has a speculative role in acetylcholine synthesis (Exton, 2002). Biochemically distinct PLD isoenzymes have been described, and multiple human genes have been identified. PLD activity was first identified in carrot (Hanahan and Chaikoff, 1947) and plants have been traditional models for PLD research. PLD was initially purified from cabbage (Tookey and Balls, 1956), and early experiments performed on purified cabbage PLD (Yang et al., 1967) established both a phosphatidylcholine phosphohydrolase activity (EC 3.1.4.4, Reaction 1) and a competing, phosphatidylcholine transphosphatidylase activity (Reaction 2).



Transfer of phosphatidyl groups from phospholipid substrates to primary alcohols (ROH) yields phosphatidylalcohols (Reaction 2). Because of their unique origin, their low abundance in biological membranes, and their metabolic stability, the formation of

phosphatidylalcohols has been used as a specific marker for PLD activity (Walker and Brown, 2004). This convenient reaction allowed the identification of PLD activities in animal species (Saito and Kanfer, 1975) and ultimately led to the molecular cloning of PLD from castor bean (Wang *et al.*, 1994). Characterization of recombinant PLD demonstrated that a single gene product conferred both enzymatic reactions. The discovery of PLD cDNA sequences was used in a reverse-genetic approach to identify mammalian PC-PLD orthologs (Hammond *et al.*, 1995; Park *et al.*, 1997; Kodaki and Yamashita, 1997). Subsequent sequence analyses defined a PLD superfamily (Ponting and Kerr, 1996; Koonin, 1996).

Enzymatic characterization of mammalian PLDs has been hindered by the inability to isolate purified enzymes. A thirty-year history of attempts to purify PLD from mammalian sources yields several consistent observations: (i) multiple PLD forms exist, (ii) PLD activity is virtually ubiquitous in tissues and cell-types, (iii) biochemical properties are often assay-dependent, and (iv) PLDs are not typically resolved by chromatography in a homogeneous manner.

Through the efforts of many laboratories, at least eight biochemically distinct, PLD isoforms have been identified in human cells (Table 1). Most forms are found in all tissues and cell-types tested. Two PC-specific PLD isoforms have been molecularly cloned from human cells and have been named PLD1 (Hammond *et al.*, 1995) and PLD2 (Lopez *et al.*, 1998). These isoforms share approximately 50 % sequence identity, and are closely related to plant and bacterial PLD superfamily members. PLD1 and PLD2 are the only mammalian PLDs with demonstrated transphosphatidylase activities.

The first PLD to be molecularly cloned was a glycosylphosphatidylinositol (GPI)-specific PLD (Scallan et al., 1991). GPI-PLDs are unrelated to the PLD superfamily discovered in plants, but rather share sequence homology with bacterial PC-specific phospholipase C enzymes. GPI-PLDs are products of two highly homologous (95 % identical) human genes. In addition, several PLD isoforms have been cloned with specificities for lysophosphatidylcholine substrate. Lyso-PLDs currently include autotaxin (Umezū-Goto et al., 2002; Tokumura et al., 2002) and NPP6 (Sakagami et al., 2005). These enzymes share approximately 40 % sequence identity and are members of a family of ectonucleotide pyrophosphatase / phosphodiesterases. Recently, a PLD has been identified that hydrolyzes *N*-acyl-phosphatidylethanolamines to simultaneously generate two potent cellular messengers: *N*-acyl-ethanolamines (e.g. anandamide) and phosphatidic acids (Okamoto et al., 2004). Molecular characterization of this enzyme, NAPE-PLD, revealed it to be a zinc metallohydrolase, unrelated to all other PLDs.

Table 1. Human PLD isoenzymes.

| Isoenzyme | Membrane Association | Substrate Selectivity | Catalytic Nucleophile | Putative Functions |
|--------------------------|-----------------------------|------------------------------|------------------------------|---|
| PLD1,2 | peripheral, acylated | PC | His | vesicle trafficking, signal transduction, mitogenesis |
| PLD3 (Hu-K4) isoform 1,2 | integral | unknown | His (?) | unknown |
| GPI-PLD1,2 | integral, secreted | GPI-surface glycoprotein | Asp/Glu (Zn ²⁺) | bone formation, triglyceride metabolism |
| autotaxin | integral, secreted | LPC, LPE, LPS, LPI, NTP, NDP | Thr | angiogenesis, LPA production, tumor metastasis |
| NPP6 | integral, secreted | LPC, sphingosyl-PC | Thr | production of LPA, sphingosine, arachidonylglycerol |
| NAPE-PLD | peripheral | <i>N</i> -acyl-PE | Zn ²⁺ | production of anandamide |

Based on primary sequence analysis, an additional class of PLD superfamily enzymes is present in humans. PLD3 (also named Hu-K4) exists as two isoforms that share limited sequence homology with PLD1 or PLD2. PLD3 was shown to be a ubiquitously expressed type II transmembrane protein, although *bona fide* phospholipase activity has not been demonstrated (Pedersen et al., 1998; Munck et al., 2005). PLD3 genes are more closely related to putative PLD isoforms from vaccinia and related poxviruses.

GPI-PLDs and lyso-PLDs are expressed as integral membrane proteins, each of which can be cleaved to release active enzyme into the blood. GPI-PLD and autotaxin are expressed in nearly all tissues, while NPP6 is largely restricted to brain and adrenal cortex. PLD1, PLD2, and NAPE-PLD are ubiquitously expressed peripheral membrane proteins.

The human PLD1 gene resides on the long arm of chromosome 3 (reverse strand locus 3q26), covering 210 kilobases of genomic DNA. Five alternatively spliced transcripts are produced from 31 exons (Figure 1). Human PLD1b1 encodes 1036 amino acids and is the dominant transcript produced in all cells tested. PLD1a1 and PLD1b1 are functionally indistinguishable. The shorter transcripts (i.e., PLD1a2, PLD1b2, and PLD1c) show negligible activity (Hughes and Parker, 2001). The human PLD2 gene resides on the short arm of chromosome 17 (locus 17p13.1), covering 16.3 kilobases of genomic DNA. Three alternatively spliced transcripts are produced from 25 exons (Figure 1). The longest transcript, PLD2a, encodes 932 amino acids and is the dominant transcript in all tissues tested. The functional properties of shorter PLD2 constructs (i.e., PLD2b and PLD2c) have not been examined. PLD2c is expressed at levels approaching that of PLD2a in heart and liver (Steed et al., 1998). The lack of catalytic domains in

PLD2c certainly suggests limited functions. PLD1 is expressed highly in human spleen, ovary, pancreas, and spinal cord, and PLD2 is expressed highly in human placenta, ovary, prostate, and thymus (Appendix A). The distribution of PLD1 and PLD2 in tissues varies between mammalian species.

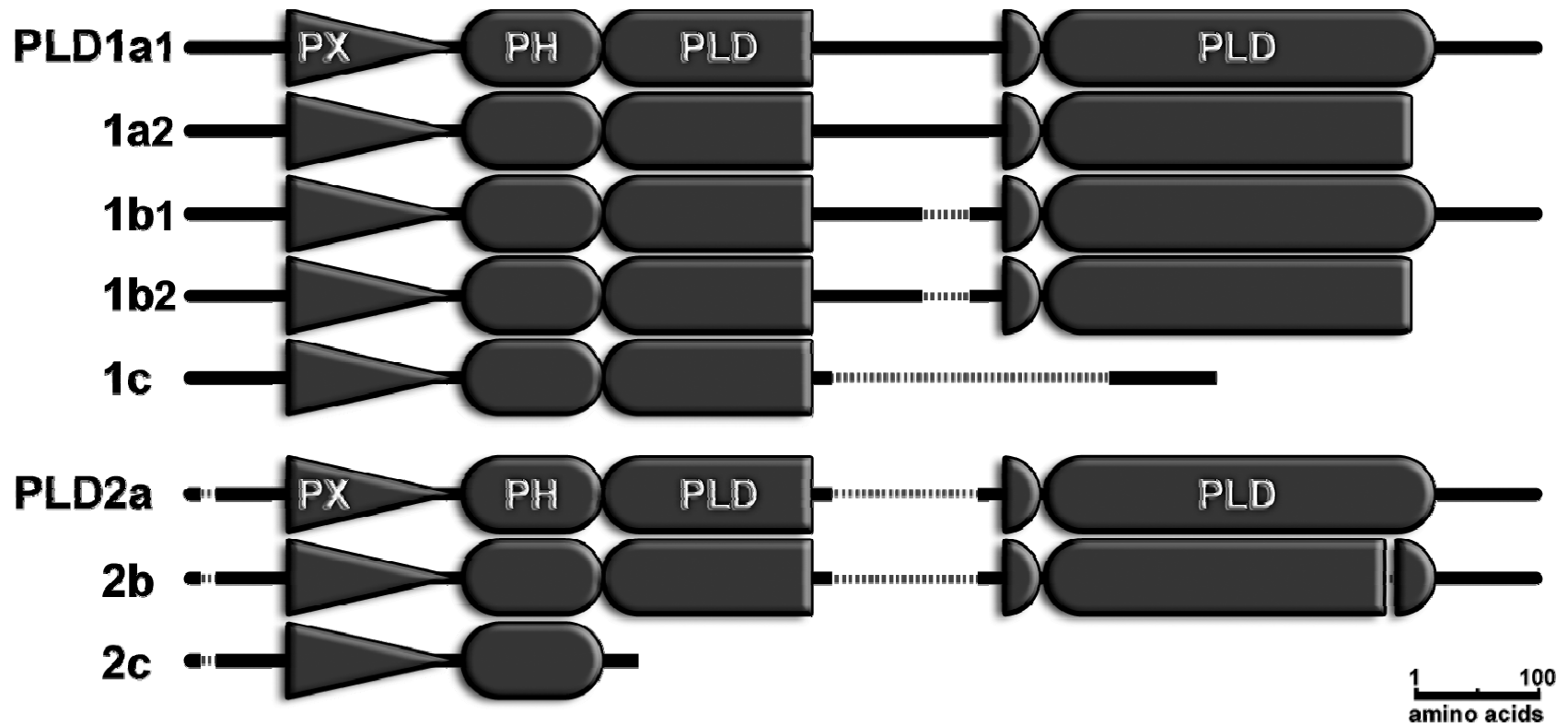


Figure 1. Alternative splicing of human PLD isoforms

PLD1 and PLD2 splice variants are depicted. Conserved domains are shown graphically, and gaps in the aligned sequences are shown as dotted lines.

Rat brain has been a traditional source for the purification of PLD. Because PLD1 and PLD2 are both enriched in brain, and because these isoenzymes are not easily resolved by chromatographic techniques, their properties were initially characterized in heterogeneous mixtures. Nonetheless, distinct PLD activities were revealed by different pH optima for catalysis, and by differential activation by detergents, fatty acids, and calcium. In fact, several groups were successful in isolating multiple, biochemically distinct PC-PLD activities from a single source (Balsinde et al., 1989; Wang et al., 1991; Huang et al., 1992; Daniel et al., 1993; Massenbourg et al., 1994).

It is important to recognize that many enzymatic properties of PLD are assay-dependent. The pH dependence of catalytic activity was shown to be dependent on the buffer system used *in vitro* (Allgyer and Wells, 1979; Inamori et al., 1993). Consequently, PC-PLD isoforms are no longer classified by their neutral or acidic activities. Also, surfactants (e.g., fatty acids, nonionic detergents) have been shown to activate PLD in lipid dispersions, but their effects often compete with one another. Kobayashi and Kanfer demonstrated that activation by detergent was directly related to the solubilization of the enzyme and that all types of detergents inhibited oleate-activated PLD (Kobayashi and Kanfer, 1991a). When PLD activity was tested using liposomes that contain phosphatidylinositol 4,5-bisphosphate (PIP₂), detergents were uniformly inhibitory (Brown and Sternweis, 1995b). Oleate demonstrably inhibits both PLD1 and PLD2 in experiments that used substrate liposomes containing phosphatidylethanolamine (Hammond *et al.*, 1995; Kodaki *et al.*, 1997). When phosphatidylethanolamine was not included with substrate in liposomes, unsaturated fatty-acids activated PLD2 but not PLD1 (Kim et al., 1999a; Sarri et al., 2003).

Structure and mechanism

A reaction mechanism involving a phosphatidyl-enzyme intermediate was proposed to explain the phosphodiesterase and transphosphatidylase activities of PLD (Yang *et al.*, 1967). An ordered, two step S_N2 -type nucleophilic substitution mechanism (ping-pong, Bi-Bi) is supported by analyses of isotopically labeled substrates and products (Yang *et al.*, 1967; Stanacev and Stuhne-Sekalec, 1970; Jiang *et al.*, 1984; Holbrook *et al.*, 1991). In fact, phosphatidyl-PLD transition-states have been trapped and characterized (Rudolph *et al.*, 1999; Iwasaki *et al.*, 1999; Leiros *et al.*, 2004). PLD-catalyzed phosphodiesterase and transphosphatidylase reactions share an initial, S_N2 -type half-reaction. A catalytic nucleophile attacks the distal phosphate of PC to generate a pentacoordinate phosphate-intermediate (Leiros *et al.*, 2004). Choline is subsequently liberated and a covalent phosphatidyl-PLD complex is generated. The exchange reaction occurs, though at a very low rate (Yang *et al.*, 1967). This phosphatidyl-enzyme intermediate decomposes to generate different products, based on the nature of nucleophile in the second half-reaction.

The second S_N2 -type half-reaction involves hydrolysis (or alcoholysis) of the phosphatidyl-PLD intermediate to release product and to regenerate PLD. The hydrolysates are phosphatidic acids, and the alcoholysates are phosphatidylalcohols. Interestingly, short-chained primary alcohols are preferred (>1000-fold) over water in this half-reaction. Using radioisotopically labeled water ($H_2^{18}O$), it was shown that this reaction proceeds via a P—O rather than a C—O bond cleavage, based on the incorporation of ^{18}O into PA products. This half-reaction appears to be irreversible. The observation that transition states accumulate (i.e., phosphatidyl-PLDs) suggest that this half-reaction is rate-limiting for the overall reaction.

Table 2. PLD superfamily members and conserved catalytic residues.

| Enzyme | Source | EC | Catalytic motif | Reference |
|-----------------------|------------------------|----------|-----------------|-----------------------------|
| phospholipase D1 | <i>H. sapiens</i> | 3.1.4.4 | AHHEKLVII-DQS | (Hammond et al., 1995) |
| SPO14 | <i>S. cerevisiae</i> | 3.1.4.4 | AHHEKLVVI-DET | (Honigberg et al., 1992) |
| phospholipase D | <i>S. antibioticus</i> | 3.1.4.4 | WMHSKLLVV-DGK | (Iwasaki et al., 1994) |
| Yersinia murine toxin | <i>Y. pestis</i> | 3.1.4.4 | WNHTKIMAS-DGT | (Cherepanov et al., 1991) |
| tyrosyl-DNA PDE | <i>H. sapiens</i> | 3.1.4.- | THHTKMMLLYEEG | (Pouliot et al., 1999) |
| DNase II | <i>H. sapiens</i> | 3.1.22.1 | RGHTKGVLLLDHD | (Krieser and Eastman, 1998) |
| PS synthase | <i>E. coli</i> | 2.7.8.8 | VLHEKGFII-DDS | (DeChavigny et al., 1991) |
| cardiolipin synthase | <i>E. coli</i> | 2.7.8.- | RQHRKMIMI-DNY | (Tropp et al., 1995) |
| polyphosphate kinase | <i>E. coli</i> | 2.7.4.1 | KIHAKLFIS-RKE | (Kuroda and Kornberg, 1997) |
| Endonuclease | <i>E. coli</i> | 3.1.21.- | IQHDKVVIV-DNV | (Pohlman et al., 1993) |
| Bfi I | <i>B. firmus</i> | 3.1.21.4 | ILHAKLYGT-SNN | (Sapranaukas et al., 2000) |
| K4 | Vaccinia virus | | VLHTKFWIS-DNT | (Krieser et al., 1998) |
| p37K | Vaccinia virus | | QNNITKLLIV-DDE | (Hirt et al., 1986) |

Primary sequence alignments of PLD superfamily members suggests a conserved role for the highly conserved H(x)K(ϕ)₄D motif, termed ‘HKD’ (Table 2, (Ponting *et al.*, 1996;Koonin, 1996), ‘ ϕ ’ represents hydrophobic amino acids). These conserved histidines and lysines are absolutely required for PLD catalytic activity (Secundo *et al.*, 1996;Sung *et al.*, 1997;Rudolph *et al.*, 1999). A conserved histidine is the initial nucleophile in the proposed reaction mechanism (Figure 2), and a phosphohistidine intermediate has been demonstrated in many superfamily members (Gottlin *et al.*, 1998;Rudolph *et al.*, 1999;Iwasaki *et al.*, 1999;Davies *et al.*, 2003;Zhu *et al.*, 2005). Homozygous mutation of a catalytic histidine in human tyrosyl-DNA phosphodiesterase (His493 to Arg) leads to an autosomal recessive form of spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima *et al.*, 2002). Conserved lysines participate in hydrogen-bond networks that coordinate the tetrahedral phosphate in the active site (Stuckey and Dixon, 1999;Leiros *et al.*, 2000b;Leiros *et al.*, 2004;Zhu *et al.*, 2005).

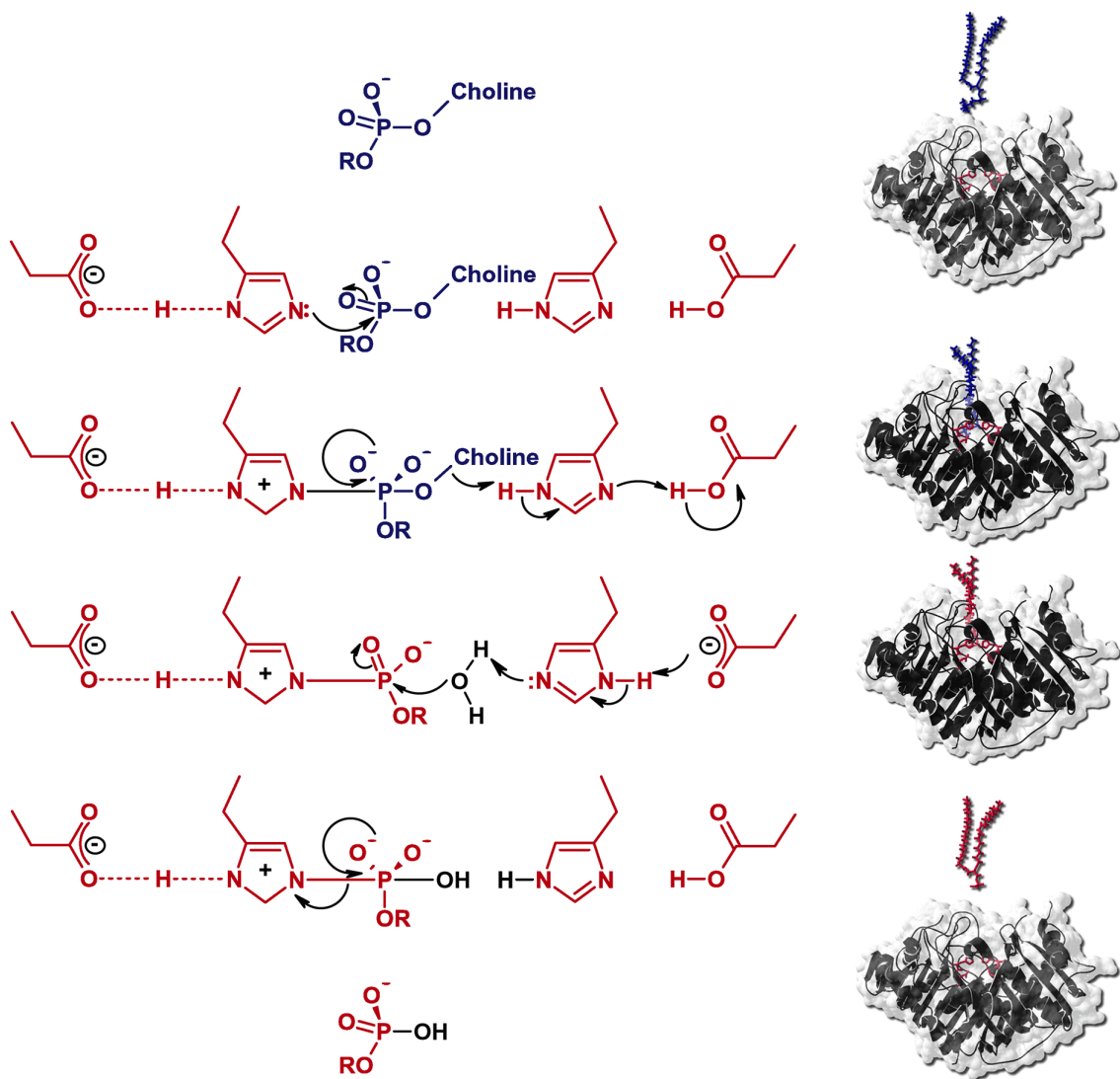


Figure 2. Reaction mechanism based on *Streptomyces* PLD structures.

A catalytic mechanism is proposed, based on structural studies of a bacterial PLD (PLD, *Streptomyces*), an endonuclease (*Nuc*, *S. typhi*), and on biochemical studies of numerous PLD enzymes. The reaction cycle begins (*top*) with the nucleophilic attack of the distal phosphorus atom of phosphatidylcholine. The diacylglycerol backbone is abbreviated to “R.” This leads to a transient, phosphatidyl-PLD intermediate and the liberation of choline. In the second half-reaction, a second active-site histidine deprotonates a water molecule during the nucleophilic attack of the phosphatidyl-intermediate. The transition state degrades to free enzyme and product (phosphatidic acid), restoring the stereochemical configuration of the phosphorus atom.

This HKD motif is part of a larger (~300 amino acid) catalytic subdomain. PLD enzymes possess duplicate catalytic subdomains (Figure 3), each with invariant HKD motifs. Two PLD domains form a characteristic bilobed structure. These paired domains produce internal symmetry, apparent in primary structure (internal sequence homology), and in tertiary structure (internal structural homology). The structure of PLD from *Streptomyces sp.* PMF revealed two tightly interacting domains with a pseudo-two-fold axis of symmetry running through a single catalytic center (RMSD ~ 2 Å). In all known structures, the H(x)K(φ)₄D sequence forms a single β-strand in the center of the β-sheet that forms each catalytic subdomain. A single catalytic site is formed at the interface of these two, opposed β-sheets. Water exposed surfaces of the β-sandwich are covered with α-helices. Surface-exposed helices show the least primary sequence conservation across the PLD superfamily while core β-structures are highly conserved.

Mammalian PLDs require both N- and C- terminal catalytic subdomains for catalytic function. PLD1 deletion mutants that possess only one catalytic subdomain are inactive by themselves. Catalytic activity is restored by the coexpression of both isolated catalytic subdomains (Xie et al., 1998). Furthermore, the physical association of these domains is disrupted by mutations in or near either HKD motif (Xie et al., 2000a). Inspection of the three-dimensional structures of PLD superfamily enzymes reveals a conserved hydrogen-bond network at the active site and a large (>1500 Å²) hydrophobic interface between the two catalytic repeats. Some distantly related enzymes possess only a single catalytic repeat. These enzymes dimerize to form active catalytic sites (Stuckey *et al.*, 1999;Grazulis et al., 2005;Zhu *et al.*, 2005). This conserved architecture suggests common ancestry with an internal gene duplication.

High-resolution structures have been determined for several diverse members of the PLD superfamily that do not have phospholipase activities. Structures of human and yeast tyrosyl-DNA phosphodiesterases (Davies *et al.*, 2002; He *et al.*, 2003), bacterial endonucleases (Stuckey *et al.*, 1999; Grazulis *et al.*, 2005), and bacterial polyphosphate kinase (Zhu *et al.*, 2005) have been determined by x-ray crystallography. While these enzymes perform cellular functions very different from PLDs, they all share chemistry (hydrolysis of a phosphodiester bond) and structure (C α pairwise RMSD 2 - 4 Å). These structural models provide valuable insight into mammalian PLD catalysis and provide mechanistic interpretations for mutational studies (Chapter IV).

A bacterial PLD enzyme was the among the first members of the PLD superfamily to be molecularly cloned (Iwasaki *et al.*, 1994). This 54 kDa enzyme is secreted from *Streptomyces antibioticus* and has putative roles in bacterial pathogenesis. Prokaryotic PLD enzymes have minimal structures, with little more than core catalytic domains and cellular export signals. Unlike eukaryotic isoforms, bacterial PLDs have broad substrate requirements. PCs are preferred substrates, but PEs, phosphatidylglycerols, phosphatidylserines, cardiolipins, sphingomyelins, and lyso-lipids are also often cleaved *in vitro* (Bossi *et al.*, 2001). Recent crystallographic work with these minimal enzymes provides structural insights into the PLD superfamily.

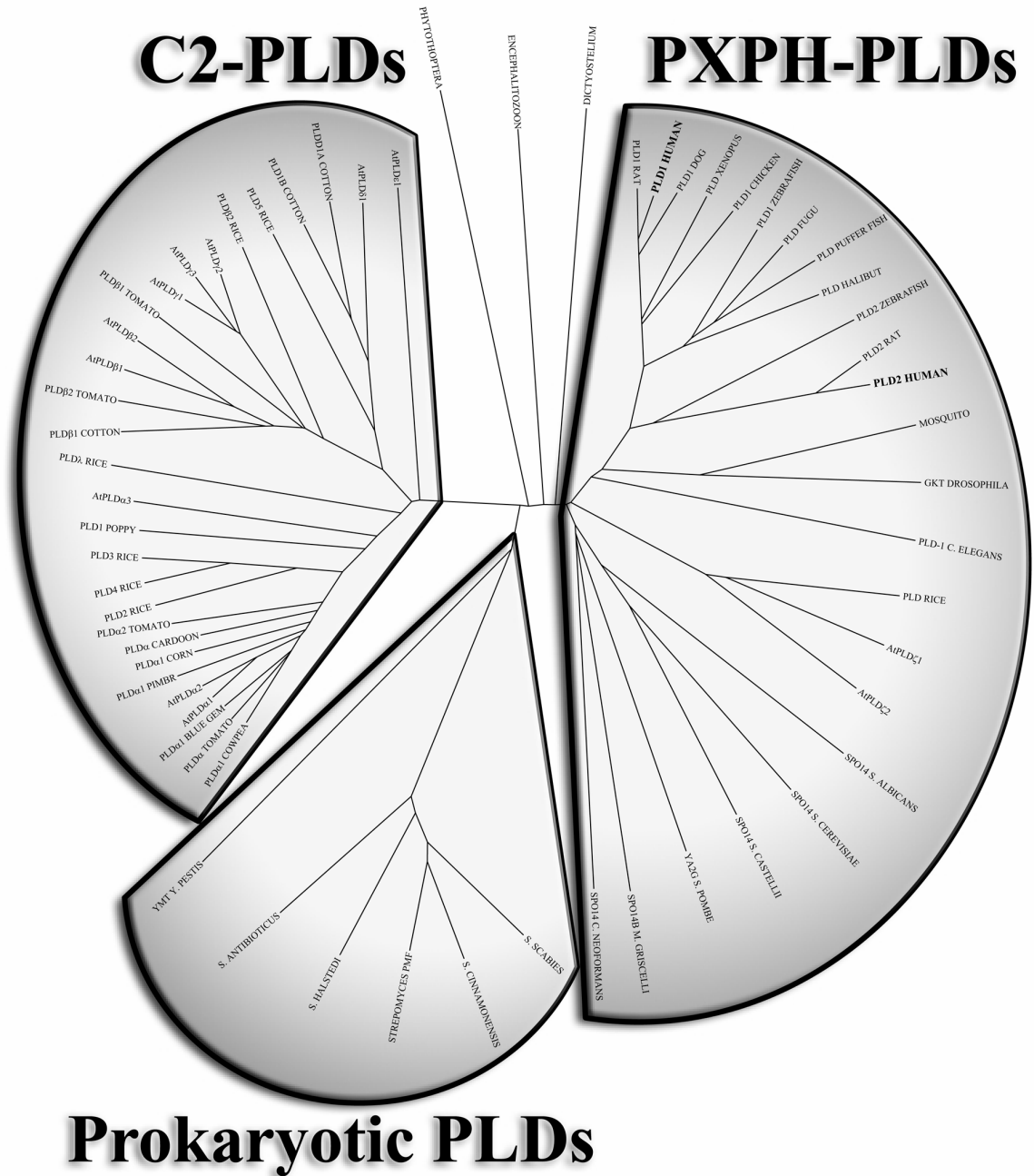


Figure 3. PLD subfamilies

PLD superfamily members were identified in sequence databases and primary sequences (amino acid) were aligned using ClustalW (Thompson et al., 1994). Unrooted dendrograms were generated using Phylip (Felsenstein, 1989).

Membrane targeting domains

Structural studies underscore the modular nature of these enzymes. Catalytic domains provide phosphodiesterase activity and accessory domains often provide specificity to the catalyzed reactions. Often, these domains function independently. In several studies, accessory domains were separated from the catalytic domains by limited proteolysis, suggesting independently folded structures (Younus et al., 2003; Zaremba et al., 2004). BfiI is a restriction endonuclease with N-terminal catalytic domains that follow the PLD superfamily fold and C-terminal domains that provide sequence-specific DNA binding. Similarly, polyphosphate kinase possesses an accessory domain that forms a lid over the catalytic site, presenting substrate to the active site. Eukaryotic PLD enzymes utilize a variety of accessory domains to interact with lipid substrates. All known eukaryotic PLDs have putative membrane-targeting domains at their N-termini. The PLD superfamily can be subcategorized based on these accessory domains (Figures 3 and 4).

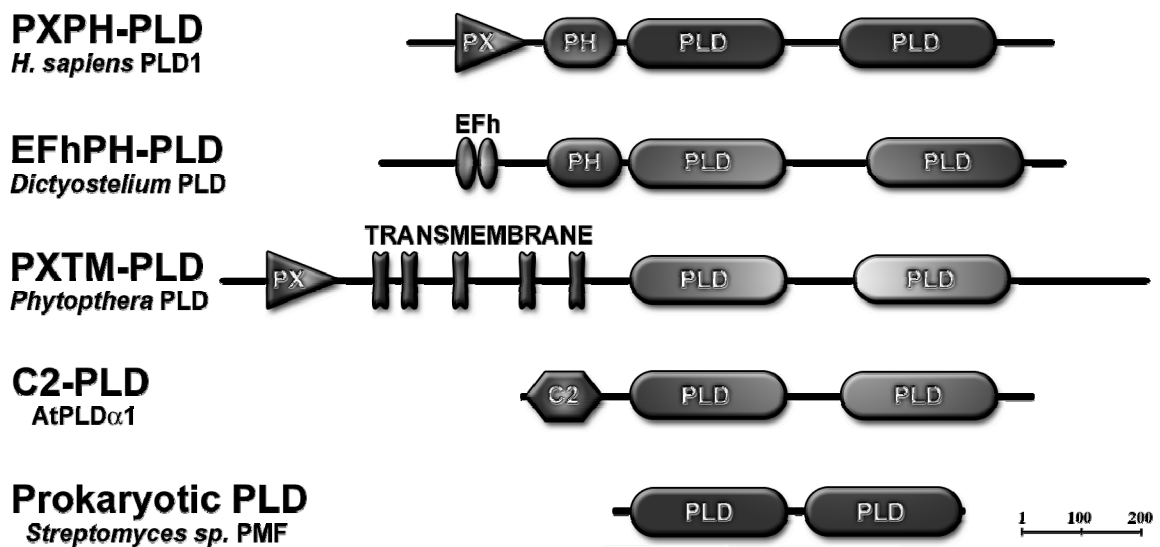


Figure 4. Domain architecture of PLD isoenzymes

C2-PLDs

The largest number of PLD isoforms is found in the plant genomes. More than eighty PLD genes have been identified in plants and several dozen have been cloned. Together, *Arabidopsis thaliana* and *Oryza sativa* (rice) possess twenty-seven PLD genes. Most plant PLD genes include a conserved, N-terminal C2 domain. These genes form a PLD subfamily, termed C2-PLDs (Elias et al., 2002). C2-domains are conserved folds of ~130 amino acids that typically bind phospholipid membranes in the presence of calcium (Hurley and Misra, 2000). All characterized C2-PLDs require calcium for activity, and cytoplasmic calcium levels correlate with PLD activation in plant tissues (Wang, 2002). In general, C2-PLDs show significant activity toward PC and PE.

C2 domains are common structural motifs, > 4000 have been recognized in >3000 proteins in public sequence databases. Initially discovered in calcium-dependent protein kinase C isoforms, C2 domains are also commonly found in lipid kinases and phospholipases. C2-like domains also exist in several lipases and lipoxygenases (Chahinian et al., 2000). Many relevant C2 domain structures have been determined: pancreatic lipase (Winkler et al., 1990), PI-PLC (Essen et al., 1996), cPLA₂ (Dessen et al., 1999), alpha-toxin/phospholipase C (Naylor et al., 1998), PI3K (Walker et al., 2000a), and 15-lipoxygenase (Gillmor et al., 1997). Most C2 domains prefer anionic lipids, though some prefer neutral lipids. Subcellular localization correlates with their lipid specificity.

Isolated C2 domains from *Arabidopsis* PLD isoenzymes bind phosphatidylinositol 4,5-bisphosphate (PIP₂) and PC in a calcium-dependent manner (Zheng et al., 2000). PLD mutants that lack C2 domains bind substrate vesicles inefficiently and exhibit

reduced catalytic activity (Pappan et al., 2004). These findings support a facilitated binding model for C2-PLDs towards aggregated substrates, analogous to the model proposed for another C2 domain-dependent phospholipase, cPLA₂ (Nalefski et al., 1994). Unlike cPLA₂, C2-PLD catalytic domains are capable of binding phospholipid vesicles even when C2 domains are removed. A membrane-scooting model has been proposed to explain the regulation of C2-PLD activity. C2-PLDs constitutively bind the phospholipid interface. Conformational changes in C2 domains (Zheng et al., 2000) and catalytic domains (Zheng et al., 2002) correlate with increased catalytic efficiency.

PXPH-PLDs

A second subfamily of PLD enzymes is defined by conserved phox homology and pleckstrin homology domains (Figure 3). Tandem phox homology (PX) and pleckstrin homology (PH) domains are present at their N-termini. This domain architecture is present in PLD orthologs identified in chordates, arthropods, nematodes, fungi, and some plants. These accessory domains define the PXPH-PLD subfamily (Elias et al., 2002). In this subfamily, a PX domain is always followed immediately by a PH domain, separated only by 5-7 conserved amino acids. This conserved, tandem arrangement suggests structural and/or functional links between the two domains. Like C2 domains, PX and PH domains are well known examples of membrane-targeting domains that bind specifically to lipid ligands in cell membranes (Hurley *et al.*, 2000). Unlike prokaryotic and C2-PLDs, all PXPH-PLDs studied so far have a marked preference for PC substrates. In addition to lipid binding properties, PX and PH domains mediate intermolecular interactions with other proteins.

While C2 domains typically bind abundant membrane lipids, PH and PX domains bind increasingly rare membrane lipids (e.g., lipids present only at specific cellular locales or in response to specific cellular stimuli). This lipid specificity is likely to result in localization of PXP-PLDs to discrete membrane domains. Wholesale deletion of these domains from PLD1 yields an enzyme with somewhat impaired ability to associate with cellular membranes (Chapter II, (Park et al., 1998;Kook and Exton, 2005)). Indeed, PLD1 deletion mutants that lack PX and PH domains distribute diffusely in serum-starved *Cos7* cells. However, these mutants are efficiently recruited to the plasma membrane upon stimulation with phorbol esters (Du et al., 2003).

The energy required to insert proteins into the lipid bilayer often requires cooperativity from multiple membrane-targeting domains. PX and PH domains have specific and non-specific interactions with membrane phospholipids. Nonspecific interactions, while often low energy, can synergize with specific interactions to have profound consequences on the net-interaction energy for membrane binding events. While PX and PH domains are both dispensable for activity and membrane localization of PLD1 and PLD2, appropriate subcellular trafficking of these enzymes requires both membrane-targeting domains.

PLD1 mutants that lack PX and PH domains exhibit slightly increased basal activity *in vivo* (Xie et al., 1998;Park et al., 1998;Sung et al., 1999b) due, in part, to enhanced expression of these mutants. Catalytic properties of PLD1 are essentially unchanged by the deletion of these domains (Chapter III). Chimaeric enzymes demonstrate that N-terminal domains from PLD2 inhibit catalytic domains from PLD1 *in vitro* (Sung et al., 1999a). Together, these findings support a proposed, inhibitory role for PX-PH domains

in PLD1 (Sung et al., 1999a). In contradistinction, PX-PH deletions in PLD2 reduce basal activity *in vivo*.

Phox Homology domains

PX domains were first recognized in NADPH oxidases (p40phox and p47phox), endocytic sorting proteins (sorting nexins, Vam7 SNARE, Bem1), PI 3-kinases (Type II), and PLDs (Ponting, 1996). There are now more than 700 PX domain-containing proteins in public sequence databases. Membrane binding properties have been examined in detail for several PX domains, including PLD1 (Stahelin et al., 2003;Stahelin et al., 2004). PX domains form a compact structure and interact with phosphoinositides via conserved binding pockets. Consistent with low sequence similarity among PX domains, PX domains exhibit different phosphoinositide binding specificities.

Membrane localization

PX domains contribute somewhat to PLD localization. Typical localization of PX domains is to endosomal and trans-Golgi network membranes. PLD1 localizes to cytosolic, vesicular structures in serum-starved Cos7 cells. Upon stimulation, PLD1 rapidly translocates to membrane fractions. Isolated PLD1 PX domains exhibit a very similar trafficking profile. Stimulation is followed by rapid translocation of these domains, from cytosolic structures to the plasma membrane (Du *et al.*, 2003;Lee et al., 2005). PLD1 mutants that lack PX domains differ from wild-type PLD1 by persisting at the plasma membrane after stimulation. Without its PX domain, PLD1 does not cycle

back to the perinuclear vesicle structures as normal after stimulation by platelet-derived growth factor or phorbol ester stimulation (Du et al., 2003).

Lipid-binding specificity

An isolated PX domain from PLD1 binds multiple lipid ligands, including PI 3,4,5-trisphosphate (PIP₃), PI 3-phosphate (PI3P), PI 5-phosphate (PI5P), PA, and phosphatidylserine (Du et al., 2003;Stahelin et al., 2004;Lee et al., 2005). PLD1 PX domains bind lipid bilayers containing cognate lipids. Phosphoinositides bind cooperatively with PA and phosphatidylserine, suggesting multiple, interacting lipid-binding sites. A cluster of basic residues in a conserved lipid-binding pocket (e.g., K117 – K121, R179) forms a polyphosphoinositide-binding site. A secondary site involves a second cluster of basic residues (e.g., R149, R150). Mutations in either binding site greatly decreases phospholipid binding *in vitro* and membrane localization *in vivo*. A R179A mutation ablates PIP₃ binding and PIP₃-dependent activity of PLD1 *in vitro*, and PDGF-dependent activity *in vivo* (Stahelin et al., 2004;Lee et al., 2005).

Interactions with effectors

The PX domains also have functional interactions with PLD regulators. Munc-18-1 associates with both PLD isoforms in brain and potently inhibits the activity of PLD1 and PLD2. Munc-18-1 interacts with PLDs via their PX domains (Lee et al., 2004). Similarly, PLD2 PX domains interact with a PLD2 inhibitor, collapsin response mediator-2 (Lee et al., 2002). A proline-rich sequence in the PLD2 PX domain mediates

a binding interaction between PLD2 and the Src homology (SH3) domain of PLC γ 1 (Jang et al., 2003)

PLD1 deletion mutants that lack PX domains are not regulated by PKCs, though otherwise identical to wild-type PLD1 when assayed *in vivo* or *in vitro* (Park *et al.*, 1998;Hoer et al., 2000). Curiously, mutation of the primary phosphoinositide binding pocket of the PLD1 PX domain selectively disrupts PKC activation *in vitro*, but not *in vivo* (Sung *et al.*, 1999b). Other PX domain mutations uncouple PLD1 from regulation by PKC and cell-surface receptors (Zhang et al., 1999;Du et al., 2000). Mutation of the primary phosphoinositide binding pocket of the PLD2-PX domain disrupts interactions with PKC ζ *in vitro* and *in vivo* (Kim et al., 2005).

Post-translational modification

A potential means of regulation by PKC is to alter the lipid binding properties of PX domains. While protein kinase C α (PKC α) does not bind isolated PLD1-PX domains *in vitro*, PKC α binds sequences located just N- and C-terminal to that domain (Kook *et al.*, 2005). *In vitro*, PKC α phosphorylates the PX domain of PLD1 at Thr 147, a site within the secondary lipid-binding pocket. (Kim et al., 1999b). In Cos7 cells, PLD2 is phosphorylated at an equivalent position (Ser 134) when PKC is activated by phorbol esters. PKC also phosphorylates the PX domain (Thr 99/100) of PLD2 at its primary phosphoinositide binding pocket (Chen and Exton, 2005). Modification of these cationic, lipid binding pockets by phosphorylation certainly changes their electrostatic properties and therefore alter their lipid-binding properties (Chapter IV). Mutation of these sites

does not alter the catalytic properties of PLD1 or PLD2. Nonetheless, lipid-binding properties and membrane localization may be modulated by phosphorylation.

Pleckstrin Homology domains

PH domains are among the most common domains found in the human genome, 469 PH domains are present in 409 human genes. Many relevant PH domain structures are known, including a large number of proteins that participate in signal-transduction pathways with PLD. Structures are known for many regulators of Arf-family GTPases (Mandiyan et al., 1999; Cronin et al., 2004a), regulators of Rho-family GTPases (Worthylake et al., 2000; Lietzke et al., 2000; Rossman et al., 2002; Snyder et al., 2002; Derewenda et al., 2004; Skowronek et al., 2004), phospholipase C- δ 1 (Ferguson et al., 1995), and protein kinase B/AKT (Thomas et al., 2002). Though not required for catalytic activity, PLD1 and PLD2 PH domains have multiple roles.

Most PH domains bind phosphoinositides *in vitro*, albeit with varying degrees of specificity. Phosphoinositides bind a conserved binding pocket formed by basic residues in the first and second β -strands of the β -sandwich PH structure. PH domains also form nonspecific contacts with certain, typically anionic, phospholipids. Membrane electrostatics often drive much of the non-specific interactions. For many PH domains, binding specificities are responsible for partitioning to the plasma membrane and, occasionally, to Golgi membranes (Hurley *et al.*, 2000).

Post-translational modification

PLD1 and PLD2 are palmitoylated at a conserved site within their PH domains. Missing in several yeast PLD isoforms, this palmitoylation site is invariant in other PXXPH-PLDs. When palmitoylation sites are removed by mutation, PLD1 and PLD2 are slightly less membrane-associated (Sugars et al., 1999; Xie et al., 2002). In serum-starved cells, subcellular localization of a palmitoylation-deficient PLD1 mutant is diffuse and cytosolic. However, treatment with serum or phorbol ester translocates PLD1 normally to the plasma membrane. Like PX domain mutants, palmitoylation-deficient PLD1 does not cycle back from the plasma membrane after stimulation (Sugars et al., 2002; Du *et al.*, 2003). Palmitoylation-deficient PLD1 is slightly less active *in vivo*, but is as active as wild-type PLD1 *in vitro* (Sugars et al., 1999; Xie et al., 2002). Palmitoylation-deficient PLD2 exhibits reduced basal activity *in vivo*, but is as active as wild-type PLD2 when stimulated by phorbol esters. In PC12 cells, palmitoylation of PLD1 is required for regulated exocytosis.

Lipid-binding specificity

Isolated PLD1 and PLD2 PH domains weakly associate with membranes and have diffuse distribution in several cell types (Sciorra *et al.*, 2002; Sugars *et al.*, 2002). The PH domain from PLD1 incompletely translocates to the membrane upon stimulation by phorbol ester and certainly does not match the localization pattern of intact PLD1 (Du *et al.*, 2003). Isolated PH domains from PLD1 and PLD2 bind phospholipid bilayers in a PIP₂-dependent manner (Hodgkin et al., 2000; Sciorra *et al.*, 2002). Both domains

principally bind PIP₂. Other anionic phospholipids (e.g., PI 3-phosphate, phosphatidylserine) are less effective.

Membrane localization

Both PLD1 and PLD2 possess conserved residues that form putative phosphoinositide binding sites. Mutation of these residues led to weakened membrane association for both isoenzymes. PLD1 mutants partially distributed to the cytosol (Zhang *et al.*, 1999; Sugars *et al.*, 2002). Mutation of the phosphoinositide binding site in PLD2 resulted in a subtle phenotype. PLD2 mutants remained at the membrane, but were excluded from detergent-resistant microdomains (Sciorra *et al.*, 2002). Deletion of the entire PH domain from PLD1 produces mutants that have catalytic activity equivalent to the wild-type enzyme, but altered subcellular localization (Hoer *et al.*, 2000; Du *et al.*, 2003). These mutants are cytosolic in serum-starved cells, and incompletely targeted to the plasma membrane upon stimulation. PLD1 mutants that lack PH domains do not translocate to the plasma membrane completely upon stimulation by phorbol ester. These mutants also fail to cycle back normally to cytosolic vesicle structures. Beyond these effects on subcellular localization, many mutations within PH domains were deleterious to catalytic activity (Zhang *et al.*, 1999; Hodgkin *et al.*, 2000; Sciorra *et al.*, 2002). However, it is not clear that these mutant enzymes are correctly folded.

Interactions with effectors

PH domains also mediate several protein-protein interactions with PLD regulators. PKC α binds an isolated PLD1 PH domain *in vitro*, and phosphorylates the PH domain of

PLD1 and PLD2 at multiple sites (Kook and Exton, 2005, Chen and Exton, 2005). Mutation of two phosphorylation sites within the PH domain of PLD2 eliminates the phorbol ester-dependent phosphorylation of the enzyme in Cos7 cells. The functional consequences of phosphorylation of this PH domain are unknown. The PH domain of PLD2 is responsible for interactions with aldolase A and C, potent inhibitors of PLD2 activity (Kim et al., 2002). This interaction is regulated by glycolytic metabolites, suggesting a regulatory mechanism for insulin-dependent PLD activity.

Amino termini

Additional regions, unique to the PXP-PLD subfamily, have variable lengths and variable primary sequences. One highly divergent region is present at the extreme N-termini of PXP-PLDs, 30 – 130 amino acids in length. Primary sequences are poorly conserved in closely related species, divergent even in the two human isoforms, PLD1 and PLD2. Deletion of fifty residues from the N-terminus of PLD1 does not change the catalytic properties or membrane-binding properties of the enzyme (Park *et al.*, 1998; Kook and Exton, 2005). This sequence includes a major binding site and phosphorylation site for PKC α *in vitro*, but PKC α still binds and activates PLD1 mutants lacking this region. Mutations to this domain have modest, if any consequences for PLD1 activity and regulation *in vitro* (Zhang *et al.*, 1999; Kim *et al.*, 1999b). PLD1 and PLD2 are tolerant of many modifications and additions (e.g., green fluorescent protein-, glutathione S-transferase-, and epitope tag-fusions). α -Helical structure is predicted for this region of PLD1, while no secondary structure elements are predicted for this region of PLD2 (Chapter IV).

‘Loop’ regions

A second divergent region, 30 – 300 amino acids inserted into the first catalytic subdomain, is present only in PLD1 and its orthologs. Biological functions of these regions are not yet clear. PLD1 possesses a 90 – 128 amino acid insertion in a conserved catalytic subdomain, just C-terminal to the first HKD catalytic motif (depending on alternative splicing, Figure 1). There are no known functional consequences for alternative splicing of this ‘loop region,’ and a number of mutations in this region have no appreciable effect on PLD1 activity and localization (Zhang *et al.*, 1999). A 116 amino acid deletion in this region moderately activates PLD1a *in vitro* and *in vivo*, but has no effect on membrane localization (Sung *et al.*, 1999b; Du *et al.*, 2003). A slightly larger deletion, 148 amino acids, abolishes PLD activity entirely. Insertion of the loop region from PLD1 into PLD2 is moderately disruptive, reducing activity ~50 % (Sung *et al.*, 1999a). These findings led to the hypothesis that this loop region contains a negative regulatory element. The loop region of PLD1b is phosphorylated by PKC α *in vivo* with unknown consequences (Kim *et al.*, 1999b). Some evidence suggests that a portion of this loop region is proteolyzed *in vivo*, producing a polydisperse PLD1 enzyme that retains catalytic activity (Chapter II).

Carboxyl termini

In contrast to N-terminal regions, the C-termini of metazoan PLD isoenzymes have highly conserved sequences. PXP Φ -PLDs from chordates, arthropods, and nematodes have a ‘KE(ϕ)₃P ϕ E ϕ WT’ sequence at their extreme C-termini. Mutations or deletions at any one of the last three positions (even conservative substitutions) dramatically reduced

PLD activity (Zhang *et al.*, 1999;Xie et al., 2000b;Liu et al., 2001a). Expression levels and subcellular localization of these mutants were not altered. Catalytic activity was rescued *in vivo* and *in vitro* by complementation with unmutated C-terminal sequences. Even the hexapeptide, 'PMEVWT,' was effective at restoring catalytic activity. As little as a modification of the terminal carboxyl group has deleterious effects on catalytic activity. In fact, C-terminal modifications have proven to be a useful tool for discovering the role of PLD in cellular signal-transduction cascades and phenotypic changes. Addition of a 29 amino acid epitope tag (e.g., V5/6His) to the C-terminus PLD1 generates a dominant-negative allele that has been used to characterize the roles of PLD1 in actin stress fiber formation, activation of mTOR and protein kinase D, and in tumorigenesis (Kam and Exton, 2001;Kam and Exton, 2004a;Kam and Exton, 2004b;Buchanan et al., 2005).

Splice variants, PLD1a2 and PLD1b2, lack C-terminal sequences altogether and have only residual catalytic activity (Figure 1). These variants remain bound to membranes, but are diffusely localized throughout the cell (Hughes *et al.*, 2001). Because this C-terminal sequence is not conserved in prokaryotic PLDs, structural models based on *Streptomyces* PLD (Chapter IV) cannot be used to predict tertiary structure for this critical region.

Polybasic regions

All characterized PXP_H-PLDs require PIP₂ for activity. While multiple N-terminal domains bind polyphosphoinositides (i.e., PX and PH domains), catalytic regulation is dependent on a 'polybasic region' located in a C-terminal catalytic subdomain (Sciorra et

al., 1999). A conserved sequence, ‘ARD ϕ ARHF ϕ QRWN,’ is found in all PXP_H-PLDs (where ‘ ϕ ’ represents hydrophobic residues). Structural models predict α -helical structure for this region, with arginine residues at the exposed surface of the enzyme (Chapter IV). A current hypothesis is that these basic residues sequester anionic polyphosphoinositides in the membrane bilayer (Papayannopoulos et al., 2005). This appears to be a common mechanism for many PLD isoforms, and sheds light on the interfacial behavior of these enzymes.

Mutations of these arginine residues (underlined) have incremental effects on PIP₂-dependent catalytic activity and PIP₂-dependent phospholipid binding (Sciorra *et al.*, 1999; Sciorra *et al.*, 2002; Du *et al.*, 2003). Single, non-conservative mutations of these residues (e.g., R \rightarrow G) result in reduced activity. Double mutants are not active *in vivo* or *in vitro*. PLD1 and PLD2 mutants (RR \rightarrow GG) retain less than 5% of wild type activity and are not activated by PIP₂. These mutants express at normal levels and are distributed normally, suggesting that global structure is not perturbed. PLD1 and PLD2 double-mutants localize to appropriate membrane structures in serum-starved cells, though slightly more diffuse in the cytosol. When cells are stimulated with phorbol esters, PLD1 mutants (RR \rightarrow GG) fail to translocate to the plasma membrane as normal.

Certain C₂-PLD enzymes are also regulated by PIP₂. C₂-PLDs possess the polybasic region identified in mammalian and yeast isoforms, though amino acid sequences differ slightly. Analogous to PXP_H-PLDs, PIP₂ binds both an N-terminal membrane-targeting domain (C₂) and a polybasic region within the C-terminal catalytic subdomain. PIP₂-dependent regulation of *Arabidopsis* PLD β requires basic residues in a polybasic region (Zheng *et al.*, 2002). In general, PIP₂ dependent PLD activity requires intact polybasic

regions. These polybasic motifs provide a mechanism for direct interaction between PLD catalytic domains and a major PLD regulator, PIP₂.

Regulation

PC-PLDs are known to be signal-activated lipases, analogous to PI- phospholipase C, phospholipase A₂, and sphingomyelinase. The activity of PLD1, in particular, is extensively regulated by a variety of cellular signals. Hormones, neurotransmitters, growth factors, and many other cellular signals (Tables 3 and 4) promote the hydrolysis of PC by PLD in many cell types and tissues. Many agonists act through cell-surface receptors that couple to heterotrimeric G-proteins to initiate signaling cascades that involve PLD. Direct interactions with PIP₂, protein kinase C, GTPases of the Rho and Arf families, and many others modulate the activity of PLD1 *in vivo* and *in vitro*. In contrast, the regulation of PLD2 is not well understood.

Interfacial activation

Phospholipases utilize lipid substrates and activators which form large aggregated structures in water. Consequently, PLDs act in environments where three-dimensional bulk interactions occur in solution and two-dimensional interactions occur at the membrane surface. This reduction in dimensionality involves partitioning of the enzyme to the bilayer interface. Modulation of the partitioning of enzymes and substrates is a common control mechanism for phospholipases, and is known as ‘interfacial activation.’ (Derewenda, 1995). Diffusion to the aggregated lipid substrate is often controlled by tethering the phospholipase to the lipid bilayer using membrane-targeting domains.

Interaction between phosphoinositides and polybasic regions of PLD catalytic domains is crucial in the activation of PLDs. Moreover, many inhibitors mediate their effects on PLD by modulating association with membranes.

Prokaryotic PLD enzymes exhibit varying amounts of interfacial behavior (Yang and Roberts, 2003). Interfacial behavior was noted very early in the study of plant PLDs (Yang *et al.*, 1967). Cabbage PLD activity shows regions of parabolic and hyperbolic kinetics towards short-chain PC species (Allgyer *et al.*, 1979). This anomalous behavior was attributed to the enzyme and substrate segregating into different phases. Human PLD1 and PLD2 isoforms prefer longer-chained PC species as substrates (e.g., palmitoyl-oleoyl PC) (Pettitt *et al.*, 2001). With critical micelle concentrations of nanomolar or less, PLD substrates exist as monomers in exceedingly small amounts. Because their substrates are aggregated, mammalian PLDs require a mechanism to adsorb the membrane surface or to disperse substrate.

Under certain assay conditions, surfactants can activate mammalian PLDs. Non-ionic detergents and long-chained fatty acids are most effective (e.g., Miranol H2M, oleate, and arachidonate). This activation is related to a surfactant's ability to disperse substrate and to solubilize PLD from microsomal preparations (Kobayashi *et al.*, 1991a). Assayed under conditions with high concentrations of surfactants, purified mammalian PLDs exhibit low affinity interaction with substrates ($K_m^{app} \sim 0.3 - 1.5$ mM, Chapter II) and do not respond to cellular PLD activators (Massenburg *et al.*, 1994). It has been reported that unsaturated fatty acids selectively activate PLD2, with only minor effects on PLD1 activity (Kim *et al.*, 1999a). Differential activation by surfactants may reveal that the two

human PLD isoforms prefer substrates in different conformations (e.g., bilayer versus micelle).

Membrane anchors

Access of substrate to the active site can be modulated by tethering an enzyme to the bilayer surface. Bacterial PLDs are typically exotoxins, secreted from pathogenic bacterial strains or released upon cell lysis. These enzymes could not function properly if tethered to the bacterial membrane. Eukaryotic PLDs are intracellular proteins that traffic between membrane structures. As a rule, eukaryotic isoenzymes possess accessory domains that interact with cellular membranes. Membrane-targeting domains are often flexibly linked to the rest of the enzyme, acting as membrane anchors (Hurley *et al.*, 2000). This arrangement confers membrane localization to the enzyme and processivity to the catalyzed reaction.

Eukaryotic PLD superfamily members use different structural motifs for the same purpose: subcellular targeting to discrete membrane domains. These enzymes employ a variety of membrane-targeting domains to enhance association with the lipid bilayer (Figure 3). C2, PX, PH, and polybasic domains are discussed at length above. C2 domains are found in many plant PLDs. Tandem PX-PH domains are found in animals, yeasts, and plants. Polybasic domains are found throughout. Slime molds provide additional examples of convergent evolution. A *Dictyostelium* PLD has putative EF-hand and PH domains at its N-terminus. PLDs found in *Phytophthora* species are anchored to the membrane by multiple transmembrane domains (Meijer *et al.*, 2005). PLD3 (Hu-K4)

isoforms are type II transmembrane proteins, tethered to the membrane by N-terminal transmembrane domains. (Munck *et al.*, 2005).

While it is clear that N-terminal membrane-targeting domains contribute to the subcellular localization and trafficking of eukaryotic PLDs, these domains are not required for catalytic activity *in vivo* or *in vitro*. N-terminal domains (PX-PH) can be removed from PLD1 and PLD2 with limited effect on membrane binding or catalytic activity. Compared to PLD catalytic domains, these accessory domains exhibit low affinity interactions with phospholipid (Table 5). In contrast, polybasic regions are essential for high affinity binding to phospholipid bilayers. Association of PLD and bulk lipid depends upon specific interactions between PIP₂ and polybasic motifs (Sciorra *et al.*, 1999;Zheng *et al.*, 2002;Du *et al.*, 2003). This association is directly related to enzymatic activation by this lipid. A simplistic interpretation is that PIP₂ activates PLD by promoting the binding of the catalytic domain to the bulk lipid phase. These requirements suggest a central role for PIP₂ in PLD activation. PIP₂ may, in fact, be a master regulator of phospholipases D and C. PIP₂ levels may coordinate the sequential actions of these signal-activated phospholipases

Table 3. Interfacial binding properties of mammalian PLDs.

Binding constants were determined using purified PLD (or isolated domains) and sucrose-loaded PE/PC vesicles containing 5 mol% PIP₂. A dissociation constant, K_s^A , is reported in bulk lipid concentration terms. These experiments are described in detail in Chapter III.

| K_s^A , bulk [lipid] | PLD isoenzyme | Reference |
|------------------------|--|------------------------------------|
| 10 μ M | PLD2, full-length | (Sciorra et al., 1999) |
| 10 μ M | PLD2, catalytic domains | (Sciorra et al., 2002) |
| 100 – 300 μ M | PLD2, PH domain | (Sciorra et al., 2002) |
| 1-2 μ M | PLD1, catalytic domains | (Sciorra et al., 2001)(Chapter II) |
| ~ 100-fold reduced | PLD1, R691G,R695G (polybasic domain mutant) | (Du et al., 2003) |

Polyphosphoinositides

The phosphoinositide requirements of many mammalian PLD isoforms have been examined in detail (Brown et al., 1993;Liscovitch et al., 1994;Hammond et al., 1997;Kodaki *et al.*, 1997;Min et al., 1998). When substrate liposomes are prepared from detergent suspensions by gel-filtration (Jiang et al., 2002), residual activity (<15%) could be measured even if PIP₂ was replaced by phosphatidylserine (the final detergent concentration was unknown). In the absence of surfactants, PIP₂ is an essential PLD activator. Phosphatidylinositol 3,4,5-trisphosphate can partially substitute for PIP₂, and other acidic phospholipids are ineffective or nearly so. Because phosphatidylinositol 3,4,5-trisphosphate is efficiently degraded in cellular membranes, it is much less abundant (~100-fold) than PIP₂. PIP₂ is likely to be the relevant activator *in vivo*.

In vitro, PLD activity is steeply dependent upon the surface concentration of PIP₂ in substrate liposomes. Activation profiles are complex, typically biphasic with respect to the surface concentration of PIP₂ and altered further by PLD effectors (Chapter III).

Deletion of N-terminal PX and PH domains do not change the PIP₂-activation profiles of PLD1 or PLD2 (Sciorra *et al.*, 1999;Hoer *et al.*, 2000). This supports the assertion that, while the PX and PH domains of PLD bind phosphoinositides *in vitro*, catalytic regulation principally depends on PIP₂ interaction with catalytic domains.

Effects of PIP₂ on PLD activity are related to the effects of PIP₂ on PLD association with bulk phospholipid. Binding isotherms demonstrate that PLDs bind phospholipid bilayers with high affinity only in the presence of PIP₂ (Chapter III, (Yokozeki *et al.*, 1996;Sciorra *et al.*, 1999;Hodgkin *et al.*, 2000;Sciorra *et al.*, 2001;Du *et al.*, 2003). Activation by PIP₂ (EC₅₀ ~ 2 – 3 mol%) mirrors its effects on vesicle-binding (half-maximal at ~ 2 – 3 mol%). Because PIP₂ reaches a surface density of at least 2 mol% on cytoplasmic leaflets of cellular membranes, this set-point is likely relevant to PLD regulation *in vivo*.

This PLD-phosphoinositide interaction has been disrupted by two, independent experimental strategies. Several primary sequence determinants for this interaction have been defined by molecular biology approaches. Mutations of certain residues in the polybasic region uncouple PLD from regulation by PIP₂ (discussed above). Also, pharmacological/biochemical methods have been used to manipulate levels of PIP₂ available to PLD. Molecules that bind and sequester (or hydrolyze) PIP₂ competitively inhibit PLD, an effect that is reversed by an addition of exogenous PIP₂. Moreover, inhibitors that compete for PIP₂ liberate PLD from liposomes in a concentration-dependent manner. Together, these findings support a central role of PIP₂ in the regulation of PLD.

Aminoglycoside antibiotics, particularly neomycin, inhibit PLD at micromolar concentrations (Liscovitch et al., 1991;Liscovitch *et al.*, 1994). Neomycin inhibits PLD activity in a dose-dependent manner, related to its affinity for PIP₂. Addition of exogenous PIP₂ reverses this inhibition. Furthermore, neomycin's inhibitory effects require PIP₂. When PLD activity is measured in the absence of PIP₂ (substrate dispersed by high concentrations of surfactant), neomycin is ineffective. By competing for phosphoinositides, aminoglycosides indirectly inhibit PIP₂-dependent PLD activity. Supporting the hypothesis that PIP₂ serves as a membrane anchor, neomycin efficiently releases PLD from rat brain microsomes (Yokozeki *et al.*, 1996). This dissociation is also dose-dependent, but requires a higher concentration of antibiotic.

By competing for PIP₂, these inhibitors are nonspecific – acting both on PLDs and PI-PLCs. Several proteins that bind PIP₂ with high affinity inhibit PLDs and PLCs. The cytoskeletal proteins, spectrin and fodrin, competitively inhibit PIP₂-dependent PLD and PLC activities (Lukowski et al., 1996;Lukowski et al., 1998). Similarly, the PH domain of β -adrenergic receptor kinase-1 inhibits PLD activity in a concentration-dependent manner (Yokozeki *et al.*, 1996;Ushio-Fukai et al., 1999). In some studies, the PIP₂-binding protein, gelsolin, shows analogous properties towards PLDs and PLCs (Sun et al., 1997;Banno et al., 1999).

Synaptojanin is a neuronal lipid phosphatase, hydrolyzing both 4- and 5- position phosphates of PIP₂. Its inhibitory effects on PLD (and PLC) activity are directly related to the dephosphorylation of PIP₂. Conversely, PI 4-P 5-kinase 1 α enhances PLD activity (Divecha et al., 2000). PI 4-P 5-kinase associates with PLD1 and PLD2 to provide local generation of PIP₂. Overexpression of PI 4-P 5-kinase 1 α has profound effects on PLD2

activity *in vivo*. Manipulation of PIP₂ levels appears to be a mechanism to coordinate PLD and PI-PLC activity in cellular membranes.

The physical interaction between PIP₂ and the polybasic region of PLD catalytic domains is thought to involve electrostatic components. Salt-bridges often form between basic residues of membrane proteins and phosphate groups of lipids to provide the forces needed to maintain a protein – phosphoinositide complex (McLaughlin et al., 2002). Consistent with such a mechanism, solutions with high ionic strength are able to extract PLD from membranes. Increasing salt concentrations inhibit PLD activity and release PLD from microsomal preparations (Allgyer *et al.*, 1979; Kobayashi *et al.*, 1991a; Brown *et al.*, 1995b).

Surface binding model

Several lines of evidence support a ‘surface binding model’ (Carman et al., 1995) for eukaryotic PLDs. The essence of this model is that the phospholipase remains tethered to the membrane throughout its catalytic cycle. This is possible if a membrane-bound enzyme undergoes conformational rearrangements upon binding activators without dissociating from the membrane surface (Figure 5).

A quiescent PLD enzyme associates with the membrane bilayer in (Figure 5A). Upon binding PIP₂ (Figure 5B), rearrangements occur at the catalytic site to promote interaction with substrate. Allosteric activators bind PLD at the membrane, dramatically enhancing catalytic efficiency (Figure 5C).

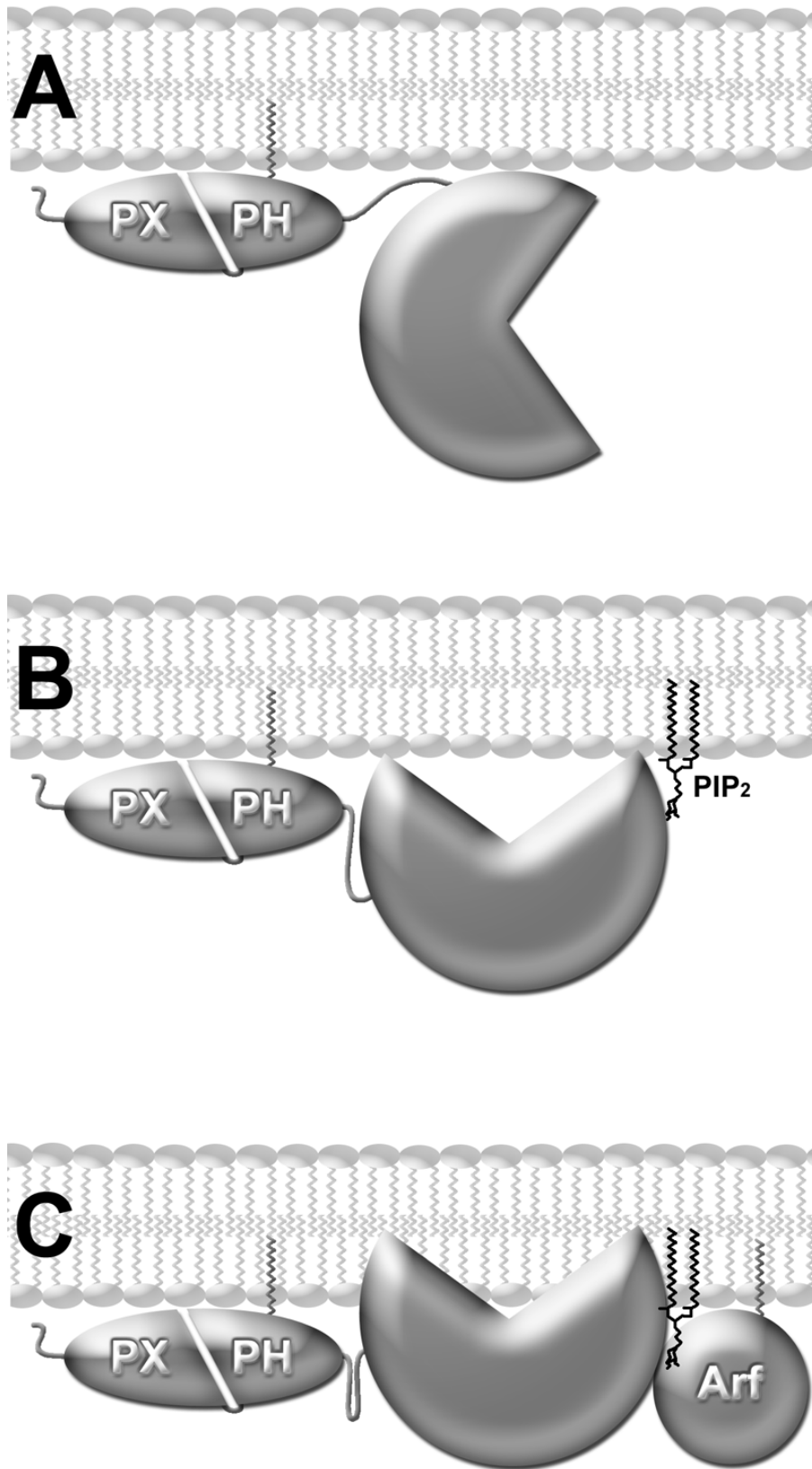


Figure 5. A conceptual model for the activation of PLD.

Several lines of evidence support this model of PLD activation. (i) Biochemical analyses of plant, animal, and yeast isoenzymes find that PLDs are constitutively bound to cellular membranes. (ii) Multiple studies have demonstrated conformational changes in PLD domains upon membrane binding. Conformational changes in the catalytic domains of *Arabidopsis* PLD β and cabbage PLD2 were revealed by circular dichroism spectroscopy and limited proteolysis, respectively (Zheng *et al.*, 2002; Younus *et al.*, 2003). Lastly, (iii) enzyme kinetics studies support the use of a two-step, surface dilution model. PLD activity depends not only on bulk concentration of lipid, but also on surface concentration of substrate (Chapter III, and (Allgyer *et al.*, 1979; Chalifa-Caspi *et al.*, 1998; Qin *et al.*, 2002)).

While partitioning to the membrane is a precondition for catalytic activity, a second binding event (specific binding to substrate) is also required for high-level activity. A sequential, two-step kinetic model uses parameters to describe each binding event. Bulk association with lipid (K_s^A) describes the first step in the model, while the interfacial Michaelis constant, K_m^B , describes subsequent interactions between a membrane-bound enzyme and its substrate.

This kinetic model allows for a mathematical description of a key observation in the regulation of PLD. Conditions that promote complete partitioning to the membrane do not necessarily produce maximal PLD activity. In fact, most activity assays are performed in conditions where the PIP₂ density is sufficient to drive the bulk association step to completion (>98% of enzyme is bound to phospholipid vesicles, Chapter III). Even when maximally bound to substrate vesicles, PLD requires additional activation to reach high level activity. Partitioning to lipid surfaces and kinetic activation are two

separate events in the activation of that enzyme. Kinetic models have been extended to take these interactions into account, and are described in detail in Chapter III.

Protein kinases

PLD is a cellular target of protein kinase C, coupled by direct and indirect pathways. Also, phosphorylation-dependent and phosphorylation-independent pathways are involved in the regulation of PLD isoforms. PKC-PLD interactions are complex, involve multiple domains, and probably include both positive and negative regulation of PLD activity.

In most cell types, calcium ionophores (e.g., A23187) and phorbol esters (e.g., 4 β -phorbol 12-myristate 13-acetate, 12-O-tetradecanoyl-phorbol-13-acetate) efficaciously enhance PLD activity. Both activators implicate calcium-dependent protein kinase C (PKC) isoforms. In fact, pharmacological downregulation of PKC (e.g., chronic stimulation, RNAi), DAG-binding site inhibitors (e.g., bryostatin I, calphostin C), and PKC catalytic inhibitors (e.g., Ro-31-8220, staurosporine, bisindolylmaleimide I, Gö6067, chelerythrine) all block the effects of phorbol esters on PLD activity. These inhibitors provide pharmacological evidence to implicate certain PKC isoforms in a number of receptor-activated signal-transduction pathways (Exton, 1997).

Experiments performed *in vitro* demonstrate that certain classical PKC isoforms ($-\alpha$, β I, $-\beta$ II) directly activate PLD1, but novel isoforms ($-\delta$, $-\epsilon$, $-\eta$) or atypical isoforms ($-\zeta$) are ineffective (Singer et al., 1996; Min et al., 1998; Sciorra et al., 2001). Purified PLD1 is potently activated by PKC α and $-\beta$. This activation does not require phosphorylation, and involves multiple PKC domains (Singer et al., 1996; Min et al., 1998; Hu and Exton,

2003). Although PKC α activates PLD2 *in vivo* (Slaaby et al., 2000;Chen and Exton, 2004), this effect has not been demonstrated *in vitro* (Chapter II, (Colley et al., 1997b;Sciorra *et al.*, 2001)). PKC regulatory domains (C1 and C2 domains) are sufficient to activate PLD1, though full effects require both regulatory and catalytic PKC domains (Hu *et al.*, 2003). *In vivo*, however, many classical and novel PKC isoforms regulate PLD activity in a phosphorylation-dependent manner (Kim *et al.*, 1999b;Mwanjewe et al., 2001;Han et al., 2002). Direct and indirect mechanisms are likely involved.

PKC α phosphorylates PLD1 at multiple sites *in vitro*, and this phosphorylation appears to inhibit phospholipase activity (Hammond *et al.*, 1997;Min *et al.*, 1998;Kim *et al.*, 1999b). In fact, activation of PLD1 by phorbol ester precedes phosphorylation by at least 30 min (Hu *et al.*, 2003). The time-course of PLD1 phosphorylation correlates more closely with PLD inactivation after a prolonged stimulation. An attractive hypothesis is that PKC phosphorylates PLD at sites within membrane-targeting domains to direct the subcellular trafficking of PLD. Without altering catalytic properties, PLD is sequestered from its activators.

PKC α directly binds multiple PLD1 domains to enhance its phospholipase activity. PKC α co-immunoprecipitates with N-terminal and C-terminal PLD1 fragments (Min and Exton, 1998;Sung *et al.*, 1999b;Kook *et al.*, 2005). Interactions with both PLD regions are required for full activation by PKC *in vivo* and *in vitro* (Chapter III). PLDs generate phosphatidic acid and, ultimately, diacylglycerol upon activation, potentially leading to the subsequent activation of conventional and novel PKC isotypes. PLD1 and PLD2

isoforms activate atypical PKC isoforms η and ζ , respectively (Mwanjewe *et al.*, 2001; Kim *et al.*, 2005). Again, direct and indirect mechanisms are implicated.

A related kinase, protein kinase N, directly activates PLD in a manner similar to PKC (Oishi *et al.*, 2001). Protein kinase N binds PLD1 *in vitro* and enhances phospholipase activity by a phosphorylation-independent mechanism. Fatty acids and the monomeric G-protein RhoA are direct effectors of PLD and protein kinase N. v -Src and a casein kinase-2-related kinase bind and phosphorylate PLD1. Epidermal growth factor receptors bind and phosphorylate PLD2 in a ligand-dependent manner (Slaaby *et al.*, 1998; Ganley *et al.*, 2001; Ho *et al.*, 2005). As before, phosphorylation of PLD1 does not, *per se*, influence phospholipase activity.

Monomeric G-proteins

Many members of the Ras superfamily of monomeric G-proteins activate PLD. Rho-family GTPases selectively activate PLD1 via defined interactions with a PLD catalytic domain. Arf-family GTPases activate PLD1 and PLD2, although direct interactions have not been demonstrated. Aside from their direct actions on PLD1, both Arf and Rho GTPases stimulate PI 4P 5-kinase to generate the essential PLD activator, PIP₂. Ras and Ral GTPases activate PLD *in vivo*, but their effects do not appear to be direct. In each case, monomeric GTPases require GTP-binding and post-translational modification (acylation) for full potency and efficacy towards PLD.

ADP-ribosylation factors

Potent PLD activators were purified from the cytosol of HL60 cells and identified as ADP-ribosylation factors (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). In subsequent work, it was shown that myristoylated Arf-1, -3, -5, and -6 equally activate PLD1 (Massenburg *et al.*, 1994; Brown *et al.*, 1995a; Tsai *et al.*, 1998). Arf-like protein 1 (Arf-1) is slightly less effective towards purified PLD (Hong *et al.*, 1998). Arf is equipotent towards PLD1 and PLD2, though PLD1 responds with much higher specific activity (Chapter II, (Lopez *et al.*, 1998)). Arf-1 binds membranes in a GTP-dependent manner, and its translocation to membranes is critical for PLD activation.

Overexpression of arfaptin, a binding protein for Arf, inhibits PLD activation *in vivo* (Williger *et al.*, 1999). Brefeldin A inhibits several guanine nucleotide exchange factors (GEFs) for Arf GTPases. Treatment with Brefeldin A inhibits the effects of many cell-surface receptors on PLD (Exton, 2002). Expression of dominant negative forms of Arf-1 and Arf-6 decoupled cellular stimuli and PLD activation (Shome *et al.*, 1998). These experiments implicate Arf in many signal-transduction networks that regulate PLD activity.

Arf-1 activates PLD *in vitro* with low potency ($EC_{50} \sim 5 \mu\text{M}$, Chapter III). This implies a low affinity interaction. In fact, a lipid bilayer may be required to promote a ternary interaction. Measured by surface plasmon resonance, Arf-1 binds GST-PLD1 with a calculated affinity of $\sim 0.7 \mu\text{M}$ (Powner *et al.*, 2002). Curiously, this binding interaction was not dependent on GTP γ S. Arf activates PLD1 and PLD2 via interactions that do not require N-terminal PLD domains. Deletion of N-terminal (PX-PH) domains has no effect on Arf-dependent activation of either PLD isoform. Several sites that are

essential for PLD activation have been mapped to the N-terminal portion of Arf-1. Indirect activation is also possible. Electron paramagnetic resonance studies demonstrate that Arf has profound effects upon membrane structure and lipid order, suggesting membrane dynamics and/or specific lipids may play a role in mediating Arf's ability to activate PLD1 (Ge et al., 2001; Ge et al., 2003).

Rho GTPases

An additional GTP γ S-dependent factor was identified in the cytosol of neutrophils based on its ability to activate purified PLD. This factor was inhibited by the GDP dissociation inhibitor, Rho GDI (Bowman et al., 1993). It was subsequently shown that Rho GTPases directly activate PLD1 but not PLD2 (Colley *et al.*, 1997b; Lopez *et al.*, 1998). To date, all members of the Rho family that have been tested with PLD1 are able to stimulate its activity. RhoA, RhoB, Rac1, Rac2, and Cdc42 activate PLD1 in a GTP γ S-dependent manner, albeit with different efficacies (Hammond *et al.*, 1997; Bae et al., 1998).

C3 exoenzyme of *Clostridium botulinum* ADP-ribosylates RhoA, and toxins A and B of *Clostridium difficile* monoglucosylate Rho GTPases. Both agents have been used to attenuate PLD responses to numerous cellular stimuli (Exton, 2002). Similarly, dominant negative mutants of RhoA, Rac1, and Cdc42 (Hess et al., 1997; Plonk et al., 1998; Walker et al., 2000b) have been used to interrupt cellular signaling to PLD.

Some primary sequence determinants for Rho activation of PLD1 have been defined by mutagenesis studies. Multiple residues, dispersed across the C-terminal catalytic domain of PLD1, are involved in an interaction with RhoA (Du *et al.*, 2000; Cai and

Exton, 2001). In models of predicted PLD structure, these residues form a coherent binding site for Rho GTPases (Chapter IV). Individual residues were identified in the activation loop and Rho-insert loop of RhoA and Cdc42 that are involved in activating PLD1 (Bae *et al.*, 1998;Walker *et al.*, 2000b;Walker and Brown, 2002).

Ras and Ral

Ras does not activate PLD *in vitro*, yet activated H-Ras strongly stimulates PLD *in vivo* (Jiang *et al.*, 1995a;Bae *et al.*, 1998;Xu *et al.*, 2003). This activation involves multiple pathways with multiple signaling intermediates, including Raf-1, PI 3-kinase, RalA, and Arf6. In fact, PLD is critical for several of the transforming effects of Ras in cells and tissues (Lu *et al.*, 2000;Buchanan *et al.*, 2005). Although RalA has no intrinsic activity towards PLD *in vitro*, Ryu and coworkers report that RalA potentiates PLD1 activation by Arf-1 (Kim *et al.*, 1998). RalA and PLD1 associate in cell lysates, but this association did not, *per se*, lead to PLD activation (Jiang *et al.*, 1995b;Luo *et al.*, 1997). A recent report identified a transient interaction between dynamin and PLD2 after epidermal growth factor receptor activation (Park *et al.*, 2004). This interaction is GTP-dependent, and correlates with a rapid response of PLD in transfected cells. A dominant negative dynamin mutant does not bind or activate PLD2.

Synergy between activators

Multiple signaling pathways converge upon PLD cells to regulate the generation of PA from the hydrolysis of PC. Accordingly, multiple signaling molecules converge upon PLD isoforms to directly modulate catalytic activity. In certain combinations, these

effectors synergize to greatly activate PLD. Very early in the study of PLD regulation, it was recognized that multiple effectors act to enhance PLD activity in a cooperative fashion (Irving and Exton, 1987; Qian and Drewes, 1989). Arf GTPases synergize with many other activators to stimulate PLD1. The requirements for synergy between Arf and other PLD1 activators are explored in detail in Chapter III.

Several proteins have no intrinsic ability to activate PLD, but are able to potentiate the effects of Arf on PLD. The ternary complex of Ral-Arf-PLD is discussed above. Other multi-activator complexes have been examined. Like RalA, G_{M2} ganglioside activator does not activate PLD by itself, but enhances the effects of Arf on PLD1 and PLD2. G_{M2} ganglioside activator enhances Arf1-stimulated activity, but has no effect on PKC-stimulated activity. Nanomolar concentrations of G_{M2} ganglioside activator are sufficient to increase the potency of Arf 5 to 10-fold *in vitro*. Interestingly, immunoprecipitation of G_{M2} ganglioside activator reveals a ternary, G_{M2} ganglioside activator-Arf-PLD complex (Nakamura et al., 1998).

Amazingly, surface plasmon resonance experiments demonstrate a quaternary complex of PKC α , Arf-1, Rac1, and PLD1 (Powner *et al.*, 2002). This PKC-Arf-Rac-PLD complex is formed by sequential binding events without regard to the order of addition. This suggests that each of these binding partners interact with PLD1 independently, and that the activator binding sites do not communicate.

Cytoskeleton

A highly conserved role of PLD is revealed by its interactions with the cytoskeleton. α , β , and γ -actin potently inhibit PLD1 and PLD2 (Lee et al., 2001; Kusner et al., 2002).

Direct binding interactions involve the C-terminal PLD catalytic domains, and do not involve PIP₂. The requirements for this binding interaction appear to be conserved across evolution (Kusner et al., 2003). Purified bacterial (*Streptomyces*), plant (*Arabidopsis* and *Brassica*), and human PLD isoenzymes bound actin with high affinity, and exhibited similar regulated properties. In each case, monomeric globular (G)-actin potently inhibited PLD activity, while polymerized filamentous (F)-actin augmented PLD activity. PLD1 localizes to the acrosomal region of bovine sperm, and PLD is thought to be required for rapid actin polymerization during capacitation (Breitbart et al., 2005). The actin-PLD binding interaction has been mapped to a region of the PLD catalytic domains that include the polybasic, PIP₂-binding region.

The same region of PLD mediates an interaction with microtubule components, α - and β -tubulin (Chae et al., 2005). Analogous to regulation by actin, monomeric tubulin inhibits PLD2, while polymerized tubulin activates PLD2. The microtubule-stabilizing agent, taxol, modestly activates PLD2, while a microtubule-disrupting agent, nocodazole, modestly inhibits PLD2. Because the domain that mediates this binding interaction contains the conserved polybasic region, the authors suggest that tubulin inhibits PLD by occluding the PIP₂ binding site in the enzyme's catalytic domain.

Production of PA by PLD is thought to stabilize microtubules. A clear demonstration of the evolutionary conservation of these interactions, PLD can be purified from tobacco by tubulin-affinity chromatography. In tobacco, PLD δ localizes with tubulin, and is reorganized by taxol treatment (Gardiner et al., 2001).

Other actin-regulating proteins, spectrin, fodrin, and gelsolin, have effects on PLD based upon their interactions with PIP₂ and were described previously. α -Actinin

associates with PLD in cardiac tissue, binding an N-terminal region of PLD2 (Park et al., 2000). Purified actinin potently inhibits PLD activity, an effect that is reversed by the addition of Arf-1.

Presynaptic terminals

Several scaffolding proteins found in presynaptic terminals are inhibitors of PLD1 and PLD2. Synaptojanin, described previously, inhibits PLD by dephosphorylating PIP₂. The clathrin assembly protein, AP180, is a potent PLD inhibitor found in brain tissue (Lee et al., 1997a). AP180 specifically binds and inhibits PLD1 *in vitro*. Also purified from brain, amphiphysin I and II are clathrin coat proteins that inhibit PLD1 and PLD2 at nanomolar concentrations via direct interactions (Lee et al., 2000a). α -, β -, and γ -synucleins bind the N-terminal PX-PH domains of PLD1 and PLD2 and inhibit PLD activity *in vivo* and *in vitro*. Mutant forms of α -synuclein that are associated with familial Parkinson's disease (A53T, and A30P) are even more potent inhibitors (Jenco et al., 1998; Ahn et al., 2002; Payton et al., 2004). Perhaps these scaffolding proteins sequester PLD at nerve terminals, coordinating the vesicular release of neurotransmitters.

Potential Roles of Phospholipase D

Vesicle trafficking and regulated exocytosis

The presence of PLD and Arf on Golgi membranes suggests a role for PLD in vesicle trafficking. PLD reportedly has a central role in the formation of coated vesicles in the Golgi (Ktistakis, 1998). PLD (*spo14p*) is not required for Golgi functions in vegetative yeast, but is critical for membrane-trafficking events in budding yeast (Bankaitis and Morris, 2003; Freyberg et al., 2003). PA levels promote the release of vesicles from the trans-Golgi network in permeabilized murine cells (Siddhanta and Shields, 1998) and the recruitment of many proteins involved in vesicle trafficking (Manifava et al., 2001). Both findings suggest that PLD participates in the regulation of these events. Moreover, PLD and its regulators colocalize at secretory granules, and cell stimulation leads to translocation to the plasma membrane. Overexpression of PLD1 was found to increase exocytosis in PC12 cells, while expression of catalytically inactive PLD1 inhibited exocytosis. Without a means to specifically inhibit mammalian PLDs, pharmacologically or genetically, the roles of mammalian PLD in Golgi functions are not definite.

One major role for PLD is the translocation of the glucose transporter, Glut-4, to the plasma membrane (Huang and Frohman, 2003; Huang et al., 2005). Microinjection of PLD into adipocytes potentiates the effects of insulin on Glut-4 translocation. Glut-4 vesicles bud from perinuclear structures and traffic to the plasma membrane to enter the general endosome recycling system. PLD1 and PLD2 are implicated in these steps and in the insulin-dependent fusion of these vesicles with the plasma membrane to activate Glut-4. PLD is also implicated in endocytotic processes. Internalization of epidermal growth

factor receptors, angiotensin II receptors, and opioid receptors depend on PLD activity (Shen et al., 2001; Du et al., 2004; Koch et al., 2004).

Vaccinia and related poxviruses replicate in infected cells, and co-opt the vesicle trafficking machinery to encapsulate and release virions. Immature virions are wrapped in an envelope derived from virus-modified trans-Golgi or endosomal membranes. These viruses supply their own PLDs, distantly related members of the PLD superfamily. The F13L protein has many of the characteristics of mammalian PLDs, including sensitivity to primary alcohols and a requirement for intact HKD catalytic motifs (Husain and Moss, 2002; Husain and Moss, 2003). F13L protein cycles between early and late endosomes and the plasma membrane, consistent with its participation in endocytic and exocytic pathways. Transfection of this protein induces the production of post Golgi vesicles, and recapitulates many of the vesicle trafficking roles of native PLDs.

In many cell types, primary alcohols act to inhibit secretion. While the production of phosphatidylalcohols is a specific marker for PLD activity, effects of alcohols on cells are not specific. In general, the use of alcohols as to inhibit PLD is problematic due to off-target effects.

Cytoskeletal regulation

PLD couples the actin cytoskeleton to signal-transduction networks. This link is bidirectional, simultaneously regulating PLD activity and cytoskeletal structure. These interactions with the actin cytoskeleton are crucial to PLD's roles in cell shape, motility, chemotaxis, cell division, and vesicle trafficking. Rho-family GTPases are also involved (Exton, 1997). Rho mediates stress fiber formation and neurite retraction. Rac is

involved in membrane ruffling and generation of lamellipodia. Cdc42 induces filopodia formation. All three stimulate PLD activity. Exogenous PLD and PA stimulated actin polymerization and stress fiber formation in fibroblasts (Ha and Exton, 1993). Expression of dominant negative PLD1 inhibited agonist-dependent stress fiber formation and α -actinin translocation (Kam *et al.*, 2001). Both lines of investigation support a role for PLD, mediating certain downstream effects of Rho GTPases on the cytoskeleton.

Direct targets of phosphatidic acid

A growing list of cellular targets for PA includes: NADPH oxidase, PI 4-phosphate 5-kinase, mTOR, atypical PKC isoforms, and protein phosphatase 1 (Table 6). A representative example is NADPH oxidase: In neutrophils, PLD participates in generation of toxic O₂ metabolites, a host defense mechanism against microorganisms (Exton, 1997). PA, generated by PLD, directly activates NADPH oxidase. NADPH oxidase is further activated by diacylglycerol and the actions of PKC and a PA-activated kinase.

Table 4. Protein targets of phosphatidic acids.

| Enzymatic Activity | Target | Reference |
|---------------------------------|---|-----------------------------|
| Kinase | Raf-1 kinase | (Ghosh et al., 1996) |
| | mTOR | (Fang et al., 2001) |
| | β ARK1,2 | (DebBurman et al., 1995) |
| | PKC α | (Limatola et al., 1994) |
| | PKC δ | (Aris et al., 1993) |
| | PKC ζ | (Nakanishi and Exton, 1992) |
| | PKN | (Khan et al., 1994) |
| | PIP5-kinase | (Jenkins et al., 1994) |
| | PAK1 | (Bokoch et al., 1998) |
| PARK | (McPhail et al., 1999) | |
| Phosphatase | PP1 γ | (Jones and Hannun, 2002) |
| | myosin phosphatase | (Ito et al., 1997) |
| | SHP-1 | (Frank et al., 1999) |
| | PDE4D3 | (El Bawab et al., 1997) |
| Phospholipase | PLC β 1 | (Litosch, 2002) |
| | PLC γ 1 | (Zhou et al., 1999) |
| | PLD, <i>S. chromofuscus</i> | (Geng et al., 1998) |
| Ion Channels/Transporter | Na ⁺ /H ⁺ exchanger | (Goel et al., 2000) |
| | calcium channel | (Siddiqui et al., 2000) |
| Membrane Transport | β -cop coatomer | (Manifava et al., 2001) |
| | Arf | (Manifava et al., 2001) |
| | NSF | (Manifava et al., 2001) |
| | Kinesin | (Manifava et al., 2001) |
| Miscellaneous | p22 ^{phox} | (Palicz et al., 2001) |
| | p47 ^{phox} | |
| | n-chimaerin | (Ahmed et al., 1995) |
| | Rac-GDI | (Chuang et al., 1993) |

Production of lysophosphatidic acid, diacylglycerol, and acetylcholine

Many potent signaling molecules are generated by the secondary metabolism of PA. In cells, PAs are rapidly hydrolyzed by lipid phosphate phosphatase to generate diacylglycerols or by phospholipase A₂ to generate lysophosphatidic acids. PLDs are thought to generate a 'late-phase' of diacylglycerol accumulation in agonist-stimulated cells (Exton, 2002). This wave of diacylglycerols follows a transient accumulation from the rapid hydrolysis of PIP₂ by PI-PLCs. Presumably, both phases of diacylglycerol production serve to activate PKC isoforms.

Lysophosphatidic acid is released from many cells, and is the major mitogenic component of serum. Lysophosphatidic acid is also generated directly by a class of lysophospholipase D enzymes. Regulation of lysoPLDs is not well understood.

The synthesis of the neurotransmitter, acetylcholine, in cholinergic neurons requires a supply of free choline. Choline liberated by the activity of PLD2 was shown to be important for acetylcholine synthesis in murine basal forebrain cells (Zhao et al., 2001). Because choline exists in cells at relatively high levels, generation of choline from PC is probably not a major source for acetylcholine synthesis.

Survival and mitogenesis

Many studies implicate PLD and its metabolites in the regulation of cell survival pathways. Many signaling networks coordinate PLD activity and mitogenic signals, and PLD appears to have opposing effects on apoptosis. In certain cells, PLD activity is pro-apoptotic. In others, PLD activity promotes cell survival and mitogenesis. Treatment of cells with exogenous PLD or PA stimulates DNA synthesis (Fukami and Takenawa,

1992;Kondo et al., 1992). Also, PLD1 activity leads to MAPK activation (Hong et al., 2001). However, treatment with actinomycin D, TNF- α , and H₂O₂ stimulates PLD activity and apoptosis in Jurkat cells (Kasai et al., 1998).

PLD has an emerging role in the signaling networks that regulate cell growth and proliferation via the mammalian target of rapamycin (mTOR). mTOR is directly activated by PA. Inhibition of PA formation by PLD reduces mTOR-dependent phosphorylation of transcription factors involved in mitogenesis (Fang *et al.*, 2001;Fang et al., 2003;Kam *et al.*, 2004b). Overexpression of PLD1 or addition of exogenous PA increased mTOR-dependent signaling. Inhibition of PLD by high concentrations of primary alcohols, RNAi, or expression of dominant negative PLD1 led to reduced mTOR-dependent signals. Furthermore, disruption of PLD1 activity was able to decouple Rho-family GTPases from mTOR. Many parallel, redundant pathways connect cellular stimuli to mTOR and its mitogenic actions, PLD appears to participate in a Rho/Cdc42-dependent branch of this network.

CHAPTER II

PURIFICATION AND CHARACTERIZATION OF PHOSPHOLIPASE D1

To date, only the secreted PLDs (i.e., GPI-PLD and autotaxin) have been purified to homogeneity. From even the earliest attempts to purify mammalian PC-PLDs (Taki and Kanfer, 1979), it was apparent that purification is hindered by enzyme aggregation, thermoinstability, and anomalous behavior during chromatographic separation. PC-PLD was successfully (2,200-fold) purified from porcine lung by six sequential chromatographic steps (Okamura and Yamashita, 1994). Because PLD2 predominates in lung (Appendix A) and because the purified preparation was stimulated by oleate, this purified isoform was likely PLD2. A PC-PLD was also partially purified (10,000-fold) from porcine brain by eight sequential chromatographic steps (Brown et al., 1995a). Biochemical properties of the purified enzyme implicate the presence of PLD1. In both cases, yields were far less than 1%. Modern methods (e.g., molecular biology and heterologous expression) now permit purification of PLD isoforms to apparent homogeneity with yields greater than 12 % of the total PLD expressed in cells.

Expression of full-length phospholipase D1

Attempts to purify PLDs from natural sources are hindered by an inability to resolve PLD isoforms by chromatography, and by a lack of high-expression sources. Several mammary carcinoma cell lines exhibit high basal PLD activity, though it is not clear

which isoform predominates (Eisen and Brown, 2002). An expression strategy was developed to generate large amounts of active PLD in heterologous sources.

Recombinant plant PLD isoenzymes are routinely purified from bacterial expression sources. Expression levels of plant PLDs in *E. coli* are typically low, as are the specific activities of the purified enzymes. Mammalian PLDs also express poorly in bacteria, and bacterially expressed PLDs lack catalytic activity. Mammalian PLD2 has been successfully expressed in yeast, but at low levels (Kodaki *et al.*, 1997). High-level expression (>10 mg/L) of cabbage PLD was achieved by expression in baculovirus-infected insect cells (El Maarouf *et al.*, 2000). In the work described in this chapter, relatively high expression levels of recombinant PLD1b were achieved in transiently transfected Cos7 cells and baculovirus-infected insect cells.

To generate sufficient quantities of purified mammalian PLD1 for biochemical and structural analyses, expression and purification methods were optimized. Multiple mammalian and insect cell lines were screened to identify an ideal expression source. Although stable cell lines have occasionally been established (Czarny *et al.*, 1999; Min *et al.*, 2001), high level expression of PLDs appears to be universally toxic to cells. Even bacteria are intolerant of PLD overexpression, cells are not viable if PLD is expressed at more than ~2-fold above basal levels (Mishima *et al.*, 1997). Optimal expression conditions appear to be a compromise between absolute expression levels and cell viability.

Transient expression of full-length PLD1 in cultured cells

PLD1 expression vectors were constructed with a PLD1b open reading frame cloned from a rat brain cDNA library (Park et al., 1997). A mammalian expression vector (pcDNA3.1HisA, Invitrogen) was modified to add a canonical FLAG® epitope (i.e., DYKDDDK) for N-terminal fusion to PLD1. A cytomegalovirus promoter in the vector backbone drives transcription in mammalian cells. PLD1b expression levels were slightly higher in Cos7 cells than in other mammalian cells tested, and liposomal transfection (Fugene6, Roche) was more successful than other techniques (calcium phosphate, DEAE-dextran, lipofectAMINE). In small-scale experiments (fewer than 10^6 cells), expression levels were equivalent to those achieved in baculovirus-infected insect cells. Unfortunately, these expression conditions did not scale up to larger experiments.

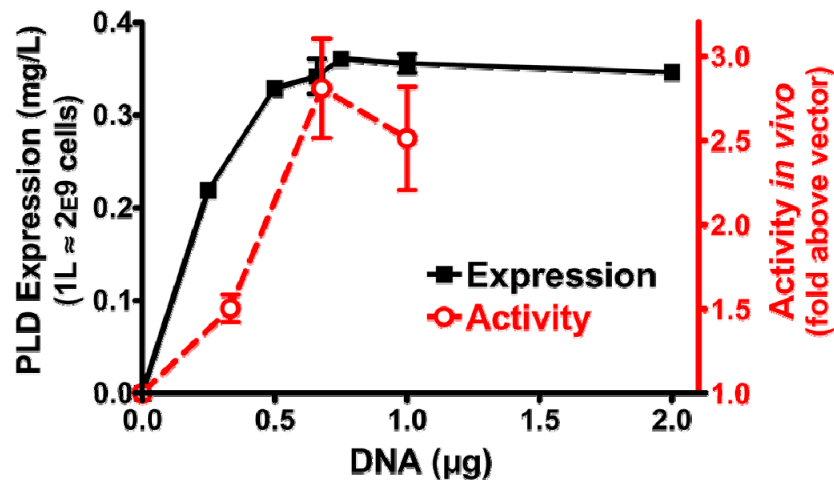


Figure 6. Expression profile for PLD1b in Cos7 cells after transient transfection

Expression was estimated by comparing α FLAG-M2 immunoreactivity in cell lysates and purified standards. Plasmid vector DNA encoding 6His/FLAG.PLD1b was combined with liposomal transfection reagent (Fugene6) and incubated with Cos7 cells for 48 h in DMEM-h supplemented with 10 % fetal bovine serum. Activity was measured in intact cells by standard methods described below (see *in vivo* activity assay using endogenous substrate).

Based on migration in polyacrylamide gels, the molecular weight of the expressed protein was ~113 kilodaltons (equivalent to the predicted molecular weight of 119 kilodaltons). Cell lysates were separated into cytosolic and crude membrane fractions in order to assess the subcellular distribution of PLD1b. Cos7 cells were harvested 48 h after transfection and washed twice with ice-cold lysis buffer (25 mM Hepes, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM of dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). Cells were then resuspended in lysis buffer and disrupted by passing through a 27-gauge needle ten times. Cell lysates were first centrifuged at 500 x g for 10 min to remove unbroken cells. The supernatant was then centrifuged at 100,000 x g for 45 min at 4 °C to separate the cytosolic and crude membrane fractions. The crude membrane fraction was washed with the lysis buffer and resuspended with a 27-gauge needle. The Bradford method was used to estimate the amount of total protein in each fraction. The amount of PLD1 present in each fraction was estimated by immunoblot, using densitometry to compare intensities of immunoreactive bands from samples and purified standards.

Recombinant PLD1 was primarily found in the membrane fraction of Cos7 cells. Less than 8 % of the overexpressed PLD1b was recovered in the cytosolic fraction. This distribution was not changed by serum-starvation or by pretreatment with cellular agonists, lysophosphatidic acid (1, 3, or 10 min) or PMA (3, 10, or 30 min). A number of detergents were screened based on their abilities to solubilize PLD from cell extracts. Nonionic detergents were employed at concentrations 1.5 – 5-fold higher their critical micelle concentrations. Triton X-100 (0.1-0.5% v/v) was slightly more effective than Nonidet P-40 (0.1% v/v) or *N*-octyl- β -D-glucopyranoside (1% v/v). Approximately 12 %

of total PLD in Cos7 lysates was solubilized by Triton X-100, a 1.5-fold enhanced yield. Still, the majority of PLD (~ 88 %) remained insoluble.

Expression of PLD1 in baculovirus-infected insect cells.

Rat PLD1b cDNA (Park *et al.*, 1997) was inserted into a baculovirus transfer vector (pBlueBacHis2A, Invitrogen), modified to possess an N-terminal 6His-FLAG® epitope. This transfer vector and linearized AcMNPV viral DNA (Invitrogen) were cotransfected into *Spodoptera frugiperda* (Sf21) cells by liposomal transfection (Cellfectin®, Invitrogen) according to the manufacturer's instructions. Recombinant virus was isolated by three rounds of plaque selection and amplified in Sf21 cells adapted to suspension culture in *Trichoplusia ni* medium-formulation Hinks (TNM-FH) supplemented with 10 % fetal bovine serum. Plasmid constructs and baculovirus DNA were sequenced to verify coding regions.

Several insect cell lines were evaluated based on their ability to express recombinant PLD1. PLD1 was not secreted into the media of infected Hi5 cells (Invitrogen) and expression levels were highest in monolayer cultures of Sf21 or Sf9 cells grown in TNM-FH media. Due to toxic effects of the PLD1 virus, protein yields were reduced at high multiplicities of infection (MOI > 1). Consequently, PLD expression was biphasic with respect to the amount of viral stock added (Figure 7).

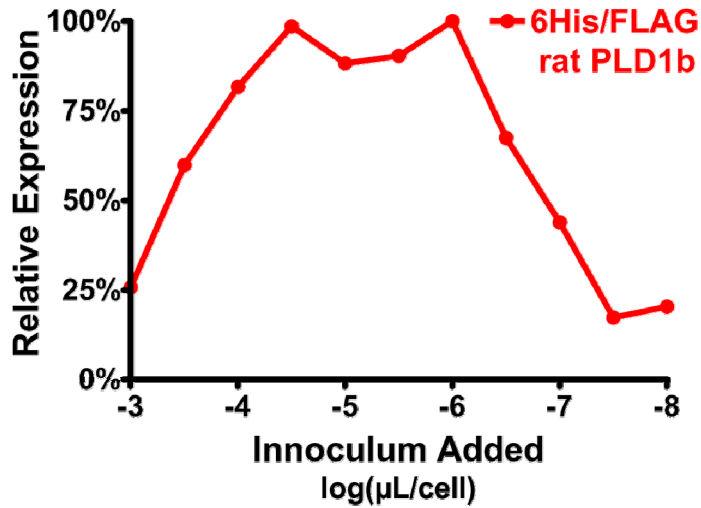


Figure 7. Expression profile for PLD1b in Sf21 cells after baculovirus infection.

PLD expression levels were estimated by comparing the immunoreactivity (α FLAG-M2) present in cell lysates and purified standards. Recombinant baculovirus was serially diluted and used to infect monolayer cultures of Sf21 cells (a limiting-dilution assay to determine baculovirus titer). Cells were lysed 72 h after infection and PLD1b expression was estimated by immunoblot and densitometry.

Properties of PLD1 expressed in insect cells were very similar to those of PLD1 expressed in Cos7 cells. PLD1 partitioned to membrane and cytosolic fractions in Sf21 cells at the same ratios measured in Cos7 cells (8 – 10 % in cytosolic fraction). Approximately 12 % of total expressed PLD1 could be extracted by detergent. Since PLD activity was higher in soluble fraction than in the particulate fraction (Min et al., 1998), it is likely that much of the overexpressed, insoluble PLD1 is misfolded or aggregated. Expression levels were equivalent, typically ~ 0.2 – 0.3 mg/L (where one L cultures typically contain 2E9 cells). However, these conditions could be easily scaled without loss of expression.

Purification of full-length phospholipase D1

Full-length PLD was purified from detergent extracts of Cos7 or Sf21 cells. A phosphate buffer system was chosen based on its ability to extract peripheral membrane proteins (Hjelmeland, 1990). High ionic strength (0.4 M NaCl) was also useful in early purification steps, but had destabilizing effects on PLD in later purification steps. Because speed and temperature are critical to purification of this protein, rapid capture was achieved by affinity methods. Denaturing conditions (8M urea) did not enhance purification. Furthermore, attempts to refold denatured PLD were unsuccessful and catalytic activity could not be recovered.

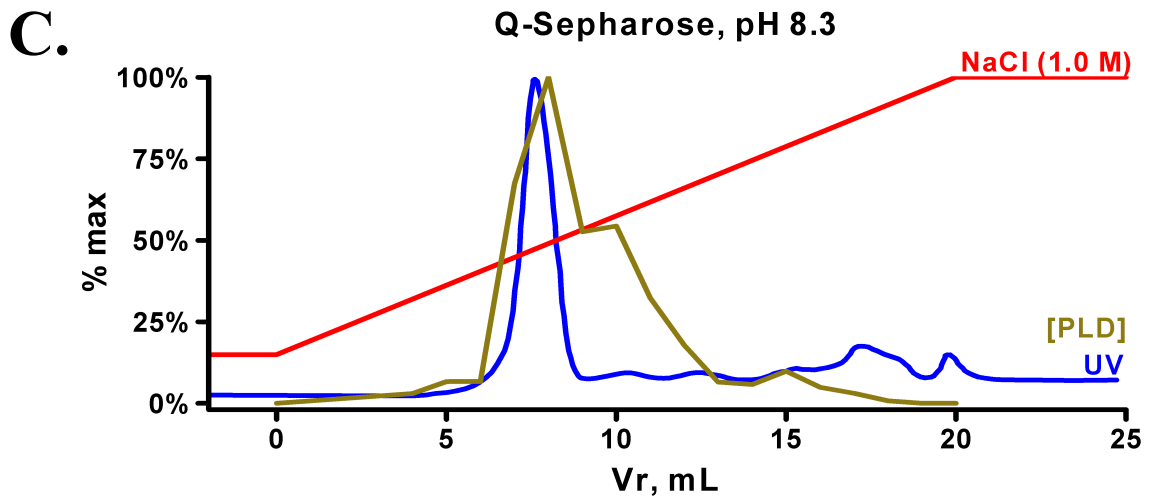
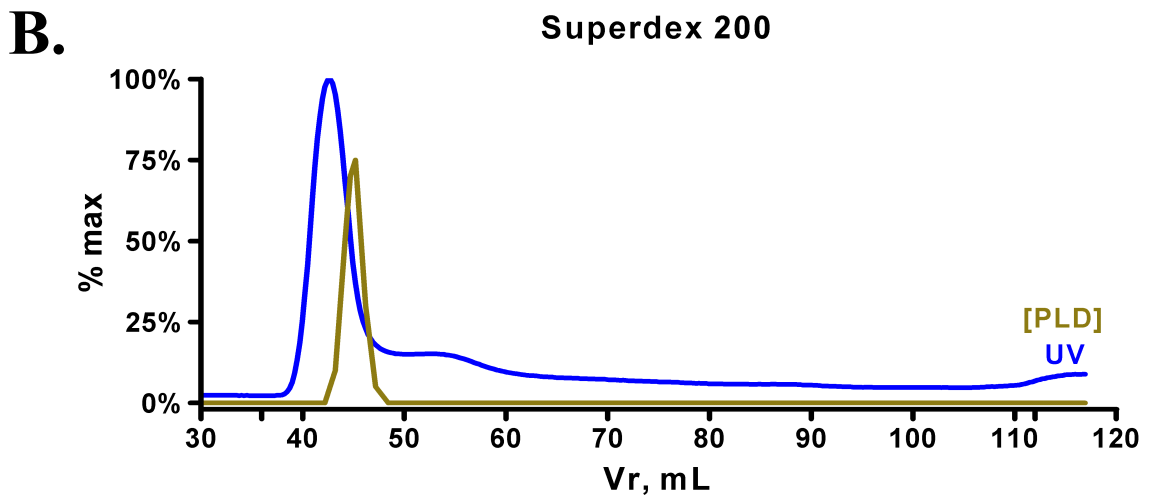
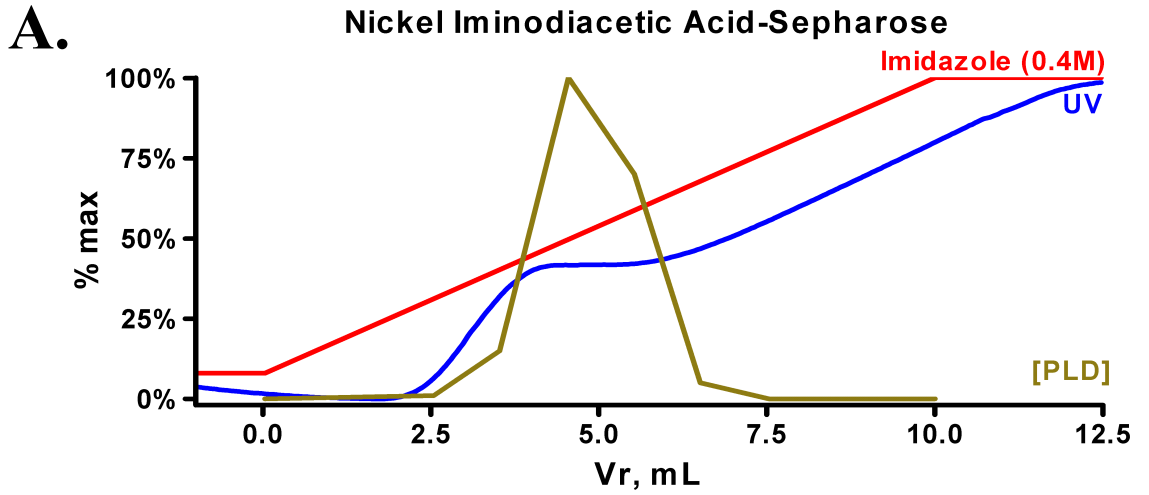
Figure 8. Purification of full-length PLD1b: FPLC Chromatograms

Soluble proteins were applied to appropriate columns by FPLC and eluted fractions were analyzed for protein content by ultraviolet absorbance and α FLAG-M2 immunoblot. Salt gradients are represented in red, ultraviolet absorbance ($A_{280\text{nm}}$) in blue, and PLD concentrations in tan.

(A) Sf21 lysates were applied to nickel-charged chelating-sepharose (1mL HiTrap chelating, Pharmacia). Nonspecific proteins were eluted by a step gradient to 30 mM imidazole in a 50 mM sodium phosphate buffer with 250 mM NaCl, 1 mM MgCl_2 , 1% (w/v) β -OG, and 0.2 mM DTT. A linear gradient (30 to 400 mM imidazole) was used to specifically elute PLD1b. Protein was exchanged to appropriate buffers for further chromatographic separations in (B) and (C). Pooled fractions (purity < 50%) from (A) were applied to size exclusion chromatography media (XK 16/60 Superdex 200 pg, Pharmacia) in (B), or to Q-sepharose (1mL HiTrap Q HP column, Pharmacia) in (C).

Proteins were resolved by size-exclusion chromatography (B) in dilute Hepes buffer, pH 7.5, 150 mM NaCl, 1% (w/v) β -OG, and 0.2 mM DTT. Calibrated by molecular weight standards in the same buffer, the void volume (V_0) of the column was approximately 36 mL (total volume, V_t , was 112 mL). PLD eluted at \sim 44 mL, an apparent molecular weight of nearly 1000 kDa. For anion-exchange chromatography (C), PLD1 was desalted and adjusted to pH 8.3 in a Hepes buffer and repeatedly applied to Q-sepharose media (cycled \sim 3 times over column). Bound proteins eluted in a linear salt gradient to 1 M NaCl.

Figure 8. PLD1 Chromatograms, Continued.



Metal-chelating chromatography (6His)

A 6His epitope was added to the N-terminus of PLD1 for purification by metal-chelating resins. A number of chelating resins were tested (Ni-iminodiacetic acid, GE Biosciences; Ni-nitrilotriacetic acid-agarose, Qiagen; Co-carboxymethylaspartate, Clontech). While the other resins were superior when batch and expanded bed methods were used, the resin purchased from GE Biosciences was superior for fast protein liquid chromatography (FPLC). This resin tolerated low concentrations of reducing agents (β -mercaptoethanol, dithiothreitol) necessary to minimize PLD1 aggregation.

More than 90% of total 6His-PLD1 was captured from clarified lysates by chelating resins, and PLD1 was efficiently eluted by imidazole. A step-gradient to 30 mM imidazole was used to elute nonspecifically-bound proteins, followed by a linear imidazole gradient to competitively elute PLD1. At pH 7.5, PLD1 eluted in a single peak with normal shape at ~180 mM imidazole in a 50 mM sodium phosphate buffer with 250 mM NaCl, 1 mM MgCl_2 , 1% (w/v) β -OG, and 0.2 mM DTT. This separation was rapid, flow-rates were limited only by the viscosity of detergent extracts at low temperatures. 6His-PLD was purified more than 100-fold with a minimal loss of protein.

Because PLD was not stable in high concentrations of imidazole, pooled fractions were immediately desalted over Sephadex G-25 Superfine gel filtration columns (GE Biosciences) and exchanged to buffers containing Hepes pH 7.5, 150 mM NaCl, 1% (w/v) β -OG, and 1 mM DTT. Purified PLD1 is highly unstable in many solutions, and readily precipitates at elevated temperatures or ionic strengths. Overnight storage at 4°C often resulted in aggregated proteins that were not useful for further purification.

Table 5. Purification of full-length PLD1 from baculovirus-infected Sf21 cells

| Purification Step | Volume (mL) | Total Protein (mg/mL) | PLD (mg) | Purity (%) | Purification (fold) | Yield (%) |
|--------------------------|------------------------|----------------------------------|---------------------|-----------------------|--------------------------------|----------------------|
| Detergent extract | 8 | 9.4 | 0.33 | < 1 | 1 | 100 |
| NiIDA | 4.5 | 0.12 | 0.28 | 52 | 130 | 85 |
| Superdex 200 | 3 | 0.05 | 0.11 | 75 | 190 | 28 |
| Q-Sepharose | | | 0.04 | 80 | 200 | 12 |

Immunopurification (α FLAG-M2)

Recombinant PLD1 was purified from Cos7 and Sf21 cells to near homogeneity using immunoaffinity methods. An internal (M2) FLAG epitope was engineered by site-directed mutagenesis as described in the Quickchange™ method (Stratagene). Commercially available immunoaffinity resins (Sigma) bind FLAG-tagged proteins with exceedingly high specificity and affinity (picomolar K_d). Bound proteins were competitively eluted by high concentrations of FLAG peptides. Under ideal conditions, immunoaffinity allowed for a one-step purification of PLD1.

Despite the ease of this method, recovery was typically low. The majority of PLD1 remained bound to the affinity gel even after competitive elution by 5 column-volumes of 3X-FLAG peptide ('MDYKDHDGDYKDHDIDYKDDDDK'). Harsh conditions (e.g., low pH) were required to elute a larger fraction of adsorbed PLD, often denaturing the enzyme. Also, binding and elution steps required long incubations, exacerbating aggregation, denaturation, and proteolysis of PLD in cell lysates.

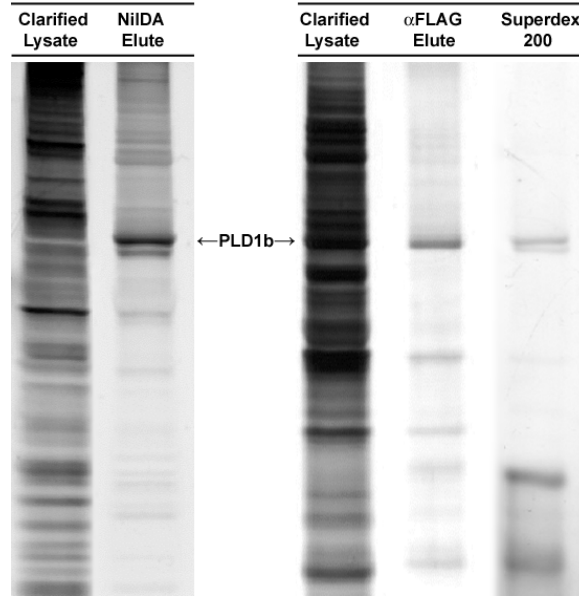


Figure 9. Purification of full-length PLD1b: Eluted Fractions

Full-length PLD1 was purified from Sf21 cells by FPLC and analyzed on silver-stained polyacrylamide (6%) gels. Peak fractions from Ni-chelating chromatography (*Left*) or sequential immunoaffinity and size-exclusion chromatography (*Right*).

Ion exchange chromatography (Q-sepharose)

Anion exchange chromatography has been particularly useful in PLD purifications. Nearly every published purification scheme includes a weak anion exchange step (i.e., diethylaminoethyl), a strong anion exchange step (i.e., quaternary ammonium), or both (Table 6). Substantial purification gained by anion exchange chromatographic separation is offset by low recovery in that step, typically less than 10%.

A strong anion exchanger, quaternary ammonium (Q)-sepharose was useful in initial or intermediate purification steps. At pH 8 – 8.5, < 30% of PLD1 adsorbed Q-sepharose media. Binding could be slightly enhanced by reapplying sample to the column multiple times. PLD1 eluted in a major peak at ~ 0.4 M NaCl and in several minor peaks at higher salt concentrations. Heterogeneous behavior was observed even when highly purified

PLD (i.e., 6His/Superdex 200 – purified PLD) was resolved by anion exchange chromatography. PLD1 required immediate buffer exchange after elution from Q-sepharose columns to stabilize the enzyme by reducing high salt concentrations.

Glutathione affinity (GSH-Sepharose)

An N-terminal glutathione-S-transferase GST fusion construct was generated by subcloning PLD1b into a the pAcGHLT baculovirus transfer vector (Pharming). GST-PLD1 was successfully purified from infected insect cells, though yields were not as great as 6His-PLD1 preparations. Interestingly, GST-PLD was impaired in its ability to respond to stimulation by PKC. A normal response to PKC could be restored by removing the GST-fusion by thrombin cleavage (observation by Hakun Kim). This result suggests that GST, a large N-terminal fusion, provides steric occlusion of PKC binding sites at the N-terminus of PLD1.

Size-exclusion chromatography (Superdex 200)

PLD elutes from gel-filtration columns in a heterogeneous manner, the majority of active enzyme eluting near the void volume of the column. Consequently, estimates of molecular weight from sizing columns were often inaccurate and contradictory (80 – 400 kDa, Table 6). Full-length PLD1 elutes at or near the void volume of every sizing column tested (Superdex 200, Superdex 75, Sephacryl S-300, Superose 8). Based on calibration curves for a XK 16/60 Superdex 200 column, PLD1 elutes with a predicted molecular weight of almost 1000 kilodaltons. This anomalous molecular weight is not likely to be the result of detergent micelles. Similar results were observed when PLD1

was purified in dilute detergent (e.g., β -OG at $\frac{1}{2}$ its critical micelle concentration) or when detergent was substituted with 10 - 20% glycerol.

While most impurities are removed during size-exclusion chromatography, several major species co-elute with PLD1. When analyzed by SDS-PAGE, a pair of bands at ~ 75 and 55 kilodaltons are routinely found with PLD. Both correspond to major proteolytic fragments of PLD1 that are found in cell lysates (*vide infra*). Despite its anomalous behavior during size-exclusion chromatography, eluted PLD1 is highly active and does not appear to be aggregated.

Table 6. Purification of mammalian PLD isoenzymes

| Ref. | Tissue Source | Purification Scheme | Purification | pH | kDa | Substrate specificity | Ca ⁺⁺ | PII ₂ | Fatty Acids | Detergent | Activators | Inhibitors |
|---------|-------------------------|--|--------------|---------|-------|--|------------------|------------------|-------------|-----------|-------------------------------|--------------------------|
| (1) | rat brain | Detergent/Sepharose 4B/DEAE/DEAE/ Sephadex G-200 | 240-fold | acidic | 200 | PC>PE ($K_m \sim 800 \mu\text{M}$) | + | | | - | | |
| (2;3) | rat brain | microsomes | | acidic | | PC>PE | + | | + | + | | pCMPS |
| (4) | rat brain | Salt/Detergent/Detergent/DEAE | 9-fold | acidic | | PC | + | | | + | | |
| (5) | neutrophils | Sucrose gradient | | acidic | | PI>PC | 0 | | | + | | |
| (6) | canine brain | Sucrose gradient/Sephacryl S-200 | | neutral | 400 | PI>PC | 0 | | | | | |
| (7;8) | MDCK | synaptic membranes | | neutral | | PC | 0 | | | + | GTPγS, phorbol esters | pCMPS |
| | | membranes | | neutral | | PC | 0 | | | + | phorbol esters | |
| | | cytosol | | neutral | | PI>PC | + | | | + | | |
| (9;10) | bovine kidney | Detergent/Heparin-Sepharose | | neutral | | PC>>PE, K_m 40-50μM, 10-fold higher V_{max} for PC | | 0 | | - | GTPγS, PE, Sphingomyelin, APS | |
| (11) | bovine lung | Detergent/MonoQ | "Peak2" | acidic | 400 | PC ($K_m < 30 \mu\text{M}$) | + | | | - | | |
| | | Detergent/MonoQ/Bio-Sil SEC-400, Aca34 | "Peak1" | acidic | 80+30 | PE>PC>PI ($K_m \sim 1.5 \text{ mM}$) | + | | | - | 0.1% β-OG | |
| (12;13) | rat intestinal mucosa | mitochondria | | acidic | | PE>PC | + | | 0 | | spermine | NO |
| (14) | porcine lung | Detergent/SO ₄ -Cellulofine/Ether-TP/ Chelating-TP/Q-Sepharose/Heparin/HA | 2,200-fold | acidic | 190 | PC ($K_m \sim 800 \mu\text{M}$) | + | | + | - | Arf, 20:4>18:1>18:2 | |
| (15) | human amnion | microsomes | | acidic | | PC ($K_m \sim 270 \mu\text{M}$) | | | + | 0 | 18:1>20:4>18:2 | high [Ca ⁺⁺] |
| (16) | bovine photoreceptor | microsomes | | neutral | | PC ($K_m \sim 510 \mu\text{M}$) | + | | | - | | |
| (17) | rat brain | Detergent extract | | neutral | | PC | | | | + | | high [Ca ⁺⁺] |
| (18;19) | rat brain | synaptic membranes | | neutral | | PC | + | | | + | | |
| (20) | HL-60 | Salt/Detergent/DEAE/Sephadex G-100/ Heparin | | neutral | | (sn-2 18:1>14:0>16:0>20:4) | + | + | | - | Arf | |
| (21-23) | porcine brain | Salt/Detergent/SP-Sepharose/HA/MonoQ/ Heparin/Phenyl-Sepharose/AcA34/ Heparin | 10,000-fold | neutral | 95 | PC | + | + | | - | Arf, Rho, cytosolic factor | |
| (24) | rat Leydig | Percoll gradient "plasma membrane" Percoll gradient "low density fraction" | | neutral | | PC | | | | + | Arf | |
| (25) | neutrophils | Q-Sepharose/Sephadex G-200 | 300-fold | neutral | 92 | PC | - | + | - | - | | GTPγS |
| (26;27) | rat brain | Detergent/Q-Sepharose/Reactive Green 19-Agarose/HTP-HA/Dialysis | 29-fold | neutral | | PC ($K_m \sim 22 \mu\text{M}$) | | | + | + | | |
| (28) | rat brain | Detergent/Heparin-Sepharose/Heparin | "Peak 1" | neutral | | PC | + | + | | | (myr)Arf | |
| | | Detergent/Heparin-Sepharose/Heparin | "Peak 2" | neutral | | PC | + | 0 | + | | GTPγS is ineffective | |
| (29) | rat brain | Salt/Heparin | | neutral | | PC | + | + | | | Arf, Rho | |
| (30;31) | bovine serum | MonoQ/Phenyl/Wheat germ lectin/MonoQ | 4,500-fold | broad | 110 | GPI-surface glycoprotein | + | | + | - | Zn ⁺⁺ , Trypsin | pCMPS |
| (32;33) | bovine brain, rat liver | Heat/DEAE/Sepharose CL-6B | | neutral | | GPI-surface glycoprotein | + | | | | | chelators |
| (34) | eosinophils | DEAE/Sephadex G-100/CM/IEF | 160-fold | acidic | 60 | PC and PAF | + | | | | | |
| (35) | rabbit kidney | microsomes | | basic | | lysoPAF | + | | | | | |
| (36) | rat brain | microsomes | | neutral | | LPC>LPE (ether-linked) | 0 | | | | Mg ⁺⁺ | |
| (37) | rat heart | microsomes | 7-fold | neutral | | N-acyl-PE | 0 | | - | + | | |

Table 6. Purification of mammalian PLD isoenzymes, Continued.

| Ref. | Recombinant PLD | Purification Scheme | Purification | pH | kDa | Substrate specificity | Ca ⁺⁺ | PII ₂ | Fatty Acids | Detergent | Activators | Inhibitors |
|---------|--------------------|---|--------------|---------|-----|--|------------------|------------------|-------------|-----------|--------------------------------------|------------|
| (38-41) | PLD1(a,b) | Sf21/Detergent/Immunoaffinity or Ni-NTA | | neutral | 120 | PC | + | + | - | - | PKC, Arf, Rho, Rac, Cdc42 | |
| (42;43) | PLD2 | Hi5/Membranes or <i>S.pombe</i> /Membranes | | neutral | 105 | PC | + | + | - | # | Arf, PE | |
| (44) | GPI-PLD | MonoQ/Phenyl/Wheat germ lectin/MonoQ | | neutral | 100 | GPI-surface glycoprotein | + | | | | | |
| (45) | lysoPLD (NPP6) | Culture supernatant/MonoQ | | basic | 50 | LPC>SphingoPC>lysoPAF | + | | | | | chelators |
| (46;47) | lysoPLD(autotaxin) | Sf9/PEG/Blue-Sepharose/ConA-Sepharose/Q-Sepharose/ Heparin/Resource-Phenyl/HA | | basic | 100 | LPC ($K_m \sim 250 \mu M$) (acyl>alkyl>alkenyl) | | | | | Co ⁺⁺ (Zn ⁺⁺) | ATP |
| (48) | NAPE-PLD | Detergent/SP-Sepharose/Q-Sepharose/Blue-Sepharose/HTP-HA | | neutral | 50 | N-acyl-PE> N-acyl-LPE ($K_m \sim 3 \mu M$) | | 0 | - | + | | |

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Characteristics of purified, full-length phospholipase D1

Purified PLD1 is highly active when reconstituted with lipid substrates. Phospholipase activity requires PIP₂ and is inhibited by detergents. Low salt (<150 mM NaCl) and reducing agents (DTT, β-mercaptoethanol) stabilize the enzyme in solution without inhibitory effects on enzymatic activity. Purified PLD1 was stable for several days at 4°C and more than 1 year at –80°C. The pI of purified PLD1 was determined to be approximately 7.5 by isoelectric focusing, equal to the reported pH optimum for enzymatic activity. PLD1 often appeared as a doublet on SDS-polyacrylamide gels, the major band at a predicted 115 kilodaltons.

Post-translational modification

There are numerous reports of serine-, threonine-, and tyrosine-phosphorylation of overexpressed PLD1. Phosphorylation typically correlates with subcellular localization of the enzyme and/or desensitization after prolonged activation. The phosphorylation status of PLD1 purified from insect cells was not examined. PLD1 is *S*-palmitoylated at a conserved position within an N-terminal pleckstrin homology domain. Palmitoylation is a reversible modification (Smotrys and Linder, 2004) important for entry into detergent-resistant membrane microdomains. The stoichiometry of this acylation is not known. Because PLD is purified and stored in the presence of reducing agents, the thioester-linked palmitoyl groups are probably lost.

PLD1 co-purifies with several proteolytic fragments. Rapid purification at low temperature reduced the amount of proteolysis, but significant cleavage occurs in intact cells. These fragments are even present after cells are rapidly lysed with boiling sample

buffer (containing SDS and protease inhibitors) and immediately resolved on denaturing gels (Figure 10). Based on migration in denaturing gels, the vast majority of proteolysis occurs in and around the poorly conserved 'loop region.' Exact sites cannot be predicted from these measurements. Interestingly, caspase-cleavage sites bracket the 'loop region,' and correlate with some predicted fragments. Unpublished results (Wright, M.H.; Farquhar, M.J.; and Hodgkin, M.N.) show that mutation of these caspase sites leads to a protease-resistant allele. PLD has been shown to both activate and inhibit Caspase 3 activity in fibroblasts and PC12 cells respectively (Lee et al., 2000b; Zhong et al., 2002). The effect of PLD1 proteolysis is not known. Isolated by sequential DEAE, heparin, and hydroxyapatite chromatographic steps, these fragments exhibit robust PLD activity (unpublished results, Provost, J.J., Ho; W.T.; and Exton, J.H.). These results, along with comigration with full-length PLD1 on sizing columns (Figure 9), suggest that processed PLD1 remains folded, and in an active form.

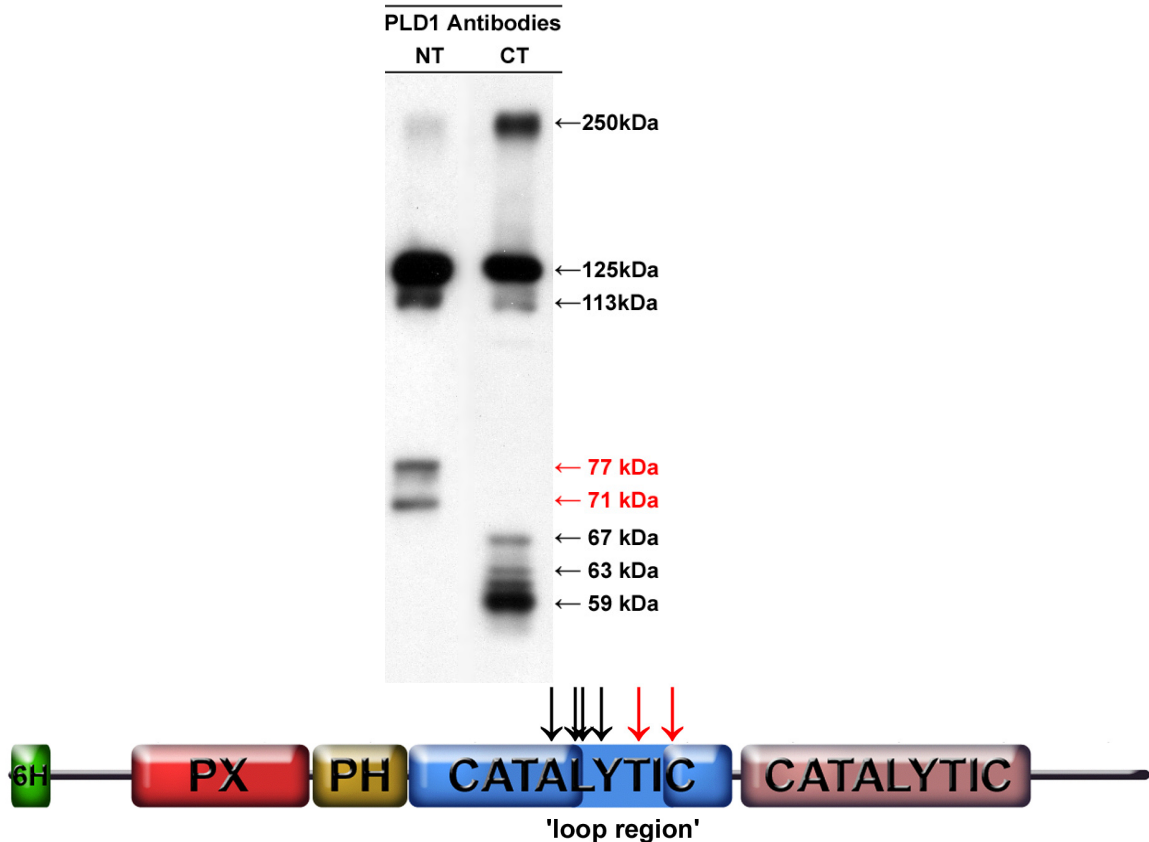


Figure 10. Proteolytic processing of PLD1

Full-length PLD1 was immunopurified (α FLAG) from transfected Cos7 cells, and analyzed by immunoblot. Polyclonal antibodies raised to N- or C- terminal PLD1 sequences were used to map the proteolytic fragments to the loop region within a catalytic subdomain. A panel of antibodies (including monoclonal) recognized the same bands in parallel experiments. PLD1-specific antibodies were generously provided by Masami Hiroyama.

Purification of phospholipase D1 catalytic domains

PLD1 deletion mutants were generated, greatly enhancing the expression of soluble PLD in heterologous cells. Removal of membrane-targeting domains from the N-terminus has significant effects on PLD1 functionality in intact cells, but *in vitro* analyses (Chapter III) show that catalytic properties are essentially unchanged. A maltose binding protein was added as a highly-soluble fusion partner to stabilize the enzyme in solution. To identify a boundary between the tandem PX-PH domain and the catalytic domains, several truncation mutants were generated and screened for expression and activity.

Based on sequence conservation, molecular modeling, and secondary structure predictions, an α -helix forms the C-terminal edge of the PH domain. This helix might be integrated into the catalytic domain fold, forming a functional contact between the domains. A deletion mutant, PLD1.d330 (amino acids 331-1036), lacks this predicted helix entirely. PLD1.d321 (amino acids 322-1036) is truncated mid-helix, and PLD1.d311 (amino acids 312-1036) retains this portion of the PH domain. Although PLD1.d321 expression levels were slightly higher, PLD1.d311 exhibited the highest activity in response to RhoA in transfected Cos7 cells. The optimal expression construct was generated with the 312-1036 PLD1b fragment and a 48 kDa fusion partner containing 6His and FLAG epitopes, a maltose binding protein, and a tobacco etch virus protease site. This construct was subcloned into a mammalian expression vector (pcDNA4HisMaxA, Invitrogen) for *in vivo* assays, and into the Gateway® vector system (Invitrogen) for the generation of recombinant baculoviruses.

PLD1.d311 expressed at levels 2.6-fold higher than PLD1 in Cos7 cells. Insect cell expression was enhanced even more, even becoming the major protein found in cell

lysates. Still, the limiting step in the purification of PLD is the ability to extract soluble protein from cell extracts (Figure 11). About 20% of PLD1.d311 was cytosolic and 26% could be extracted with detergent (74% remained in crude membrane / particulate fractions). PLD1.d311 also performed well during gel-filtration. About half of PLD1.d311 eluted in the void-volume of a Superdex 200 column, half at the expected retention volume for a 130 kDa protein (95% eluted from a Sephacryl S-300 column at the appropriate retention volume). Together, these properties allow rapid purification of catalytically active PLD1 to near homogeneity. Yields were enhanced 50 to 100-fold over full-length PLD1 yields.

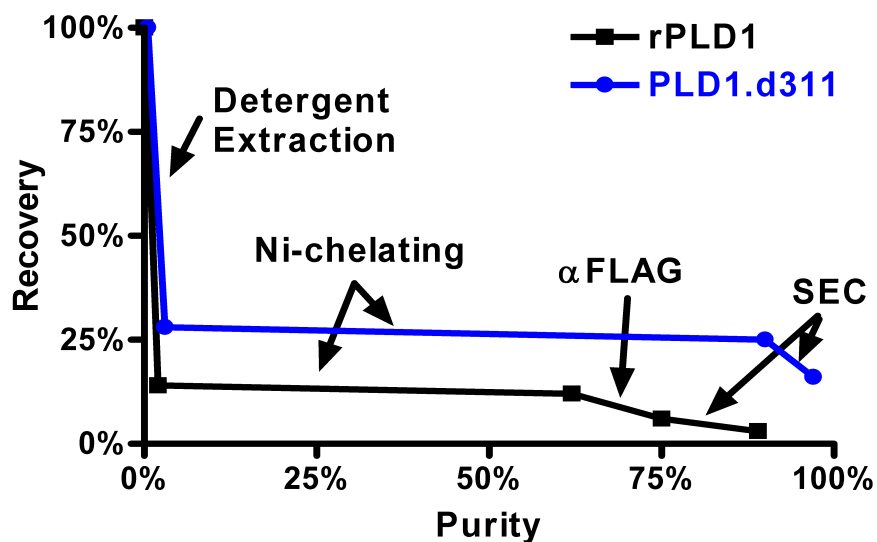


Figure 11. Purification of PLD1

Purification profiles of full-length PLD1 and PLD1.d311 are compared. The majority of both enzymes are lost in the initial, detergent-extraction step. A larger proportion of PLD1.d311 is solubilized, leading to enhanced yields overall.

PLD1.d311 could be purified in the absence of detergent, though β -OG stabilized the enzyme in solution. Dilute detergent reduced aggregation, and allowed the enzyme to be concentrated by ultrafiltration to nearly 30 mg/mL. Salt concentrations above 150 mM were destabilizing. The fusion protein was removed from the purified protein, using a molar excess of tobacco etch virus protease (>4 h at 6°C). Proteolytic removal of the maltose binding protein fusion was accompanied with heavy losses due aggregation.

Phospholipase D activity *in vitro*

Enhanced yields of purified enzyme allowed a detailed characterization of the phospholipase activity of PLD (Chapter III). Activity was measured *in vitro* by established methods (Brown *et al.*, 1995b). PLD-catalyzed reactions are monitored by the production of free choline from exogenous phosphatidylcholine. Dipalmitoyl-[³H-methyl]phosphatidylcholine substrate is presented to the enzyme in PE-dominated phospholipid vesicles.

Small unilamellar vesicles (SUVs, 200Å diameter) are prepared from lipid dispersions by repeated sonication at high power (80W RMS). Standard vesicle composition was PE:PC:PIP₂:cholesterol at molar ratios of 10:1:0.62:0.14 in Hepes buffer pH 7.5, 160 mM KCl, 6mM EGTA, and 0.2 mM DTT. Most SUV preparations were stable for 4 to 5 h at room temperature, and were prepared fresh for enzymatic assays. Purified PLD was reconstituted with substrate vesicles, calcium, magnesium, and activators at defined concentrations. After a 30 min incubation at 37°C, proteins and phospholipids were precipitated and free [³H-methyl]choline was measured by scintillation counting.

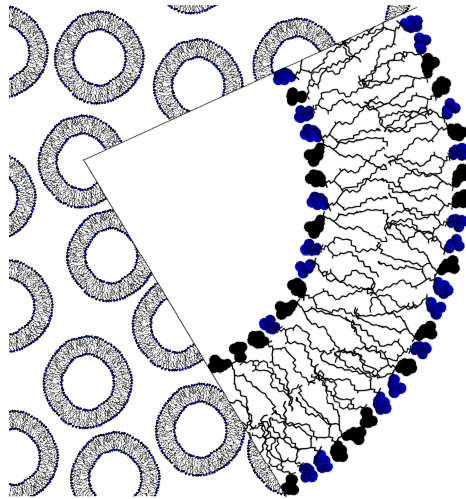


Figure 12. Small-unilamellar vesicles

Phospholipids form regular, bilayer structures in solution. PLD substrates were prepared in SUVs with highly curved bilayers. The outer leaflet is available to PLD in the reaction mixture, while the inner layer remains sealed within the vesicle.

To allow steady-state kinetic characterization of PLD1 activity, it was necessary to identify assay conditions that allow accurate estimates of enzymatic rates. Vesicle concentrations were kept in excess of PLD concentrations (assuming ~2500 phospholipids per vesicle). Because the lipid substrate forms an ordered bilayer structure, only the outer leaflet is available to PLD. Approximately 40% of the substrate partitions to the inner leaflet of small-unilamellar vesicles and is therefore inaccessible. In this assay, enzymatic rates became substrate-limited when as little as 40 to 50 % of the substrate was hydrolyzed. Reactions were linear with time and enzyme concentration over a limited range (Figure 13). Initial rates were determined from measurements between 5 and 25% PC hydrolysis. In practice, a 100 to 200-fold range of enzymatic rates could be measured reliably.

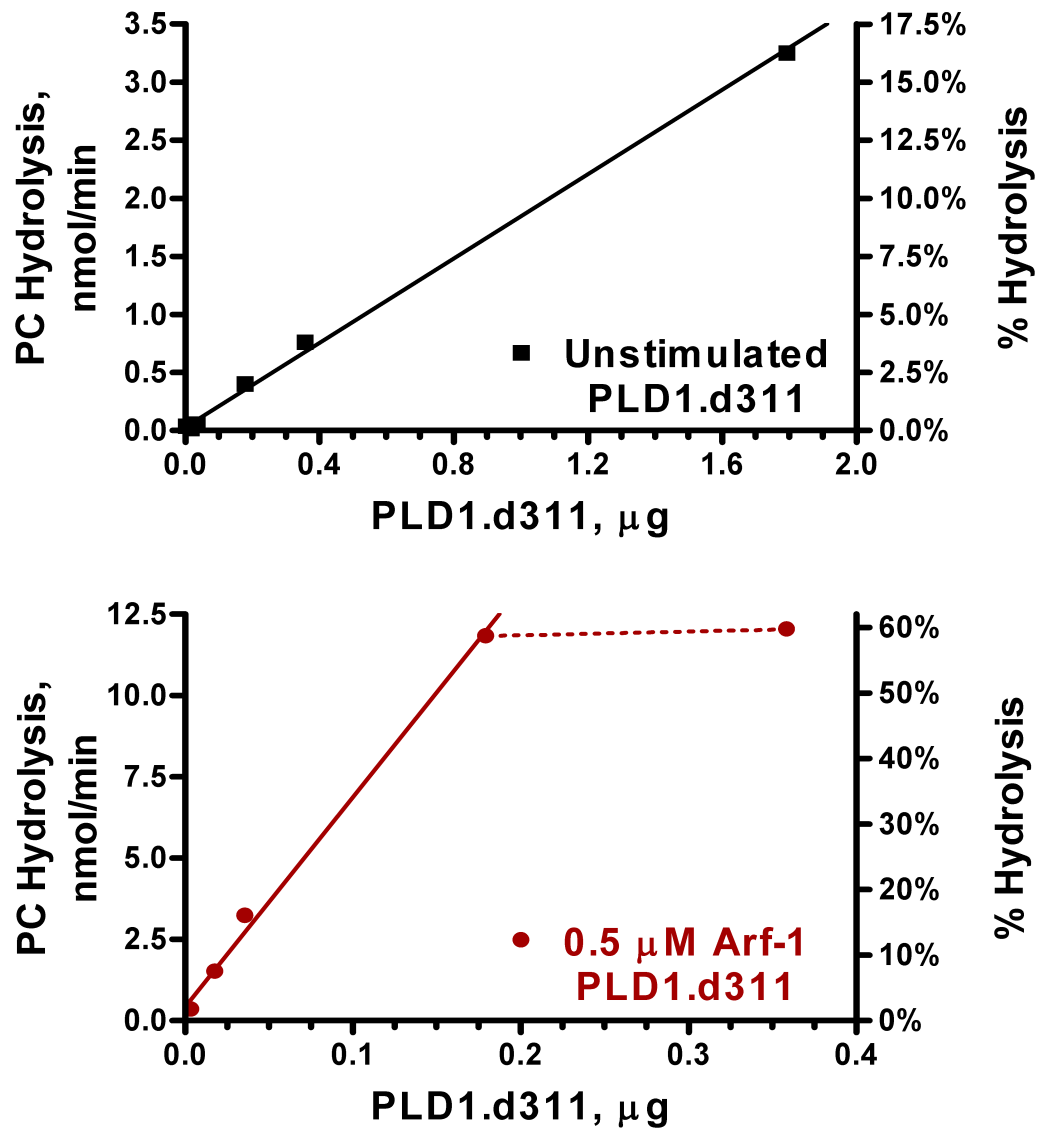


Figure 13. Linear range of *in vitro* activity assays

PLD1.d311 was added at indicated amounts to reaction mixtures (60 μL) containing substrate vesicles. Rates were linear with enzyme concentration until limited by substrate availability or until PLD concentration exceeded vesicle concentration (not shown).

Under conditions described in detail in Chapter III, PLD1.d311 exhibits saturation kinetics toward PC substrate. The surface concentration of PC was manipulated by adjusting the PE/PC ratio in vesicles containing 5 mol% PIP₂. PLD1.d311 activity exhibited classical Michaelis-Menten behavior in PE-dominated vesicles, but activity was reduced in PC-dominated vesicles (Figure 14). A poor substrate for mammalian PLDs, PE is known to enhance PLD activity towards PC (Nakamura et al., 1996). In fact, PLD1 does not hydrolyze PC vesicles without the incorporation of other lipids (e.g., PIP₂, oleate). Kinetic assays were performed with substrate vesicles containing PC at less than 40 mol%.

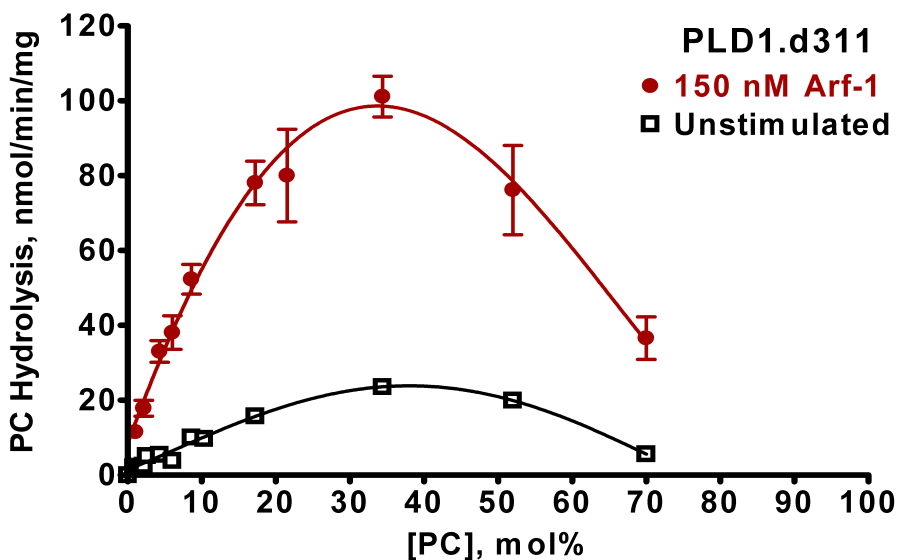


Figure 14. Substrate dependence of PLD1.d311 activity.

Specific activity of PLD1.d311 is assayed with increasing surface concentrations of substrate. Phospholipase activity is measured *in vitro* in the standard assay modified to vary the PC content of the lipid vesicles. Total lipid concentration was held constant at 116 μ M, PIP₂ was held at 5 mol%, and the PC/PE ratio was adjusted to achieve the indicated PC concentration.

Purification of phospholipase D1 amino-terminal domains

The N-terminal membrane-targeting domains of PLD1 were purified to homogeneity from bacteria and insect cells using several strategies. Characterization of a minimal PX-PH domain can provide insight into the structure and function of the holo-enzyme. These domains are critical for subcellular trafficking of PLD1, and in interactions with many positive and negative regulators. *In vitro* studies with these domains were used to identify a direct interaction with $G_{\beta\gamma}$, revealing a novel form of PLD regulation (Preininger *et al.*, in review).

Epitope tags confer properties to the attached protein that allow efficient purification and detection (Kolodziej and Young, 1991). A panel of fusion partners was screened for their use in protein purification (e.g., 6His, hemagglutinin, myc, V5, Xpress, FLAG, glutathione-S-transferase, maltose binding protein, chitin binding domain). By themselves, each fusion partner has strengths and weaknesses. Expression constructs were engineered with combinations of fusion partners to benefit from the advantages of each epitope. Ultimately, a 6His/maltose binding protein/FLAG/tobacco etch virus protease site tag was constructed for N-terminal fusion to PLD1 fragments. Other research groups have arrived at similar solutions. High-throughput structural initiatives at NCI (Frederick), UC-Berkeley, and U. of Wisconsin-Madison have all recently adopted a 6His/MBD/TEV fusion protein strategy. 6His tags are useful for rapid capture of recombinant proteins, maltose binding proteins stabilize hydrophobic proteins in solution (Waugh, 2005), and tobacco etch virus protease is highly specific and active at low temperatures.

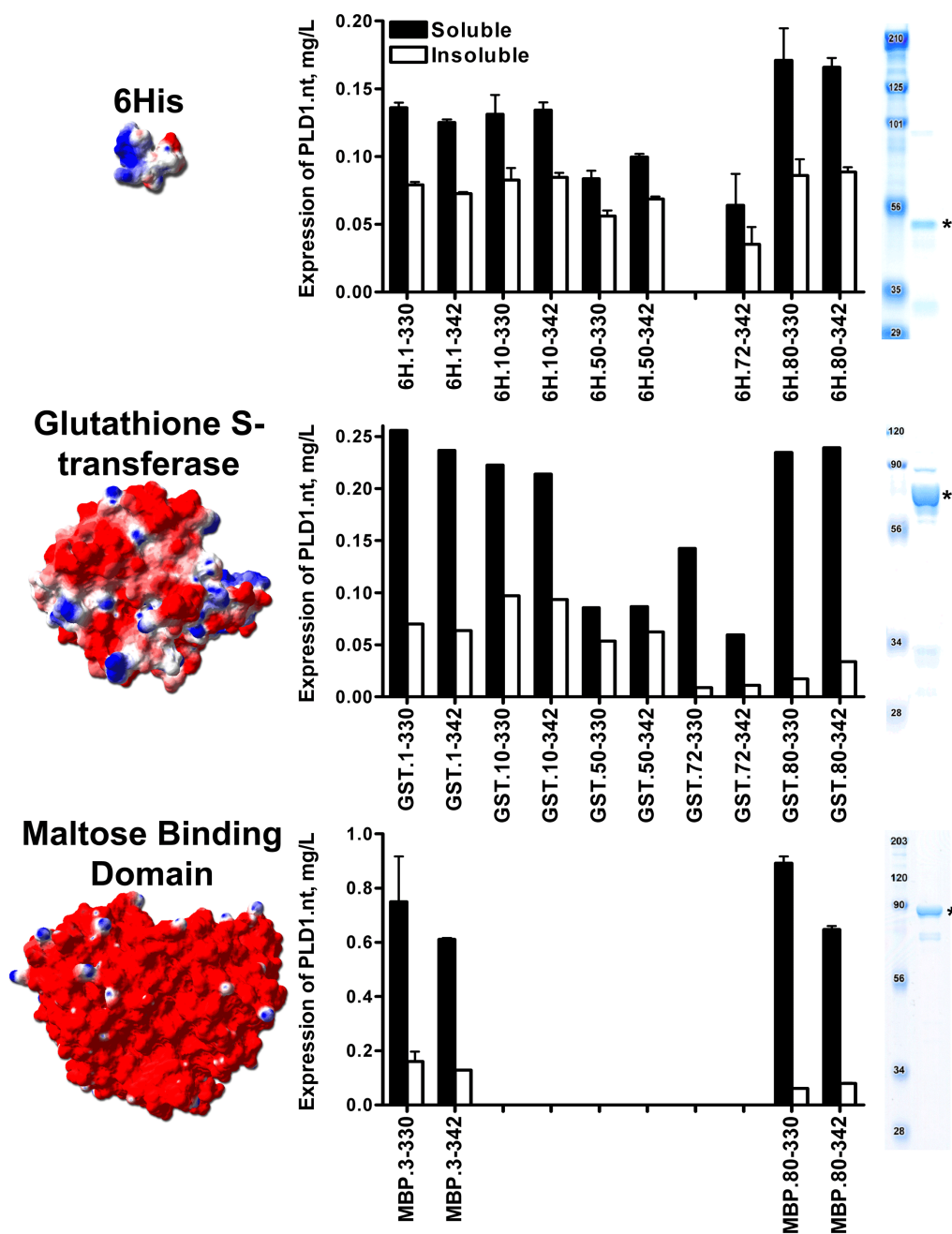


Figure 15. Expression on N-terminal fragments in *E. coli*.

(*Left*), Space-filling models demonstrate differences in the size and surface charge of PLD fusion partners. (*Middle*) Soluble and insoluble fractions were analyzed for PLD content after expression of various N-terminal fragments. (*Right*) Representative coomassie stained gels show successful purification of PLD1 fragments.

N-terminal constructs were expressed in *E. coli* and purified by FPLC. Highest expression levels were achieved in BL21 DE3 cell derivatives (Rosetta, Novagen) that carry supplemental tRNAs for rare codons (these codons are present in PLD1 sequences). Ideal expression conditions involved low copy vectors and mild induction (0.3 mM IPTG) at low temperature (18°C). N-terminal constructs that contain the entire N-terminus (amino acids 3-342) or constructs that possess only the tandem PX-PH domain (amino acids 80-330) were more soluble than other constructs (Figure 15). Intermediate-length fragments (e.g., amino acids 50-330) express at low levels and are probably misfolded.

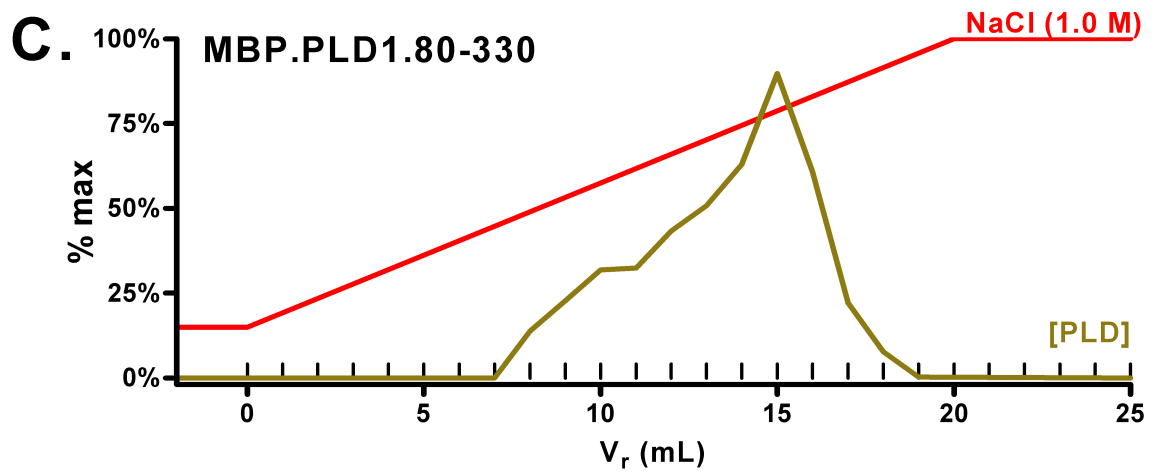
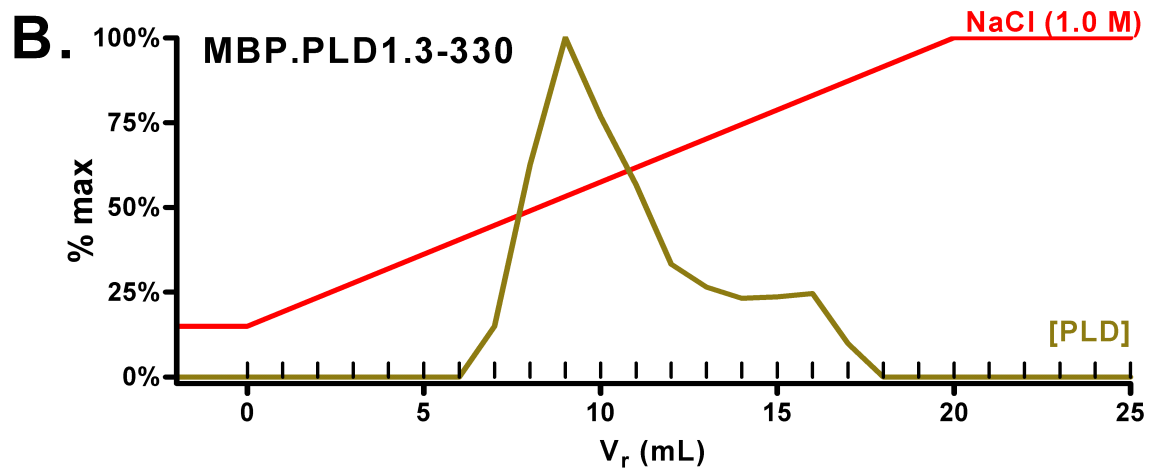
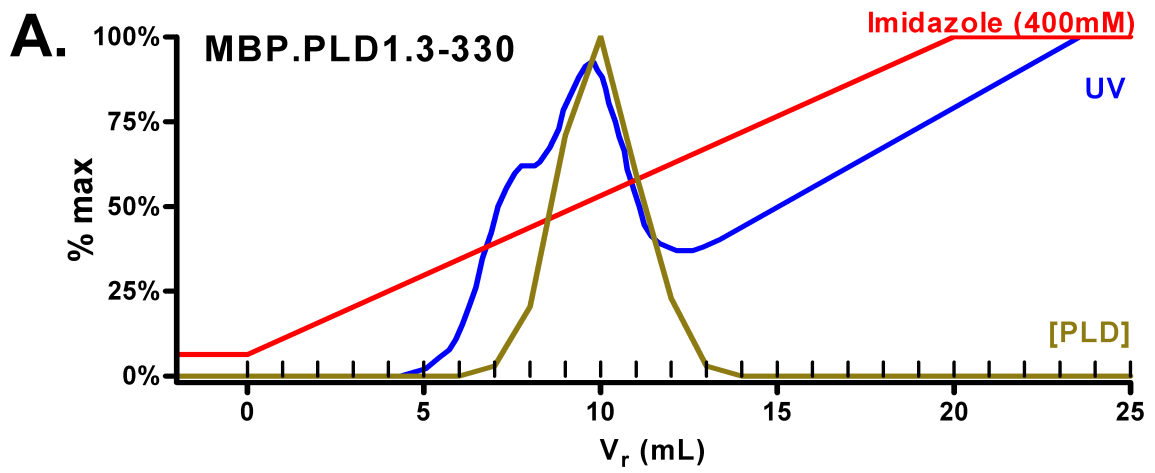
6His-tagged fragments are purified by nickel-chelating chromatography. The majority of contaminants can be eluted with a step gradient to 50 mM imidazole before eluting 6His/MBP-PLD1 fragments (typically elute at ~200 mM in a linear imidazole gradient (Figure 16). About 50 % of the eluted protein is a soluble aggregate that is resistant to tobacco etch virus protease.

MBP.PLD1.3-330 and MBP.PLD1.80-330 have pI's of 8.0 to 8.3 on isoelectric focusing gels. However, these proteins have very different elution profiles when purified by anion exchange chromatography. Both PLD fragments bind Q-sepharose with high affinity. At pH 8.3, MBP.PLD1.3-330 elutes from Q-sepharose columns at ~ 450 mM NaCl, while MBP.PLD1.80-330 elutes at ~ 600 mM NaCl. While many properties of PLD1 PX and PH domains are known, little is known about the residues (amino acids 1-80) that precede them. Deletion analysis of this N-terminal region suggest that this domain contains structural elements that are important for proper folding.

Figure 16. Purification of N-terminal PLD1 fragments

N-terminal fragments of PLD1 were expressed in *E. coli* and purified by FPLC. Salt gradients are represented in red, ultraviolet absorbance ($A_{280\text{nm}}$) in blue, and PLD concentrations in tan.

(A.) MBP.PLD1.3-330 was successfully purified (to >90% purity) by a single, Ni-chelating chromatographic separation. MBP.PLD1.3-330 eluted in a second major peak in a linear gradient from 30 to 400 mM imidazole. A 6His tagged maltose binding protein (without the PLD fusion) was also generated by these cells, perhaps by premature translation termination. These contaminants were largely removed by prolonged (5 to 10 column volumes) washes at 50 to 80 mM imidazole. MBP.PLD1.3-330 (B.) and MBP.PLD1.80-330 (C.) were purified further by anion exchange chromatography over Q-sepharose columns.



Purification of phospholipase D2

PLD2 expresses at much higher levels than does PLD1. 6His-PLD2 was expressed in Sf21 insect cells by baculovirus infection. One-step purification of PLD2 (>50% pure) was achieved using nickel-chelating chromatography. Despite reports that this enzyme is constitutively active, the purified enzyme had similar *specific* activity to unstimulated PLD1. Perhaps the elevated basal activity observed in transfected cells is due to high expression levels. *In vitro*, PLD2 responds weakly to Arf-1, and is unresponsive to PKC α (Figure 17).

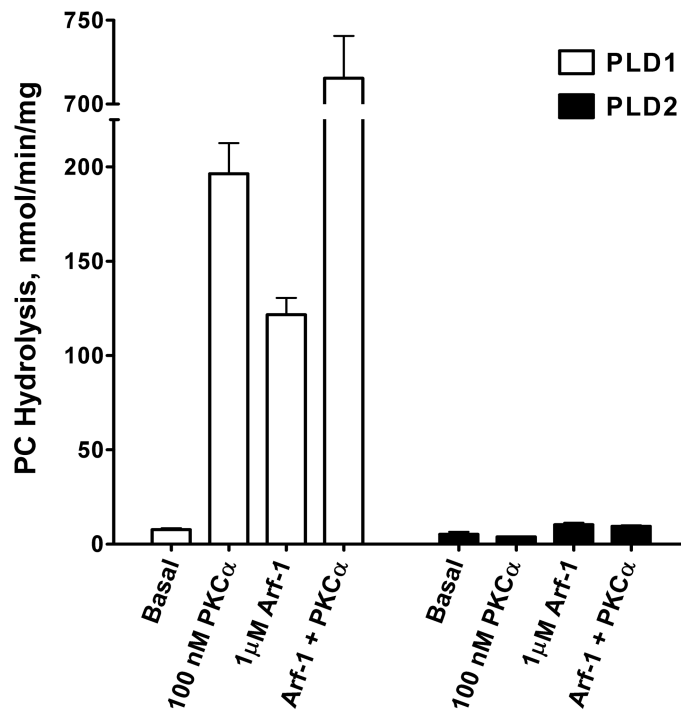


Figure 17. Regulated activity of purified PLD1 and PLD2 *in vitro*

Purified PLDs were reconstituted with activators at the indicated concentrations. PC hydrolysis was determined by standard methods, and reported as specific enzyme activity. Estimates of PLD purity (from stained gels) and total protein concentrations (by Bradford method) were used to determine enzyme concentrations.

CHAPTER III

REGULATION OF PHOSPHOLIPASE D1

“Causa latet, vis est notissima” – The cause is hidden, but the effect is well known.
Ovid (*Metamorphoses* IV, 278)

Rationale

Because of the critical roles of PLD and its products, the enzymatic activity of PLD is tightly regulated by a variety of hormones, neurotransmitters, growth factors, cytokines, and other cellular signals. The PLD1 isozyme is under elaborate control *in vitro* and *in vivo* (Exton, 2002). PIP₂ is an essential PLD1 activator and most characterized eukaryotic PLD isoforms are regulated by PIP₂. Phosphatidylinositol 3,4,5-trisphosphate can partially substitute for PIP₂, but other acidic phospholipids are ineffective or nearly so (Liscovitch *et al.*, 1994; Jiang *et al.*, 2002). Bulk interaction between PLD and lipid vesicles is dependent on PIP₂ *in vitro* (Sciorra *et al.*, 1999; Sciorra *et al.*, 2001; Zheng *et al.*, 2002; Du *et al.*, 2003). While multiple PLD domains bind polyphosphoinositides directly (Figure 18), PIP₂ interaction with a conserved polybasic region within a C-terminal PLD catalytic subdomain appears to be responsible for regulation by this lipid (Sciorra *et al.*, 1999; Zheng *et al.*, 2002; Du *et al.*, 2003).

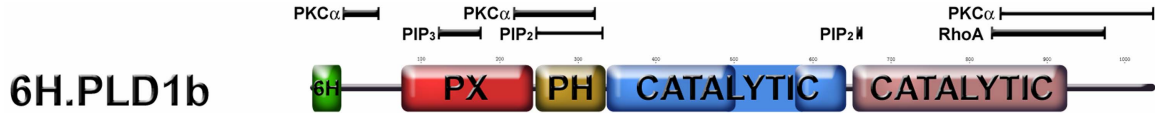


Figure 18. PLD1 binding sites for major regulators

Sequences involved in intermolecular interactions are outlined above a schematic representation of PLD1b. Binding sites were characterized in the following studies (Hodgkin *et al.*, 2000;Cai *et al.*, 2001;Du *et al.*, 2003;Stahelin *et al.*, 2004;Kook *et al.*, 2005).

Current work suggests that there are distinct, yet interacting binding sites for major regulators. Mutational studies have identified PLD1 domains and amino acid sequences involved in interactions with many PLD1 effectors (Figure 1A). PLD1 activity is regulated by conventional protein kinase C isozymes (PKC α , - β , and - γ). PKC α activates PLD1 via direct protein-protein interactions between PKC regulatory domains (Singer *et al.*, 1996) and multiple PLD domains (Sung *et al.*, 1999b;Kook *et al.*, 2005). PLD1 is also regulated by members of Rho and Arf subfamilies of the Ras GTPase superfamily. RhoA activates PLD through direct interaction with a C-terminal PLD catalytic subdomain (Sung *et al.*, 1997;Du *et al.*, 2000;Cai *et al.*, 2001). Members of the Arf subfamily enhance PLD activity *in vitro* (Brown *et al.*, 1993) and *in vivo* (Rumenapp *et al.*, 1995), but a direct interaction has not been demonstrated.

A novel PLD1 expression construct allowed high-level expression and efficient purification of highly active enzyme (>100-fold enhanced yields). Sufficient amounts of PLD1 now permit detailed characterization of PLD1 activity *in vitro*. To understand how allosteric modulators regulate catalysis, we performed kinetic analyses of regulated PLD1

activity. We report that PLD1 effectors enhanced PLD activity differently. PKC α activation of PLD1 involves N- and C-terminal PLD domains. Rho GTPases were *binding activators* and Arf-1 was a *catalytic activator*. When PLD1 is simultaneously stimulated by multiple effectors, the combined response was greater than the sum of the responses to individual effectors (Singer et al., 1995; Hammond *et al.*, 1997). A kinetic description of PLD1 activation by allosteric modulators reveals a mechanism for apparent synergy between activators. Consistent with this interpretation, we found that Arf-1, a *catalytic activator*, was required for synergistic activation of PLD1.

PIP₂ had concentration-dependent effects on membrane association of PLD1 and biphasic effects on PLD activation. Surprisingly, synergy between activators was steeply dependent upon PIP₂ concentration. A narrow range of PIP₂ concentration and a combination of Arf and *binding activators* are required to produce synergistic activation of PLD1. Cellular locales where these signaling molecules converge on appropriate membranes experience dramatic remodeling of the lipid membrane, hydrolyzing PC to PA.

These studies address open questions about the complex prerequisites for PLD activation. A greater understanding of activation mechanisms may help to pharmacologically dissect signaling pathways that converge upon PLD1. The present study reports the first detailed kinetic description of regulated phospholipase D activity towards PC substrate. Mammalian PLD activity is regulated by elaborate networks of upstream signaling molecules *in vivo* and PLD1 is directly activated by multiple classes of proteins and lipids *in vitro* (Exton, 2002). Basic components of this signaling network were examined by reconstituting purified PLD1 with varied amounts of substrate and

allosteric modulators. Steady-state kinetic analysis of PLD activity revealed important differences between PLD1 effectors. Activators exploit different properties of PLD catalysis to enhance activity. Distinct modes of activation provide a mechanistic basis for synergistic relationships between PLD1 effectors.

A systematic investigation of PLD1 activation revealed several strict requirements for synergy between activators. Synergy requires appropriate lipid composition and particular combinations of protein effectors. These requirements are revealed in each of the kinetic terms that describe PLD1 activation. Phosphoinositides regulate the association of PLD1 and the lipid bilayer. This form of activation is represented by the dissociation constant, K_s^A . Synergy also requires modulation of k_{cat} by Arf-1 and modulation of K_m by PKC or Rho GTPases.

Prior studies have characterized the enzymatic activities of bacterial and plant PLD enzymes in detail (Waite, 1999; Leiros *et al.*, 2004). The inability to generate sufficient amounts of purified mammalian PLD has hindered the biochemical characterization of this enzyme. A novel PLD1 N-terminal truncation mutant, PLD1.d311 with greatly enhanced expression and stability was purified to homogeneity from recombinant sources. This mutant retains full enzymatic activity (Figure 1). Unexpectedly, this catalytic domain fragment was regulated by all classes of PLD allosteric modulators.

Purification of PLD1 and PLD1.d311

Full-length PLD1b (amino acids 1-1036, Figure 19) and PLD1.d311 (an N-terminally truncated PLD1b, amino acids 312-1036) were expressed in baculovirus infected insect cells and purified as described in the 'Methods' section of this chapter. Both proteins

contain N-terminal 6His tags and efficiently bind to immobilized nickel columns. Full-length PLD1 failed to purify in a homogeneous manner over subsequent chromatographic steps. N-terminally truncated PLD1 (*PLD1.d311*) was expressed as a maltose binding domain fusion protein and purified by metal chelation and size exclusion chromatography. Typical yields of purified PLD1.d311 (30-40 mg/L insect cells) were much greater than those of full-length PLD1 (0.2-0.5 mg/L insect cells).

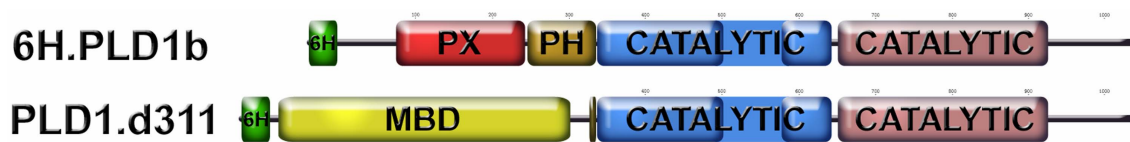


Figure 19. Domain architecture of PLD1 and PLD1.d311

A schematic representation of full-length, rat PLD1b (amino acids 1-1036, *top*) and rPLD1b.d311 (amino acids 312-1036, *bottom*). N-terminal 6-His (*6H*) tags were added to both constructs to facilitate purification. PLD1.d311 retains both catalytic repeats, but pleckstrin homology (*PH*) and phox homology (*PX*) domains have been replaced with a maltose-binding domain (*MBD*) from *E. coli*.

Protein kinase C α , RhoA, Rac1, and Cdc42 were purified from baculovirus infected insect cells and recombinant Arf-1 was purified from bacteria. Purified proteins were evaluated by SDS-PAGE followed by colloidal coomassie staining (Figure 20) and immunoblot.

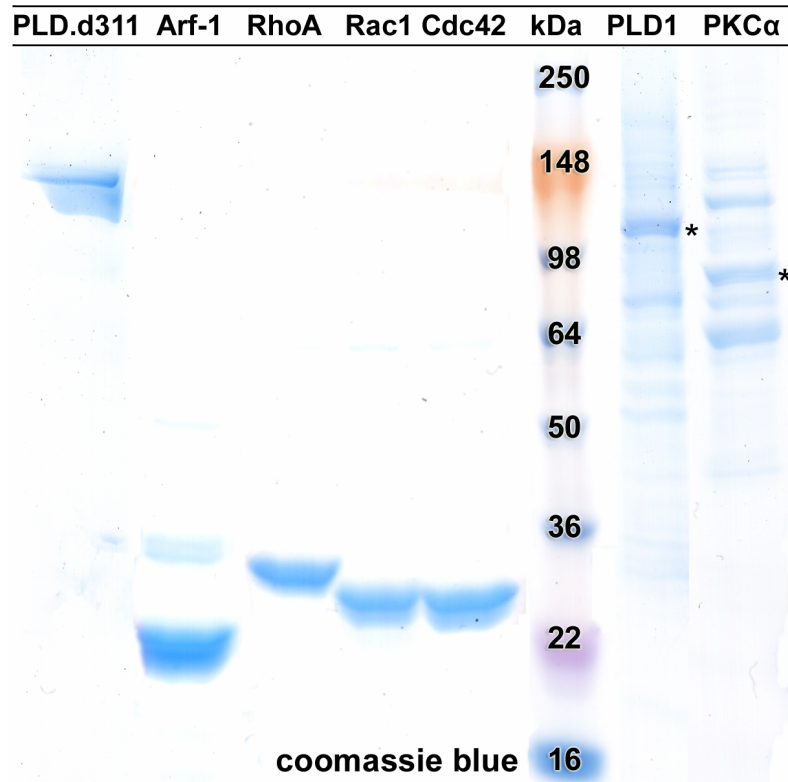


Figure 20. Purified PLD1, PLD1.d311, and activators

Phospholipase D1 protein and recombinant PLD activators were purified as described in ‘Methods.’ Samples (1.5 μ g total protein) were analyzed by SDS-PAGE using a 4-20% denaturing gel and stained by coomassie blue. Purified proteins were verified by immunoblot (data not shown) and immunoreactive bands are indicated by asterisks.

Regulated phospholipase D1 activity

To examine the enzymatic properties of purified PLD, we used standard *in vitro* methods (Brown *et al.*, 1995b) to assay activity towards dipalmitoyl-PC substrate. PLD was reconstituted with purified effectors and lipid vesicles containing radiolabeled substrate. PLD1.d311 retained the full enzymatic activity of full-length PLD1 and was regulated by all classes of PLD activators (Figure 21). PLD1.d311 exhibited slightly

elevated basal activity in the absence of activators, similar to other N-terminally truncated PLD mutants (Park *et al.*, 1998; Sung *et al.*, 1999b).

Recent work has mapped major sites of interaction with PKC α to the extreme N-terminus and PH-domain of PLD1 (Figure 18). Surprisingly, potent activation by PKC α was still observed when these major PKC binding sites were deleted from the N-terminus of PLD1. Maximal PLD1.d311 response to PKC α was 16 % of the maximal response of full-length PLD1 to PKC α (Figure 21 and Table 7). Monomeric G-protein activators were equally effective towards PLD1 and PLD1.d311, both in terms of potency and maximal activation.

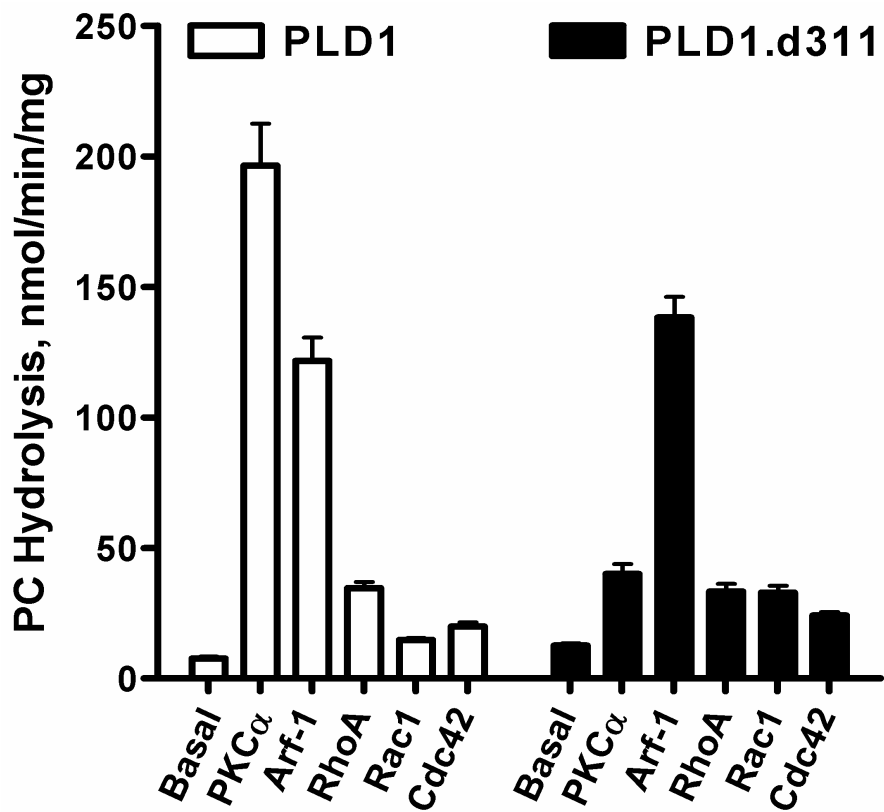


Figure 21. Regulated activity of purified PLD1 and PLD1.d311

Specific activities of full length (PLD1, *left*) and truncated (PLD1.d311, *right*) phospholipase D1 were determined by [^3H]phosphatidylcholine hydrolysis *in vitro* using standard methods (Brown *et al.*, 1995b). Enzymatic activity of purified phospholipase D (5-10 nM) was measured in the presence of 10 μM GTP γS alone (Basal) or reconstituted with GTP γS and maximally effective concentrations of purified activators. Data are presented as mean initial rates \pm standard error, n = 3 to 11 independent experiments.

Full-length PLD1 and PLD1.d311 were both strongly activated by Arf-1·GTP γ S (Figure 21). Arf-1 stimulated PLD1 activity in a concentration-dependent manner and its effects did not saturate at even 10 μ M Arf-1 (data not shown). Other activators were more than 30-fold more potent than Arf-1 (Figure 22). No other activator equaled the degree of activation elicited by Arf-1. Three members of the Rho GTPase subfamily potently stimulated PLD1, but with low maximal stimulation. GTP γ S-loaded RhoA, Rac1, or Cdc42 stimulated PLD1 and PLD1.d311 2- to 5-fold above basal activity (Table I). Normal ($n_H \approx 1$) concentration-dependent effects were observed for each of the activators tested, although very high (μ M) concentrations of PKC α , RhoA, Rac1, or Cdc42 had reduced ability to activate PLD1 (Figure 22).

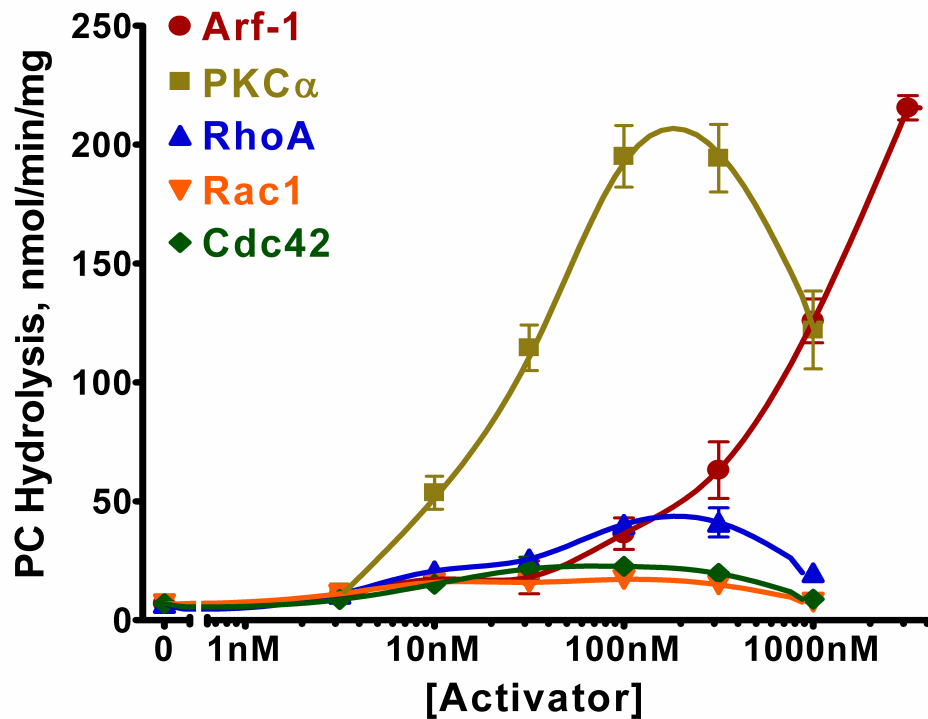


Figure 22. Allosteric activation of PLD1

Increasing concentrations of purified activators were reconstituted with partially purified, full-length phospholipase D1 (5 nM). Phosphatidylcholine hydrolysis was measured under standard conditions described in 'Methods.' Arf-1, PKC α , RhoA, Rac1, and Cdc42 were examined for their ability to activate PLD1. Specific activities are presented as mean initial rates \pm standard error.

Synergy between PLD1 activators

At maximally effective concentrations, RhoA and PKC α stimulated PLD1 activity no more than PKC α alone. At lower PKC α concentrations, the combined effects of PKC α and 100 nM RhoA were roughly equal to the sum of the effects of each activator alone (Figure 23A). The combined effect of PKC α and Arf-1 exceeded the additive effects of the activators alone, indicating synergy (Figure 23A). Co-stimulation with Arf-1 enhanced the maximal response to PKC α but did not change PKC α potency towards PLD1. Arf-1 (1 μ M) synergized with PKC α to stimulate PLD1 100-fold above basal activity, more than twice the maximal response predicted for an additive effect.

Arf-1 also synergized with RhoA to activate PLD1 (Figure 23B). Arf-1 (1 μ M) and RhoA (100 nM) combined to enhance PLD1 activity almost 50-fold, twice the response predicted for an additive effect. Arf-1 exhibited synergistic relationships with all PLD1 effectors tested. Combinations without Arf-1 did not produce synergistic responses.

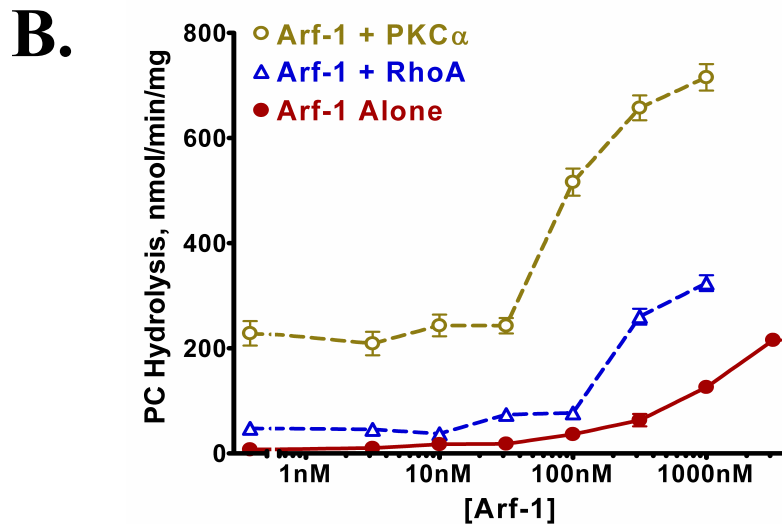
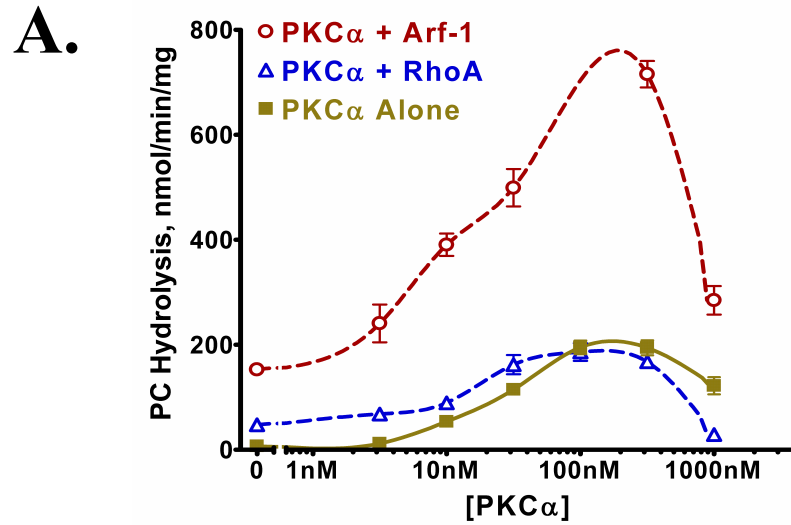


Figure 23. Synergistic activation of PLD1

Increasing concentrations of purified activators were reconstituted with partially purified, full-length phospholipase D1 (5 nM). Phosphatidylcholine hydrolysis was measured under standard conditions described in ‘Methods.’ *A*, Simultaneous activation of PLD1 by increasing concentrations of PKC α and maximally effective concentrations of Arf-1 or RhoA. *B*, Synergistic activation of PLD1 by increasing concentrations of Arf-1 and maximally effective concentrations of PKC α or RhoA. Specific activities are presented as mean initial rates \pm standard error.

Table 7. Activation of phospholipase D1 *in vitro*

Activity of purified phospholipase D1 (1 nM) was stimulated by purified activators.

| Activator(s) | PLD1 (<i>full-length</i>) | | PLD1.d311 | |
|-----------------------|-----------------------------|--------------------|------------------|--------------------|
| | EC ₅₀ | Max | EC ₅₀ | Max |
| | <i>nM</i> | <i>nmol/min/mg</i> | <i>nM</i> | <i>nmol/min/mg</i> |
| Unstimulated | | 7 ± 1 | | 13 ± 1 |
| PKCα | 32 ± 1 | 204 ± 17 | 56 ± 22 | 34 ± 4 |
| Arf-1 | | >500 | | >500 |
| RhoA | 29 ± 2 | 44 ± 5 | 67 ± 43 | 37 ± 4 |
| Rac1 | 5 ± 2 | 17 ± 1 | 19 ± 19 | 31 ± 6 |
| Cdc42 | 9 ± 4 | 23 ± 2 | 30 ± 20 | 23 ± 1 |
| Arf-1 + RhoA § | 346 ± 21 | 346 ± 21 | | |
| Arf-1 + PKCα § | 731 ± 24 | 731 ± 24 | | |
| PKCα + RhoA † | 14 ± 4 | 177 ± 9 | | |
| PKCα + Arf-1 † | 22 ± 12 | 716 ± 25 | | |

§ Concentrations of *Arf-1* were varied, *RhoA* or *PKCα* were held constant at 300 nM.† Concentrations of *PKCα* are varied, *RhoA* was held constant at 300 nM, *Arf-1* at 1 μM.**PLD1.d311 activity *in vivo***

The N-terminus of PLD1 contains several membrane-targeting domains that determine the subcellular localization and trafficking of the enzyme in Cos7 cells. Despite lacking these N-terminal domains, PLD1.d311 was highly active when expressed in Cos7 cells (Figure 24). Consistent with our findings *in vitro*, PLD1.d311 activity was stimulated by RhoA *in vivo* but its response to PKC was blunted. To examine Rho-dependent stimulation of PLD in cultured cells, full-length PLD1 or PLD1.d311 was transiently coexpressed with a constitutively activated RhoA mutant (RhoA.G14V). Phorbol myristate acetate stimulation of Cos7 cells activates PLD1, presumably via regulation of classical PKC isoforms (Exton, 2002). Full-length PLD1 was expressed at levels about one-third of PLD1.d311 expression and exhibited robust stimulation by RhoA.G14V (1.6-fold over control) and by PMA (1.6-fold over control).

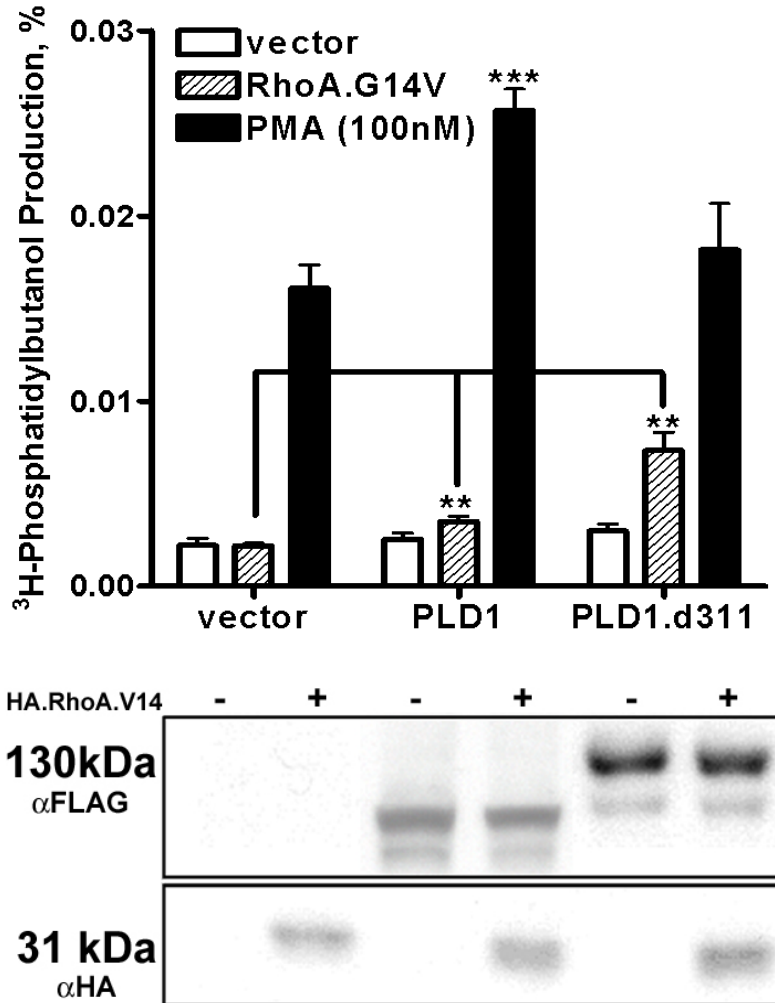


Figure 24. Activity of PLD1 and PLD1.d311 *in vivo*

Recombinant phospholipase D was transiently expressed in Cos7 cells and activity was measured *in vivo* by the transphosphatidylation of [³H]oleic acid-labeled endogenous substrate to 1-butanol. PLD constructs (0.6 μ g DNA) were cotransfected 1:1 with RhoA.G14V or empty vector as ballast. Transfected cells were serum starved (18 h) and treated (15 min) with phorbol myristate acetate (100 nM) as indicated. [³H]Phosphatidylbutanol production is presented as mean rates \pm standard error for three independent experiments performed in triplicate. ** indicates significant ($P < 0.01$) increases relative to control (vector + RhoA.V14) and *** indicates a significant ($P < 0.01$) increase relative to control (vector + PMA). Representative immunoblot of transfected cells is shown below.

Steady-state kinetic analysis

A detailed kinetic characterization of PLD1 activity was performed to gain insight into the enzyme's regulation. It has previously been shown that mammalian PLD preparations obey Michaelis-Menten kinetics towards substrate (Nakamura *et al.*, 1996;Chalifa-Caspi *et al.*, 1998;Hoer *et al.*, 2000), but allosteric modulation of PLD kinetics has not been studied. We report the first use of saturation kinetics to characterize the activation of PLD1 by purified effectors. The ability to generate large quantities of PLD1.d311 permitted us to perform comprehensive enzymatic studies.

Previous studies suggest that many PLD enzymes exhibit interfacial behavior (Chalifa-Caspi *et al.*, 1998;Qin *et al.*, 2002;Yang *et al.*, 2003). Macroscopic Michaelis constants are dependent upon interactions with substrate and with bulk lipid. Surface-dilution kinetic models account for aggregated substrates, describing both three-dimensional interactions with lipid interfaces and two-dimensional surface interactions with specific substrate (Carman *et al.*, 1995). Because detergents strongly inhibit PLD1 *in vitro* (Brown *et al.*, 1995b;Nakamura *et al.*, 1996;Min *et al.*, 1998;Hoer *et al.*, 2000;Jiang *et al.*, 2002) and participate in the transphosphatidylolation reaction as nucleophiles (Yang *et al.*, 2003), kinetic parameters cannot be calculated from mixed-detergent-lipid micelle experiments.

Association of phospholipase D and the phospholipid interface was examined by measuring the binding of purified PLD1.d311 to sucrose-loaded phospholipid vesicles (Figure 25). Vesicle-bound PLD1.d311 was separated from free enzyme by ultracentrifugation and analyzed by immunoblot (Buser and McLaughlin, 1998). The catalytic domain of PLD1 (PLD1.d311) displayed high affinity for phospholipid surfaces.

The PIP₂ content of the vesicles was crucial for the binding interaction with PLD1.d311. For vesicles prepared with 5 mol% PIP₂, we report a dissociation constant, K_s^A , of about 2 μ M bulk lipid. Sucrose-loaded vesicles prepared without PIP₂ bound PLD1.d311 weakly. Prior reports demonstrated that other phosphoinositides can partially substitute for PIP₂ in promoting PLD association with lipid vesicles and that membrane association is dependent upon conserved arginine residues within a C-terminal polybasic region (Sciorra *et al.*, 1999; Sciorra *et al.*, 2001; Zheng *et al.*, 2002; Du *et al.*, 2003).

These data demonstrate that a limiting step in PLD1 catalysis (i.e. partitioning to the lipid interface) is saturated at \sim 10 μ M bulk lipid. Interestingly, conditions that promote maximal (>98%) binding of PLD to phospholipid vesicles do not stimulate PLD activity to maximal rates. This indicates that activation of PLD1 involves effects on both bulk and interfacial binding steps. The *surface binding model* (Carman *et al.*, 1995) describes a two-step association of PLD1 with its substrate, where PLD1 non-specifically binds the vesicle surface before specifically binding substrate (Figure 26). To examine subsequent interfacial events in PLD1 catalysis, total lipid concentration was held constant at saturating levels (116 μ M, >98% bound enzyme).

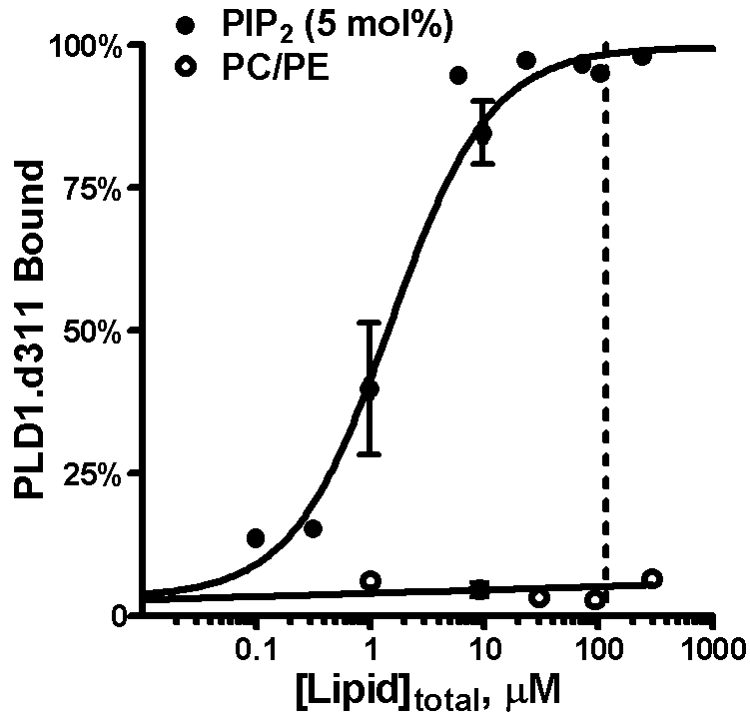
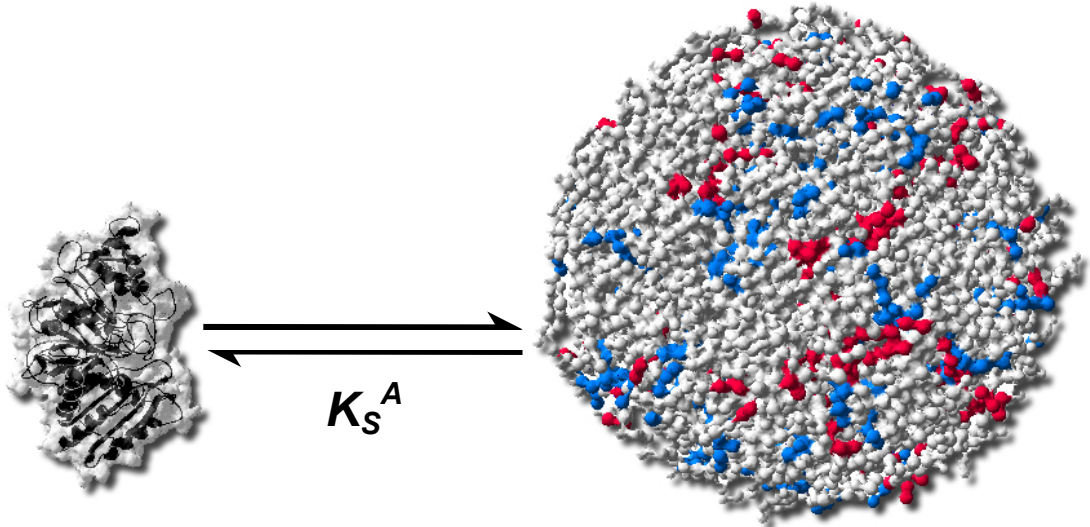
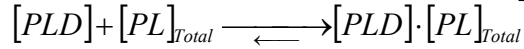
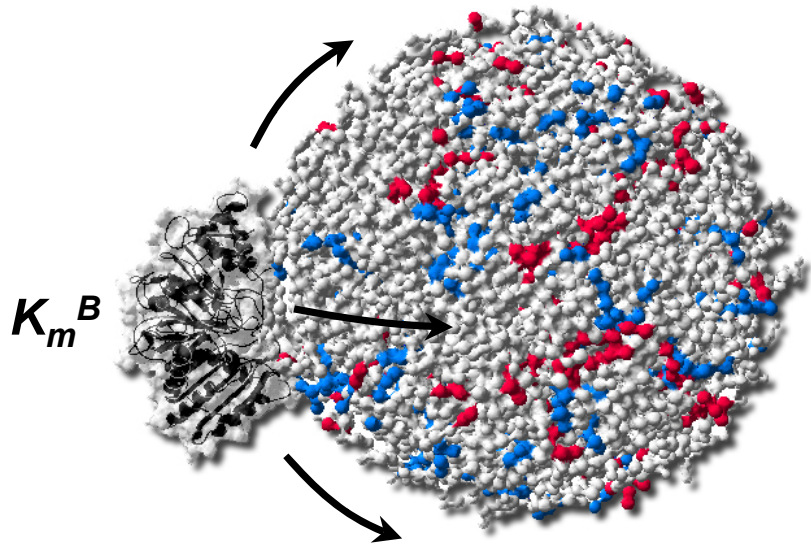
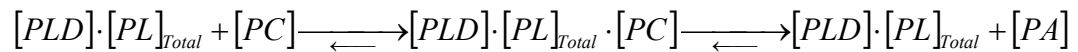


Figure 25. PLD1.d311 binds phospholipid vesicles with high affinity

Association of PLD1 catalytic domains with phospholipid vesicles is dependent upon PIP₂. PLD1.d311 binding to sucrose-loaded vesicles was performed using the method of Buser and McLaughlin (Buser *et al.*, 1998). Vesicles were prepared with and without PIP₂ (5 mol%). Bound PLD1.d311 was separated from free enzyme by ultracentrifugation. Supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblot. Data are presented as mean \pm standard error for three independent experiments.

A.**Association with bulk lipid****B. Interfacial association with substrate****Figure 26. Surface binding model of PLD activity**

A two-step kinetic model of PLD activity that involves (**A.**) a non-specific binding interaction with the phospholipid bilayer, and (**B.**) specific interactions with phosphatidylcholine at the vesicle surface. $[PL]_{Total}$ refers to total lipid concentration.

Kinetic parameters of PLD catalysis were determined by initial velocity experiments measuring dipalmitoyl-PC hydrolysis in PE-dominated vesicles, as described in 'Methods.' Phospholipase activity was dependent on the surface concentration of substrate (Figure 27 and Table 8) and the experimental data fit Michaelis-Menten relationships by least squares and Eadie-Hofstee analyses ($r^2 > 0.7$). All reported kinetic constants were determined by non-linear regression analysis. The interfacial Michaelis constant, K_m^B , describes two-dimensional surface interactions between PLD and its substrate, PC.

Apparent K_m^B values for dipalmitoyl-PC were about 33 mol% (39 μ M) in the absence of protein activators, similar to the reported value of 42 μ M for PLD purified from bovine kidney (Nakamura *et al.*, 1996). A V_{max} value of 32 nmol PC hydrolyzed $\text{min}^{-1}\text{mg}^{-1}$ PLD1 was obtained for the unstimulated enzyme. PLD1.d311 was only slightly activated, with a V_{max} of 46 nmol $\text{min}^{-1}\text{mg}^{-1}$. PLD1 activators enhanced catalytic efficiency, k_{cat}/K_m , both by reducing K_m^B and by enhancing catalytic potential, k_{cat} (Table 8). PLD1 activators could be discriminated based on their effects on these kinetic parameters.

PKC α had profound effects upon PLD1 kinetics, enhancing catalytic efficiency 75-fold. A *mixed activator*, PKC α produced dramatic effects on both K_m^B and k_{cat} . A maximal rate (V_{max}) of 453 nmol $\text{min}^{-1}\text{mg}^{-1}$ was calculated for PKC α -stimulated PLD1. PLD1.d311 maximal rate was not enhanced by PKC α . Kinetic analyses reveal that PKC α regulates the catalytic efficiency of PLD1.d311 via *binding activation* (IUPAC-IUBMB, 1982). While marked effects on K_m^B remain, deletion of N-terminal PLD1 domains are reflected in a loss of PKC α effects on k_{cat} . While full effects of PKC α on PLD1 activity

require both N- and C- terminal domains, C-terminal interactions with PKC α had marked effects on PLD1.d311 catalysis (Tables 7 and 8). These experiments provide new insights into PKC α regulation of PLD1.

Arf-1·GTP γ S was equally effective towards PLD1 and PLD1.d311. In both cases, Arf-1 enhanced PLD catalytic efficiency with only minor effects on K_m^B . A *catalytic activator* (IUPAC-IUBMB, 1982), Arf-1 increased PLD1 catalytic potential (k_{cat}) in a concentration-dependent manner. 150 nM Arf-1 increased PLD's maximal rate 4-fold while K_m^B values were only modestly reduced, \sim 40% (Table 8). 10 μ M Arf-1 enhanced k_{cat} values more than 10-fold (data not shown). To allow direct comparisons between activators and to compare synergistically activated conditions, Arf-1 concentrations were set at 150 nM throughout these experiments. These conditions allowed accurate estimates of initial enzymatic rates where PLD activity was within the linear range of the assay (PC hydrolysis between 5 and 25% of total substrate).

Figure 27. Kinetic properties of PLD activity

Specific phospholipase activities of purified PLD1 and PLD1.d311 were assayed with increasing concentrations of substrate. Phospholipase activity was measured *in vitro* in the exogenous substrate assay (Brown *et al.*, 1995b) modified to vary the PC content of the lipid vesicles. PIP₂ and total lipid concentrations were held constant, while the PC : PE ratio was adjusted to achieve the indicated substrate concentration. **A**, Activity of purified, full-length PLD1 was measured in the presence of 10 μM GTPγS (unstimulated), or reconstituted with GTPγS and PKCα, Arf-1, or RhoA. **Right**, Eadie-Hofstee linear transformation of the same data.

B, Activity of purified, PLD1.d311 was measured in the presence of 10 μM GTPγS (unstimulated), or reconstituted with GTPγS and PKCα, Arf-1, RhoA, Rac1, or Cdc42. **Right**, Eadie-Hofstee linear transformation of the same data. Concentrations of activators were 150 nM Arf-1, 300 nM PKCα, 300 nM RhoA, 300 nM Rac1, and 300 nM Cdc42.

Figure 27. Kinetic properties of PLD activity, continued

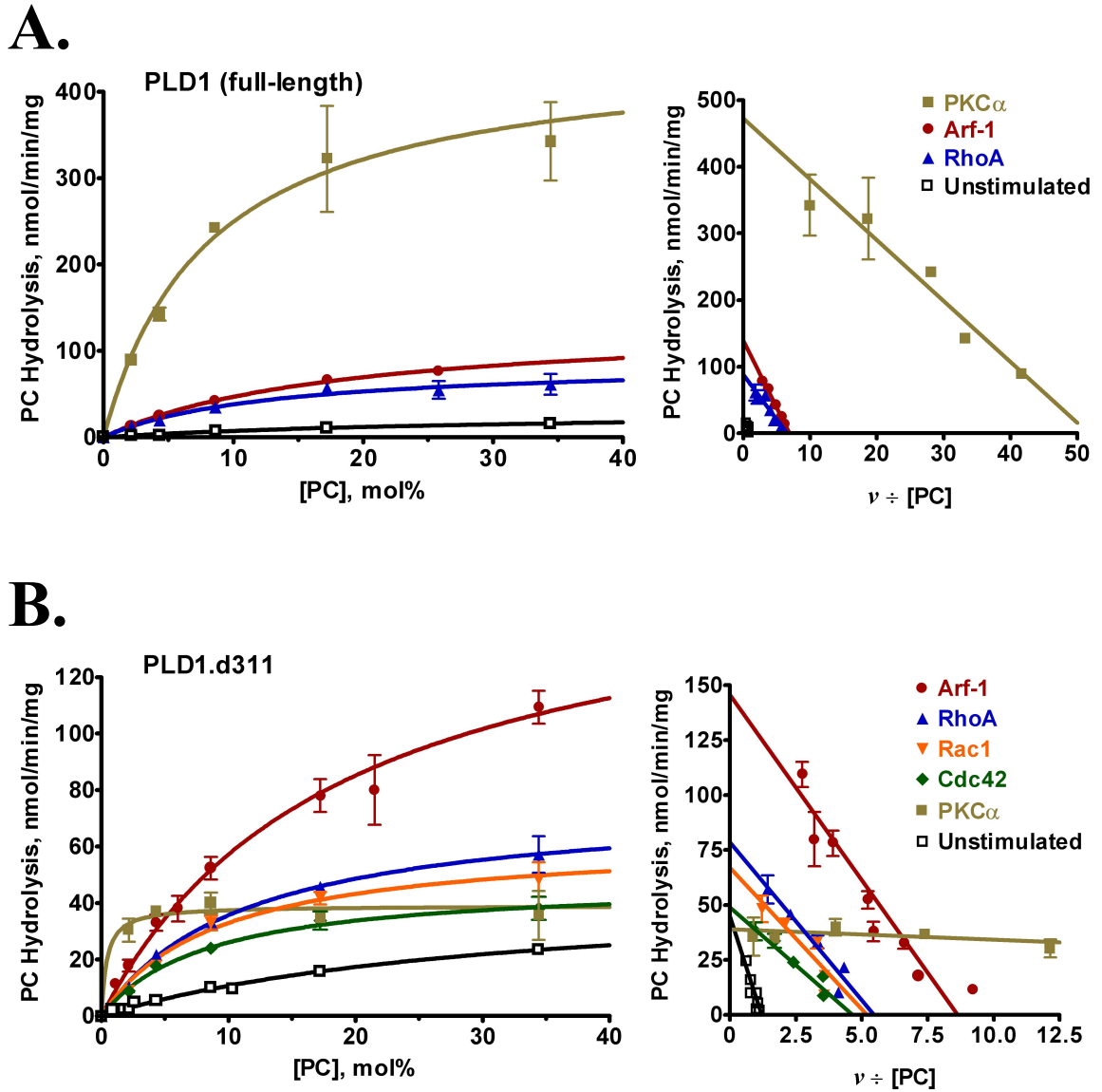


Table 8. Kinetic properties of PLD activity

Effects of allosteric activators on K_m and k_{cat} values of purified phospholipase D1 towards PC. Apparent K_m and k_{cat} were calculated as described in ‘Methods.’

| Activator(s) | PLD1 (<i>full-length</i>) | | | PLD1.d311 | | |
|---------------------------------------|-----------------------------|--------------------------------------|---|------------------------|--------------------------------------|---|
| | K_m^B <i>mol%</i> | k_{cat} <i>min⁻¹</i> | k_{cat} / K_m^B <i>mol%⁻¹min⁻¹</i> | K_m^B <i>mol%</i> | k_{cat} <i>min⁻¹</i> | k_{cat} / K_m^B <i>mol%⁻¹min⁻¹</i> |
| Unstimulated | 33 ± 12 | 4 ± 1 | 0.1 | 32 ± 8 | 6 ± 1 | 0.2 |
| PKCα | 6 ± 4 | 56 ± 10 | 9 | < 1 | 5 ± 1 | 11 |
| Arf-1 | 19 ± 6 | 16 ± 3 | 0.8 | 19 ± 4 | 22 ± 2 | 1.2 |
| RhoA | 13 ± 3 | 11 ± 1 | 0.8 | 11 ± 3 | 10 ± 1 | 0.9 |
| Rac1 | | | | 9 ± 2 | 8 ± 1 | 0.9 |
| Cdc42 | | | | 8 ± 2 | 6 ± 1 | 0.8 |
| Arf-1 + PKCα | | | | < 1 | 26 ± 2 | 48 |
| Arf-1 + RhoA | | | | 2 ± 1 | 27 ± 2 | 12 |
| Arf-1 + Rac1 | | | | 4 ± 2 | 22 ± 3 | 5 |
| Arf-1 + Cdc42 | | | | 3 ± 2 | 22 ± 3 | 6 |
| PKCα + RhoA | | | | 1 ± 1 | 6 ± 1 | 6 |
| PKCα + Rac1 | | | | 1 ± 2 | 6 ± 1 | 6 |
| PKCα + Cdc42 | | | | 2 ± 1 | 7 ± 1 | 4 |

Rho-family GTPases regulated PLD1 catalysis via allosteric interactions that promote *binding activation*. It is understood that RhoA activates PLD1 through direct interaction with a C-terminal PLD catalytic subdomain (Sung *et al.*, 1997; Du *et al.*, 2000; Cai *et al.*, 2001). Our own data show that RhoA-dependent activation of PLD1 is not altered by deletion of N-terminal domains (Table 8). Maximally effective concentrations of RhoA-GTP γ S enhanced the catalytic efficiencies of PLD1 and PLD1.d311 equally. The primary kinetic effect of RhoA was on the apparent interfacial Michaelis constant, K_m^B . Effects of closely related GTPases, Rac1 and Cdc42, were also examined (Figure 27B). Like RhoA, Rac1 and Cdc42 were *binding activators*. K_m^B values were reduced 3- to 4-fold while k_{cat} values remained essentially unchanged. *Binding activators* stimulated

PLD1 activity to limiting rates even at low concentrations of PC, shifting the substrate-dependence curve leftward without increasing the maximal rate.

Synergy is a product of catalytic activation and binding activation

Kinetic analyses of PLD1.d311 activity revealed that synergistic responses were hybrid responses, composed of the kinetic properties of each activator. Arf-1 and PKC α combined to activate PLD1.d311 with catalytic activator effects on k_{cat} and binding activator effects on K_m . The substrate-dependence curve was shifted leftward, reflecting a 50-fold reduction in K_m^B , and vertically, reflecting a 4-fold increased k_{cat} (Figure 27 and Table 8). Moreover, the combined effects of PKC and Arf on K_m^B values were equivalent to the effects of PKC alone, and the effects of combined activators on k_{cat} values were equivalent to the effects of Arf alone. Together, these effects led to cooperative effects on catalytic efficiency (k_{cat}/K_m) and led to synergy between these activators.

Rho GTPases did not synergize with PKC α . Kinetic properties of PKC α -stimulated PLD1.d311 were not significantly changed by the addition of RhoA, Rac1 or Cdc42 to the reaction mixture (Figure 28 and Table 8). Arf-1 synergized with RhoA, Rac1, and Cdc42 (Figure 28 and Table 8). Arf-1 contributed enhanced catalytic potential, while Rho GTPases contributed reduced K_m^B values. Shown in Figure 24B, simultaneous stimulation by Arf-1 and RhoA led to a synergistic response with properties of each activator. K_m^B values were reduced 14-fold, k_{cat} enhanced 4-fold. PLD1.d311 initial rates reached levels nearly 3-fold greater than can be explained by additive effects of RhoA and Arf-1. Synergy between Arf-1 and Rac1 or Cdc42 was slightly less robust.

Combinations of Arf-1 and Rac1 (Figure 28C) or Arf-1 and Cdc42 (Figure 28D) stimulated PLD1.d311 to levels 2-fold greater than can be explained by additive effects.

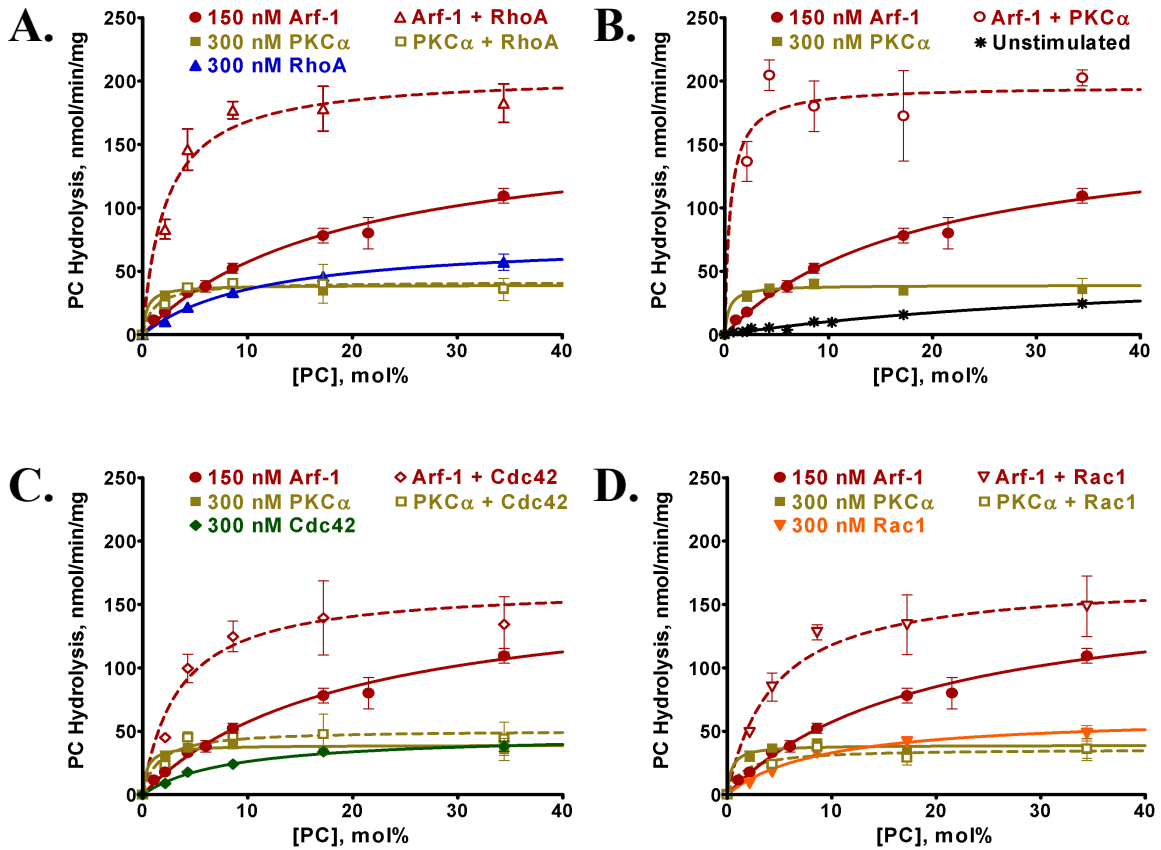


Figure 28. Synergistic activation of PLD1 is dependent upon Arf-1

Synergistic activation of PLD1 is dependent upon Arf-1. Substrate dependence of purified PLD1.d311 was examined as described in the legend of Figure 27. *A*, Phospholipase activity was measured without activators, or stimulated by PKC α , Arf-1, or PKC α and Arf-1. *B*, RhoA; *C*, Rac1; and *D*, Cdc42 were combined with Arf-1 or PKC α to stimulate PLD1.d311.

Phosphatidylinositol 4,5-bisphosphate is an essential PLD1 activator

The activity of purified PLD1.d311 was measured over a range of PIP₂ concentrations, the parameters of the assay are described in relevant figure legends. In the absence of PIP₂, PLD1.d311 was inactive and did not respond to activators. PIP₂ stimulated PLD1.d311 activity in a concentration-dependent manner (Figure 29). In the absence of protein activators, PLD activity was detected at less than 2 mol% PIP₂ and achieved maximal velocity at ~5 mol% PIP₂. Shown in Figure 25, binding of PLD1.d311 to phospholipid vesicles required PIP₂, and binding was essentially complete (>98%) for vesicles prepared with 5 mol% PIP₂. Together, these data are consistent with the interpretation that PIP₂ activates PLD1.d311 catalysis by recruiting the enzyme to the membrane.

Regulated PLD activity was also steeply dependent on PIP₂. PIP₂ potentiated the effects of all PLD1 activators (Figure 29). Activity rose sharply with increasing PIP₂ concentrations. PIP₂ effects were biphasic towards PKC α -, RhoA-, Rac1-, and Cdc42-stimulated PLD1.d311 activity. Maximal enzymatic rates were achieved at 8-12 mol% PIP₂, and concentrations greater than 35 mol% rendered PLD1.d311 unresponsive to activators. RhoA, Rac1, and Cdc42 stimulated PLD1.d311 equally, and had identical PIP₂-dependent profiles (Figure 29). Rho GTPases did not synergize with PKC α to activate PLD1.d311 (Figure 30). At optimal PIP₂ concentrations (8-12 mol%), effects of PKC α and Rho GTPases were nearly additive. At other concentrations of PIP₂, PKC α effects dominated.

150 nM Arf-1 stimulated PLD.d311 activity 4 to 6-fold above basal activity, with a PIP₂-dependence profile very similar to the profile of unstimulated PLD1.d311 (Figure

30 A,C). Arf-1 strongly synergized with all other activators (Figure 30C). PLD1 activators synergistically activated PLD1.d311 only under optimal PIP₂ concentrations. Synergy between Arf-1 and PKC α was greatest at 8 mol% PIP₂, while synergy between Arf-1 and Rho GTPases was greatest at ~5 mol%.

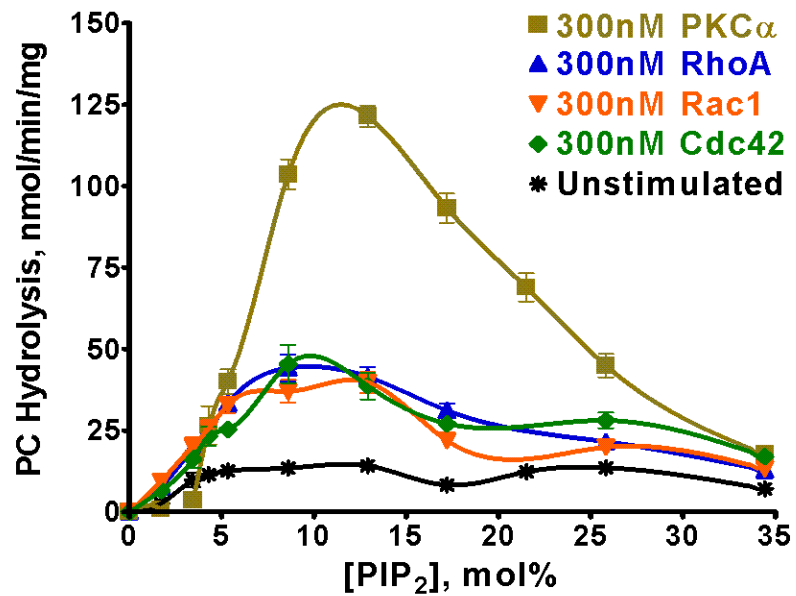


Figure 29. PIP₂ is essential for PLD1 activity

Specific phospholipase activity of PLD1.d311 was assayed with increasing concentrations of PIP₂. Phospholipase activity was measured in vitro in the standard assay (Brown *et al.*, 1995b) modified to vary the PIP₂ content of the lipid vesicles. PC and total lipid concentrations were held constant, while the PIP₂ : PE ratio was adjusted to achieve the indicated PIP₂ concentration. Phospholipase activity of purified PLD1.d311 (10 nM) was measured in the presence of 10 μ M GTP γ S alone (unstimulated), or reconstituted with GTP γ S and activators at maximally effective concentrations.

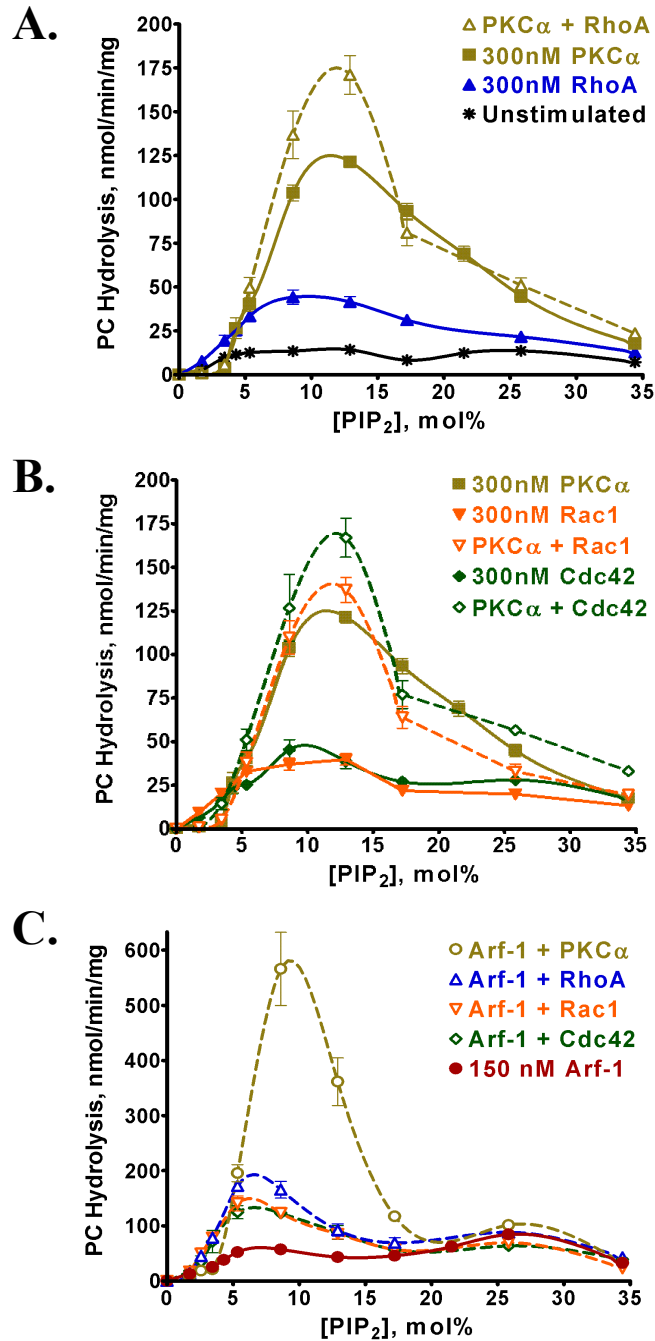


Figure 30. Synergy is dependent upon both Arf-1 and PIP₂

Specific phospholipase activity of PLD1.d311 was assayed with increasing concentrations of PIP₂ as described in the legend of Figure 29. *A.,B.*, PKC α was combined with RhoA, Rac1 or Cdc42. Activators were added at maximally effective concentrations. *C.*, Arf-1 is combined with PKC α , RhoA, Rac1, or Cdc42.

PIP₂ performs multiple roles in PLD1 activation. Clearly, PIP₂ is required for recruitment of PLD catalytic domains to the membrane interface (Sciorra *et al.*, 1999; Hodgkin *et al.*, 2000; Sciorra *et al.*, 2001; Zheng *et al.*, 2002; Du *et al.*, 2003). PLD1 mutations that disrupt a PIP₂ binding site led to impaired membrane association and catalytic activity (<5% of wild-type activity *in vitro* and *in vivo*). In the surface-binding kinetic model, this effect of PIP₂ occurs at the first binding step and is characterized by the binding constant, K_s^A . PIP₂ may also affect interfacial events, orienting the catalytic site at the membrane or stabilizing activated PLD conformations (Zheng *et al.*, 2002). In addition, PIP₂ may activate PLD via independent effects on substrate conformation (Ge *et al.*, 2001) and on PLD1 activators. All PLD1 activators bind PIP₂ in lipid membranes and are themselves activated by PIP₂ (Lee and Bell, 1991; Terui *et al.*, 1994; Zheng *et al.*, 1996). Through multiple mechanisms, PIP₂ levels determine the activity of PLD1 and determine synergistic interactions between activators.

The proposed kinetic interpretation of synergism between PLD1 activators has several important corollaries. At limiting concentrations of substrate ($[PC]_0 \ll K_m$), initial velocity of the PLD-catalyzed reaction is directly proportional to initial substrate concentration ($v_0 \approx [PC]_0 V_{max}/K_m$). This relationship is consistent with observations that synergy between activators is pronounced at low PC concentrations. At high substrate concentrations ($[PC]_0 \gg K_m$), the reaction displays first-order kinetics and approaches maximal velocity ($v_0 \approx V_{max}$). Under such conditions, *binding activators* have little effect on enzymatic rates and minimal capacity to synergize with other activators.

In a cellular context, binding activators like Rho, Rac, and Cdc42 may have little or no effect on PLD1 activity in PC-rich membrane domains. At nearby membrane

subdomains with reduced PC content, the same activators may have dramatic stimulatory effects on PLD1 activity. The proposed kinetic model of PLD1 activation predicts these counterintuitive responses.

Signal Integration by PLD1

Input from multiple PLD1 activators produces responses in a context-dependent manner. As a coincidence detector, PLD1 can constrain activity to cellular locales where these signaling molecules converge. Synergy requires appropriate concentrations of substrate, PIP₂, catalytic activators, and binding activators. Working together, these molecules can elicit substantial PLD1 activity, hydrolyzing PC to generate PA.

A descriptive parameter, denoted “ κ ,” was generated to describe functional interactions between PLD effectors (Equation 4). With this formalism, κ is a ratio of the response to combined activators relative to the sum of the responses to individual activators: $\varepsilon_{(a,b)} = \kappa (\varepsilon_{(a)} + \varepsilon_{(b)})$. *Degree of activation*, $\varepsilon_{(a)}$, relates PLD functional output (enzymatic rate) to a signaling input (effector concentration) for a given effector, “*a*” (IUPAC-IUBMB, 1982). $\varepsilon_{(a,b)}$ denotes the enzymatic response to simultaneous stimulation by effector “*a*” and effector “*b*”. κ values of unity indicate strictly additive effects ($\varepsilon_{(a,b)} = \varepsilon_{(a)} + \varepsilon_{(b)}$). Synergistic effects produce κ values greater than one (combined response greater than the sum of the effects of individual activators, $\varepsilon_{(a,b)} > \varepsilon_{(a)} + \varepsilon_{(b)}$). A κ value of 4 represents a synergistic response, four-times greater than an additive response.

PKC α did not interact with Rho, Rac, or Cdc42 to produce synergistic responses ($\kappa \leq 1$, not shown). Combinations including Arf-1 led to synergy ($\kappa > 1$) only when PC and

PIP₂ concentrations were optimal (Figure 31). Synergistic activation is related to differences in K_m values between individual activators, synergy is greatest at substrate concentrations less than K_m for Arf-1-stimulated PLD (~19 mol% PC). Consequently, synergy was greatest at the lowest substrate concentrations tested (Figure 8A).

Arf-1 + PKC α and Arf-1 + RhoA displayed the strongest synergistic interactions, reaching rates 3.5-fold greater than those predicted for additive effects (Figure 31A). Rac1 and Cdc42 synergized with Arf-1 to generate responses with κ values ~2. Differences between the properties of RhoA, Rac1, Cdc42 became apparent when these activators were combined with Arf-1. Functional differences between Rho GTPases reveal subtle differences in intermolecular interactions between PLD1 and these activators.

Synergy was steeply dependent on PIP₂ levels. Synergy between Arf-1 and PKC α was greatest at ~8 mol% PIP₂ and synergy was sensitive to small changes in PIP₂ surface concentration (Figure 31B). A 2-fold increase or decrease in PIP₂ levels eliminated synergy altogether. Synergy between Arf-1 and Rho GTPases was similarly PIP₂-dependent, but these combinations required less PIP₂ (3 mol% compared with 8 mol%).

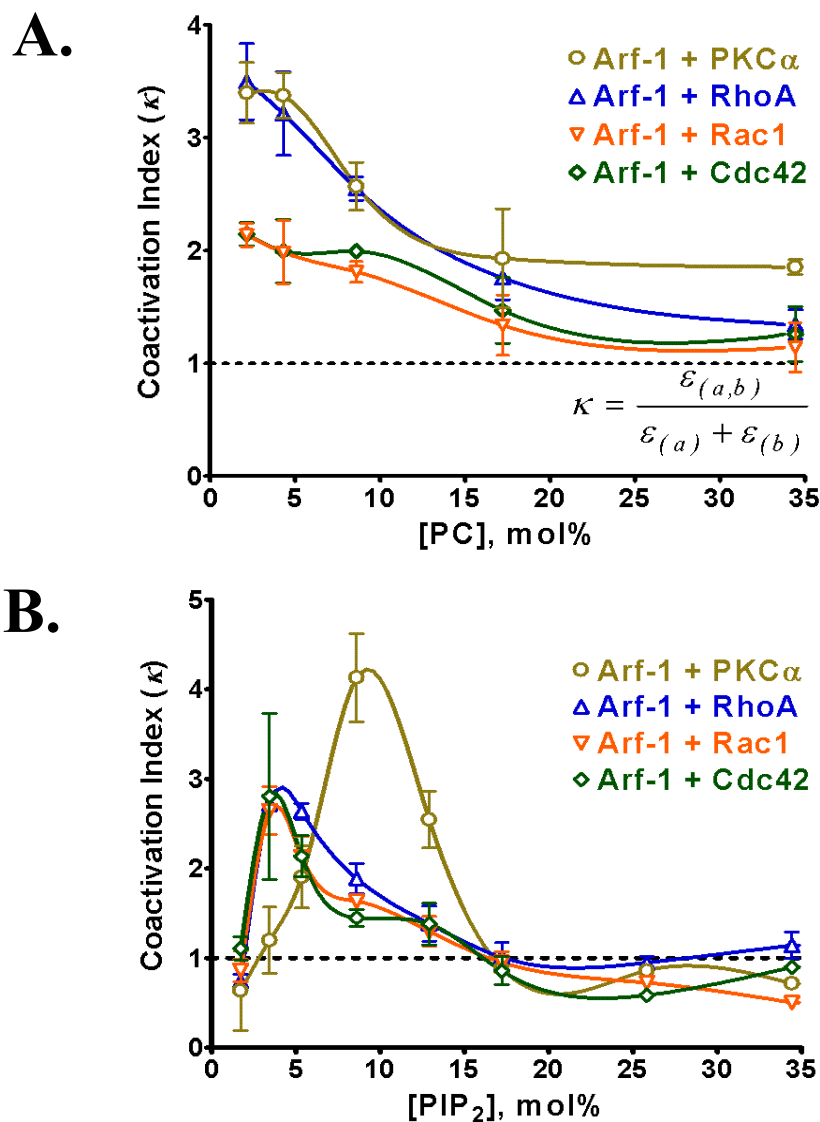


Figure 31. Synergistic effects of PLD1 activators.

Synergy is expressed as a ratio “ κ ” of the response to combined activators relative to the sum of the responses to individual activators. Additive responses have κ values of unity (*dashed lines*). **A**, Synergy between effectors was evaluated over a range of substrate (PC) concentrations. **B**, Synergistic responses to effectors were evaluated over a range of PIP₂ concentrations. Concentrations of activators were 150 nM Arf-1, 300 nM RhoA, 300 nM Rac1, 300 nM Cdc42, and 300 nM PKC α .

These requirements for PLD1 regulation are consistent with the enzyme's proposed role in membrane trafficking and exocytosis. PLD1, Arf-1, PKC α , and Cdc42 localize in late endosomes and trans-Golgi structures (Erickson et al., 1996; Westermann et al., 1996; Hiroyama and Exton, 2005). Secretory functions require PLD, Arf-1, PIP₂, and Cdc42 (Ktistakis et al., 1995; Roth, 2004; Matas et al., 2004). Extensive crosstalk between signaling networks coordinates PLD1 activators. Golgi-associated GTPase-activating proteins coordinate the activation state of Rho, Cdc42, and Arf (Miura et al., 2002; Dubois et al., 2005). PLD, PKC, Arf, and Rho subfamily GTPases activate PIP₂ synthesis (Oude Weernink et al., 2004). PIP₂ stimulates PLD1 and PLD1 activators (Lee et al., 1991; Terui et al., 1994; Zheng et al., 1996).

An important further development in this area is greater definition and structural characterization of the sites at which PLD1 activators interact with the enzyme. This and the elucidation of the full structure of this enzyme will enable understanding of the molecular mechanisms by which activators alter the catalytic mechanism.

Nota bene: Much of the work presented in the chapter has been published previously in (Henage et al., 2005).

Methods

Materials

The chemicals used for all experiments were of the highest grade. Myristic acid was purchased from Sigma Aldrich, n-octyl- β -D-glucopyranoside (β -OG) and guanosine 5'-3-O-(thio)triphosphate (GTP γ S) were from Calbiochem. Phosphatidylethanolamine (PE), PC, and PIP₂ were purchased from Avanti Polar Lipids. [methyl-³H]PC was purchased from Perkin-Elmer Life Sciences.

Partial purification of PLD1

The full open reading frame of rat phospholipase D1b (Min *et al.*, 1998) in the pBlueBacHis2B (Invitrogen) baculovirus transfer vector was modified to generate an N-terminal FLAG epitope by site directed mutagenesis as described in the QuickChange™ method (Stratagene). Baculovirus was generated by cotransfection with linearized AcMNPV viral DNA (Invitrogen) in *Spodoptera frugiperda* Sf21 cells. Recombinant virus was isolated by three rounds of plaque selection and amplified in Sf21 cells adapted to suspension culture in *Trichoplusia ni* Medium-Formulation Hinks (TNM-FH) with 10% fetal bovine serum. Plasmid constructs and baculovirus DNA were sequenced to verify coding regions. Adherent Sf21 cultures (7×10^8 cells) were infected at a multiplicity of infection of 1. After 72 h, cells were harvested by centrifugation and resuspended in 10 mL Lysis Buffer A (50 mM sodium phosphate buffer pH 7.5, 250 mM NaCl, 15 mM imidazole, 1 mM MgCl₂, 1% (w/v) β -OG, 0.5 mM DTT, 2 mM PMSF, Complete protease inhibitor cocktail (Roche)). Cells were disrupted by sonication 6 x 10 sec at 6W RMS on ice. Insoluble material was removed by centrifugation (40,000 x g for

30 min at 4°C) and lysate was further clarified (100,000 x g for 1 h at 4°C). Recombinant PLD1 protein (124 kDa) was purified over a HiTrap Chelating HP nickel imino-diacetic acid (NiIDA) column (GE Biosciences) in Buffer A (50 mM sodium phosphate buffer pH 7.5, 200 mM NaCl, 30 mM imidazole, 1 mM MgCl₂, 1% (w/v) β-OG, 0.5 mM DTT), eluting at 180 mM imidazole in a linear imidazole gradient. Pooled fractions were desalted by gel filtration and stored at -80°C in 10% glycerol.

Purification of PLD1.d311

N-terminally truncated rat PLD1.d311 (amino acids 312-1036) was amplified by PCR from rPLD1b (Park *et al.*, 1997) and subcloned into pENTRdTOPO (Invitrogen) in frame with an N-terminal 6HIS / maltose binding domain (*maltE*) fusion protein amplified from pSV282 (Laura Mizoue, Vanderbilt University). PLD1b.d311 was transferred to pDEST8 baculovirus expression vectors by homologous recombination using GATEWAY® methods (Invitrogen). Baculovirus encoding the PLD1.d311 construct was generated in Sf21 insect cells using the Bac-to-Bac® system (Invitrogen) and screened for optimal expression conditions. Plasmid constructs and baculovirus DNA were sequenced to verify coding regions. Adherent Sf21 cultures (1 x 10⁹ cells) were infected at a multiplicity of infection of 0.1 to 0.5 in TNM-FH media with 10% fetal bovine serum. After 72 h, cells were harvested by centrifugation and resuspended in 20 mL Lysis Buffer A. Recombinant PLD1.d311 protein (131 kDa) was purified as described for full-length rPLD1b above. Purified protein eluted from HiTrap Chelating HP NiIDA columns at 170 mM imidazole in a linear imidazole gradient. Pooled fractions were immediately desalted over a Sephadex G-25 Superfine gel filtration

column (GE Biosciences) and exchanged to Buffer B (30 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM DTT). Aggregated protein and minor contaminants were resolved from the purified fractions by size-exclusion chromatography over a 16/60 Superdex 200 pg column (GE Biosciences). PLD1.d311 (>95% pure) eluted with a retention volume of 68 mL. Pooled fractions were concentrated to >1 mg/mL by ultrafiltration, frozen in 10% glycerol, and stored at -80°C . Purified PLD retained high enzymatic activity for 2-3 days at 4°C , and for >1 year at -80°C .

Purification of myristoyl-ADP ribosylation factor-1

Human Arf-1 was coexpressed with human N-myristoyltransferase 1 in BL21(DE3) *Escherichia coli*. Four-liter cultures were grown in LB broth (150 $\mu\text{g}/\text{mL}$ carbenicillin and 50 $\mu\text{g}/\text{mL}$ kanamycin) at 37°C and 250 rpm. At $\text{OD}_{600} = 0.7$, myristic acid was added to 50 mg/L. Expression was induced at $\text{OD}_{600} = 0.9$ with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside. Cultures were incubated for three additional hours at 27°C and harvested by centrifugation. Cells were resuspended in 10 mL lysis buffer (20 mM Tris-Cl pH 8, 20 mM NaCl, 1 mM MgCl_2 , 100 μM GDP, 1 mM DTT, 10 mg/mL lysozyme, 2 mM PMSF, complete protease inhibitor cocktail) and lysed by sonication (6 x 30 sec pulses at 6W RMS on ice). Insoluble material was removed by centrifugation (40,000 x g for 30 min at 4°C) and lysate was further clarified (100,000 x g for 1 h at 4°C). Supernatant was diluted to 50 mL in Buffer C (20 mM Tris-Cl pH 8, 20 mM NaCl, 1 mM MgCl_2 , 1 mM EDTA, 100 μM GDP, 1 mM DTT). Clarified lysate was applied to an 85 mL DEAE Sepharose FF column (GE Biosciences) and eluted at 110 mM NaCl in a linear NaCl gradient. Active fractions were identified by activation of PLD *in vitro* and

concentrated to 5 mL by ultrafiltration. Recombinant Arf-1 was applied to a 26/60 Superdex 75 pg column (GE Biosciences) in Buffer D (20 mM Tris-Cl pH 8, 150 mM NaCl, 1 mM MgCl₂, 1 mM DTT). Purified (~95%) Arf-1 eluted with a retention volume of 196 mL. Active fractions were concentrated to 0.5 mg/mL by ultrafiltration, frozen in 5% glycerol, and stored at -80°C.

Purification of geranylgeranylated RhoA, Rac1, and Cdc42

Baculoviruses encoding N-terminal His-tagged human RhoA, Rac1, and Cdc42 have been described previously (Walker *et al.*, 2002). Adherent Sf21 cultures (3 x 10⁸) cells were infected at a multiplicity of infection of >1. After 72 h, cells were harvested by centrifugation and resuspended in Lysis Buffer B (50 mM sodium phosphate buffer pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 1% (w/v) β-OG, 10 μM GDP, 2 mM PMSF, complete protease inhibitor cocktail). Cells were disrupted by sonication 6 x 10 sec at 6W RMS on ice. Lysate was clarified by centrifugation at 100,000 x g at 6°C for 60 min. Recombinant GTPases were purified over 1 mL HiTrap Chelating HP NiIDA columns (GE Biosciences) and eluted with linear imidazole gradients. Active fractions (300 - 360 mM imidazole) were identified by activation of PLD *in vitro* and exchanged to a storage buffer containing 25 mM phosphate buffer pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 0.5% (w/v) β-OG) over a Sephadex G-25 Superfine gel filtration column (GE Biosciences). Purified (>95%), highly active geranylgeranyl- RhoA, Rac1, and Cdc42 were frozen in 5% glycerol, and stored at -80°C.

Partial purification of protein kinase Cα

Rat PKC α was expressed in baculovirus infected Sf21 cells and purified essentially as described for PKC β II in (Walker *et al.*, 2000b).

Binding of PLD1.d311 to sucrose-loaded vesicles

The sucrose-loaded vesicle binding assay was adopted, with minor modifications, from the procedure of Buser and McLaughlin (Buser *et al.*, 1998). Large unilamellar vesicles were prepared by extrusion (Lipex Biomembranes, Inc., Vancouver) of lipid dispersions through two 0.1 μ M polycarbonate filters (Nuclepore). Lipid composition was identical to standard *in vitro* assay preparations, *vide infra*, or modified to replace PIP₂ with PE. Vesicles were loaded with: 176 mM sucrose, 50 mM Hepes pH 7.5, 3 mM EGTA, 3 mM MgCl₂, 3 mM CaCl₂. Sucrose-loaded vesicles were washed and resuspended in an isotonic buffer containing KCl. Sucrose-loaded vesicles were incubated with 10 nM PLD1.d311 for 30 min at 25°C and sedimented by ultracentrifugation, 100,000 x g for 45 min at 20°C. PLD1 present in supernatant (PLD_{sup}) and pellet (PLD_{pellet}) was estimated by immunoblot and vesicle-associated PLD1.d311 was calculated according to Equation 1:

$$PLD_{vesicle} = \frac{(\beta)PLD_{pellet}(\beta - 1)PLD_{sup}}{\alpha + \beta - 1} = \frac{(B_{max})[PL]_T}{K_s^A + [PL]_T} \quad (1)$$

‘ α ’ is the fraction of sedimented vesicles determined by scintillation counting, and ‘ β ’ is the fraction of PLD immunoreactivity (HRP-conjugated α FLAG-M2 antibody, Sigma) in the supernatant fraction in the absence of lipid (accounts for PLD that precipitates without lipid). $[PL]_T$ is the total concentration of lipid, and K_s^A is a dissociation constant describing bulk association with lipid.

Measurement of PLD activity *in vivo*

Activity assays were performed as described in (Walker *et al.*, 2004). HA-tagged RhoA.G14V was obtained from Guthrie Research Institute (www.cdna.org). Rat PLD1b and PLD1.d311 were subcloned into pcDNA3.1 (Invitrogen). PLD1 and activators were transiently expressed in Cos7 cells by liposome transfection (Fugene6, Roche). PLD constructs (0.6 µg DNA) were cotransfected 1:1 with RhoA.G14V or empty vector as ballast. Transfected cells were serum starved (18 h) and treated (15 min) with phorbol myristate acetate (100 nM) as indicated. Expression of heterologous proteins was verified by SDS-PAGE and immunoblot.

Measurement of PLD activity *in vitro*

Activity assays were performed with exogenous substrate as described previously (Brown *et al.*, 1995b). Briefly, PLD activity was measured by the release of [methyl-³H]choline from [choline-methyl-³H]dipalmitoyl-PC. 1-10 nM PLD was reconstituted with phospholipid vesicle substrates as described in the text, typically composed of 10 µM dipalmitoyl-PC, 100 µM PE (bovine liver), 6.2 µM PIP₂ (porcine brain), and 1.4 µM cholesterol. Lipid solutions were dried under a gentle stream of nitrogen and then resuspended in 100 mM Hepes pH 7.5, 160 mM KCl, 6 mM EGTA, 0.2 mM DTT. Small unilamellar vesicles were prepared by bath sonication (6 x 1 min intervals at 80W). All assays were conducted for 30 min at 37°C in 50 mM Hepes pH 7.5, 80 mM KCl, 3 mM EGTA, 0.1 mM DTT, 3.6 mM MgCl₂, 3.6 mM CaCl₂, and 10 µM GTPγS. Reactions were stopped by addition of trichloroacetic acid and bovine serum albumin. Free [methyl-³H]choline was separated from precipitated lipids and proteins by centrifugation and was analyzed by liquid scintillation counting. The enzymatic reactions were linear

with time and protein concentration. Initial rates were determined from measurements between 5 and 25% PC hydrolysis. Data are presented as mean initial enzymatic rates \pm standard error (nmoles PC hydrolyzed per min per mg PLD) measured in 3–12 independent experiments performed in duplicate.

Analysis of kinetic data

Apparent dissociation constants and rate constants were determined from best-fit parameters by nonlinear regression (sum-of-squares) using Equation 2 (Carman *et al.*, 1995).

$$\left. \frac{d[\text{choline}]}{dt} \right|_{(t \rightarrow 0)} = \frac{k_{cat} [PL]_T [PC]_0 [PLD]}{K_s^A K_m^B + K_m^B [PL]_T + [PL]_T [PC]_0} \quad (2)$$

Concentration-response data were fit to Equation 3, where “MAX” and “MIN” refer to maximum and minimum rates, “ n_H ” is Hill’s coefficient, and “[a]” represents the concentration of an allosteric effector.

$$\frac{d[\text{choline}]}{dt} = \frac{MIN + (MAX - MIN)}{1 + 10^{(n_H \log EC_{50} - \log[a])}} \quad (3)$$

Synergy is expressed as a ratio, “ κ ,” of the response to combined activators relative to the responses to individual activators (Equation 4). Degree of activation, “ ε ” (IUPAC-IUBMB, 1982), describes the increase in PLD catalytic rate due to the effects of individual activators, “a” and “b,” or activators in combination, “a,b.”

$$\kappa = \frac{\varepsilon_{(a,b)}}{\varepsilon_{(a)} + \varepsilon_{(b)}} \quad (4)$$

Calculations were performed using Prism v4.0 (GraphPad Software).

CHAPTER IV

PRELIMINARY STUDIES OF PHOSPHOLIPASE D STRUCTURE

“Facilius enim per partes in cognitionem totius adducimur”
We are more easily led part-by-part to an understanding of the whole.
Seneca (*Epistularum Moralium ad Lucilium*, LXXXIX)

Rationale

Phospholipase D1 has been implicated in many critically important cellular functions and in several human disease states. As yet, the enzyme's potential as a therapeutic target remains entirely unexplored. Unanswered questions about PLD structure and catalysis are, in part, responsible for the complete lack of pharmacological agents targeted to phospholipase D. Structural knowledge of PLD1 catalytic and regulatory domains will lead to an understanding of the enzyme's regulation and its mechanism of action. Primary sequence requirements for interactions with PKC, Rho GTPases, and PIP₂ have recently been defined, although the secondary and tertiary structures of these domains have not. Structural analysis of these domains will provide crucial information about the allosteric modulation of phospholipase D activity.

Spectroscopic methods are paired with computational approaches to evaluate the fold of PLD domains. Advances in the purification of recombinant PLD1 (Chapter II) make biophysical approaches possible. X-ray crystallography and electron paramagnetic resonance spectroscopy were employed to study structural properties of isolated PLD domains. Homology-based molecular modeling provides insight into the structure of

conserved PLD domains, predicting tertiary structure for nearly 60% of PLD1b sequences.

Crystallographic analysis of PLD1 catalytic domains

PLD1.d311 was purified from insect cells to near homogeneity (>95% pure by coomassie-stained gels) by sequential nickel-chelating (NiIDA-sepharose, GE Biosciences) and size-exclusion (Superdex 200) chromatography steps (Chapter II). Detergent and salt were removed, and purified protein was concentrated to ~20 mg/mL by ultrafiltration. Although much of the protein was lost to insoluble aggregates during concentration steps, ~ 40 mg of purified MBP.PLD1.d311 was used to screen crystallization conditions.

Using commercial (Crystal Screens I and II, Hampton Research), 294 conditions were screened by sitting-drop vapor-diffusion over 6 months at 4°C. MBP.PLD1.d311 precipitated in more than 60% of the conditions tested. In collaboration with researchers at The Ohio State University (Martin Caffrey and Vadim Cherezov) and the University of Alabama at Birmingham (Larry Delucas and Lisa Nagy), MBP.PLD1.d311 was screened in 1152 single-lipid, cubic-phase conditions at 20°C. While none of these conditions led to successful crystallization of PLD, the search could be narrowed to those conditions where PLD did not precipitate.

Diffraction crystals have been successfully obtained from several PLD enzymes; *Yersina pestis* (Rudolph et al., 1999), *Streptomyces antibioticus* (Suzuki et al., 1999), *Streptomyces sp.* PMF (Leiros et al., 2000a), sunflower and cowpea (Abergel et al., 2001). Structures have been solved for *Streptomyces sp.* PMF PLD (Leiros et al., 2000b)

using heavy-atom derivatives and by atomic replacement. Other PLD crystals have not yet produced three-dimensional structures.

Several enzymes in the PLD superfamily specifically bind tungstate ions, potential heavy-atom ligands for PLD1. Tungstate binds at their catalytic sites, mimicking phosphates in substrate transition-states. Like vanadate, tungstate is a competitive inhibitor of many phosphodiesterases. In activity assays, tungstate inhibited PLD with low potency ($IC_{50} \approx 200 \mu\text{M}$). At 10 mM, tungstate completely blocked basal and Arf-stimulated PLD activity *in vitro* (Figure 32). This suggests that tungstate will be useful for phase-determination should diffracting crystals be obtained.

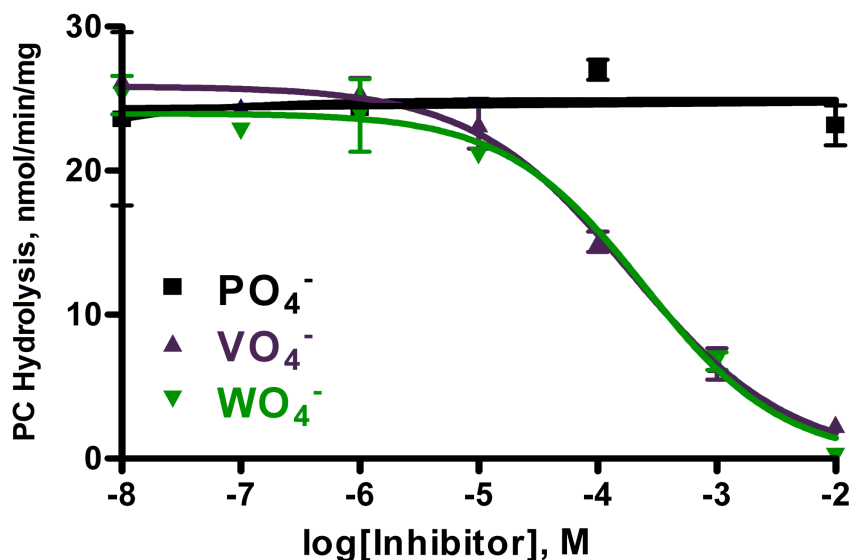
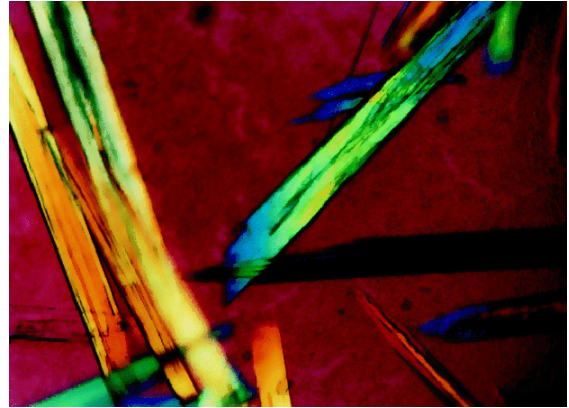
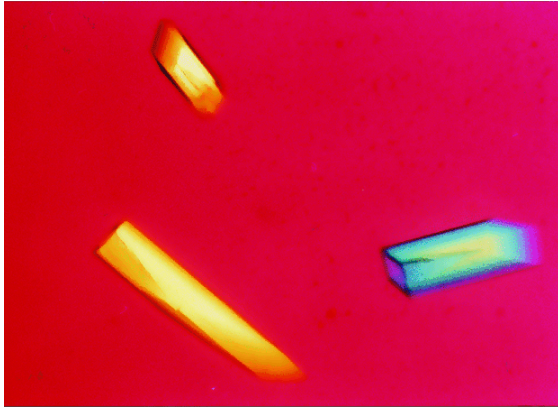
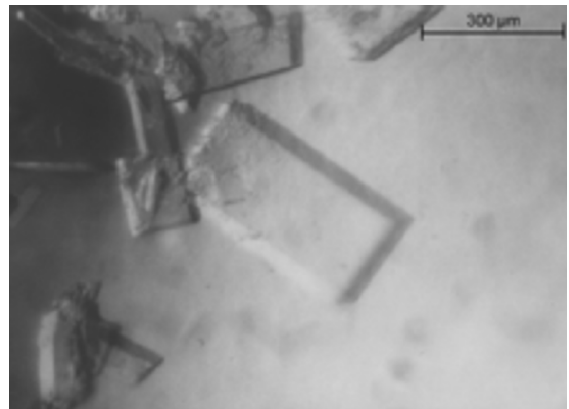
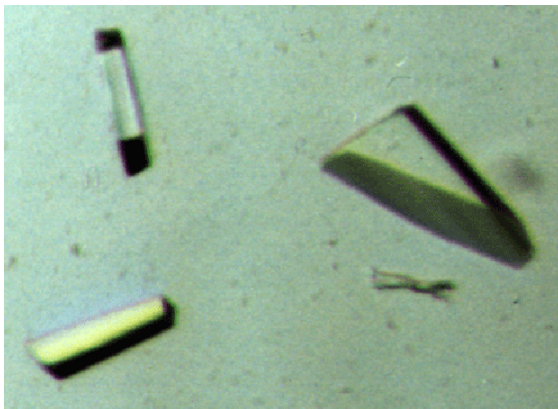


Figure 32. Competitive inhibition by transition metals

PLD1.d311 activity was inhibited by high concentrations of tungstate and vanadate but not phosphate. PLD activity was incubated with 150 nM Arf-1 and increasing concentrations of inhibitors. Unstimulated PLD activity was inhibited by these agents at similar concentrations (not shown).

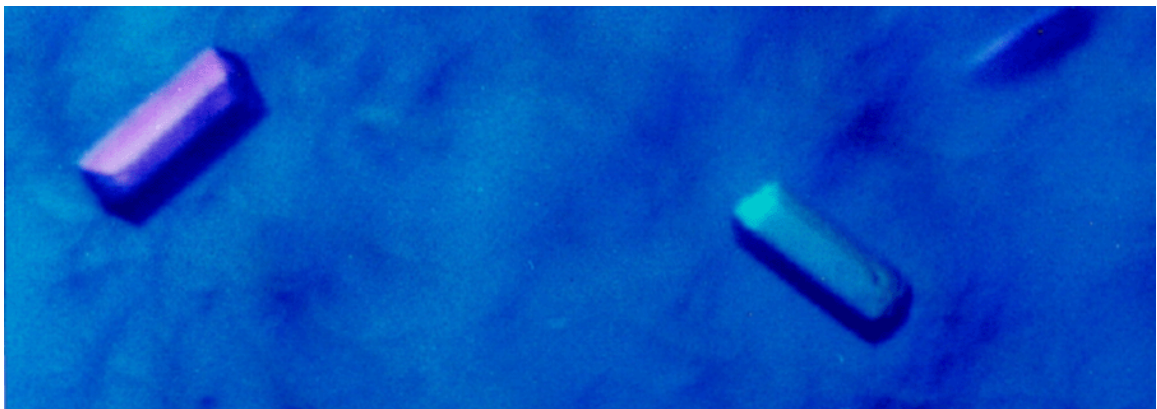


Crystals of PLD from *Streptomyces antibioticus* (Suzuki et al., 1999)



Crystals of PLD from sunflower (Abergel et al., 2001)

Crystals of PLD from *Streptomyces* PMF (Leiros et al., 2000a)



Crystals of PLD (murine toxin) from *Yersinia pestis* (Rudolph et al., 1999)

Figure 33. Gallery of phospholipase D crystals

Site-directed spin-labeling and electron paramagnetic resonance spectroscopy

Multiple laboratories have failed to successfully solve the three-dimensional structure of a eukaryotic phospholipase D by x-ray crystallography. Should such a structure become available, interpretation of SDSL-EPR experiments will be greatly enhanced. A static structure of PLD can provide a framework for interpretations of conformational dynamics and protein-protein interactions. Because EPR provides time-resolved structural information, conformational dynamics are revealed.

Structural studies of multiple signaling molecules in GPCR signaling cascades reveal that spatial disorder in crystal structures correlates with protein-protein interaction sites ($G_{\beta\gamma}$ in (Sondek et al., 1996), GPCR in (Palczewski et al., 2000), and monomeric-GTPases in (Milburn et al., 1990)). Because protein crystallography cannot provide a direct view of protein motions, these critically important sequences are absent in crystallographic structures. The SDSL-EPR approach is ideally suited to examine dynamic sequences and has been particularly successful in identifying functionally important domains in membrane proteins (GPCR in (Hubbell et al., 2003), ion channels in (Liu et al., 2001b), and phospholipases (Malmberg et al., 2003)).

A proven technique for probing the structure of membrane proteins, site-directed spin-labeling (SDSL) electron paramagnetic resonance (EPR) provides information about protein structure and conformational dynamics (McHaourab et al., 1996). These methods can be used to map surface interfaces between PLD1 and its activators and to assess the orientation of relevant domains. Site-directed labeling (EPR and fluorescence) approaches are uniquely compatible with the lipid environment native to PLD1. Also,

SDSL-EPR experiments require small quantities of protein (10-100 pmol) for structural characterization.

The approach involves the modification of a reactive sulfhydryl moiety with a nitroxide spin-label (Figure 34). An unpaired electron allows the nitroxide to act as an EPR reporter group. To incorporate spin-labels at specific sites within a protein, site-directed mutagenesis is used to introduce target residues. In this approach, all native, reactive cysteines are mutated so that the introduction of novel cysteines targets MTS-nitroxide spin-labels to specific positions within the protein. To incorporate spin-labels at specific sites within a protein, site-directed mutagenesis is used to introduce target residues. Nitroxide derivatized to cysteine is typically no more perturbing than other single amino acid substitutions (McHaourab et al., 1996).

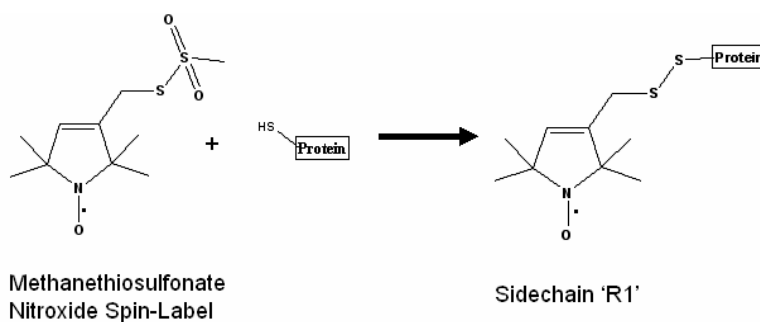


Figure 34. Thiol-specific nitroxide spin-label for SDSL-EPR experiments

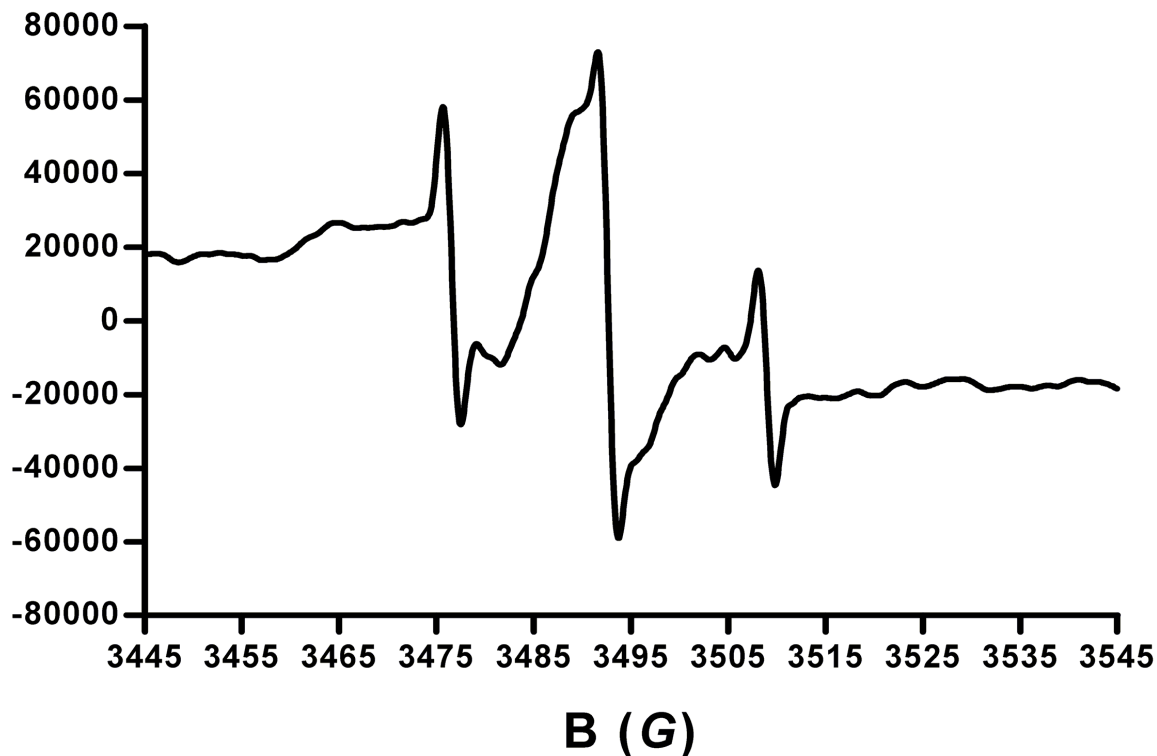


Figure 35. EPR spectra for spin-labeled PLD1.d311

Pilot experiments were performed to demonstrate the feasibility of SDSL studies. PLD1.d311 was expressed in insect cells and purified to near homogeneity as described above. Native PLD1.d311 cysteines were labeled 12 hrs with 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate (R1, Toronto Research Chemicals). Free label was removed by size-exclusion chromatography and ultrafiltration. Spectra were collected at X-band (9.8 GHz) with a Bruker EMX spectrometer at 4°C.

Experiments were performed to assess the effects of cysteine mutagenesis on PLD1 function. Even in the extreme case of a cysteine-null PLD1 mutant (13 Cys→Ala mutations), catalytic activity is preserved (Figure 36). While expression of the cysteine-null PLD1 is somewhat reduced in Cos7 cells, expression in baculovirus infected insect cells was sufficient for successful purification. Mutation of native cysteines to alanines (or serines) resulted in modestly reduced expression levels in Cos7 cells. About 5% of PLD expression is lost per mutation.

Expression and purification are greatly enhanced by N-terminal deletions and fusion to soluble, maltose binding protein partner. The *E. coli* maltose binding protein (MalE) is natively a protein that is excreted into the periplasmic space where the redox environment is favorable to the formation of disulfide bonds. MalE has evolved to be resistant to this environment, and has no native cysteines. This property makes the MBP.PLD.d311 an excellent platform for SDSL-EPR studies of PLD catalytic domains.

Mutants that exhibit altered regulated activity provide information about the primary structure determinants of PLD1 regulation, while EPR experiments examine the secondary and tertiary structures of those same domains. Periodicity in solvent accessibility (quantified by collision parameters (II) between spin-labels and paramagnetic centers in the solvent), polarity (quantified by the spectral breadth ($2A_{zz}$)) and mobility (quantified by the inverse central resonance line width ($(\Delta H_0)^{-1}$)) are used to identify residues at the membrane surface, map secondary structure, and identify tertiary contacts. Reconstituted with lipid vesicles and/or activators, these experiments assess the orientation of PLD domains toward the membrane and may identify sites of direct contact with regulators. Changes in these parameters (II , A_{zz} , $(\Delta H_0)^{-1}$) upon PLD activation report conformational rearrangements involved in PLD1 regulation.

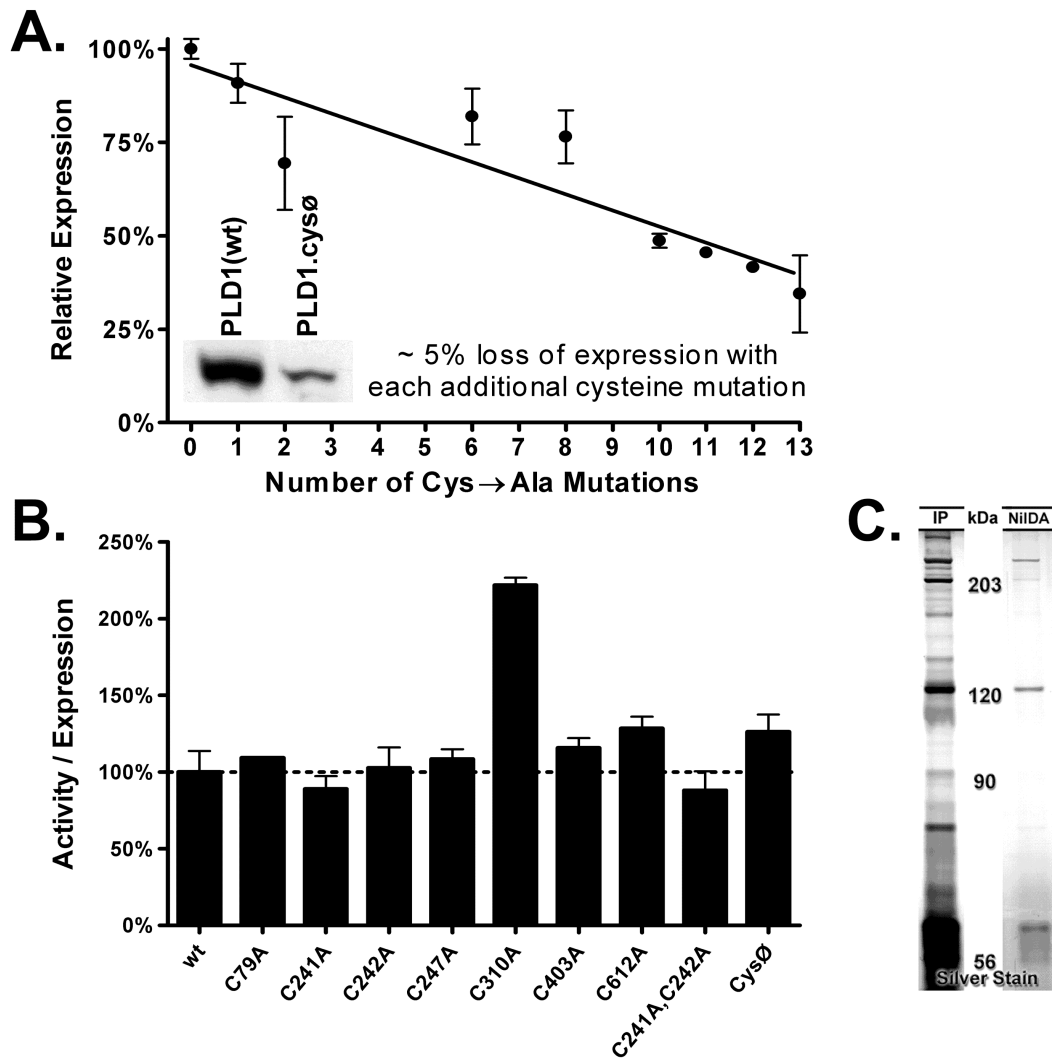


Figure 36. Characterization of full-length PLD1 cysteine mutants

Wild-type and Cys→Ala mutant PLD1b constructs were transiently expressed in Cos7 cells. (A.) Relative expression levels were estimated by immunoblot and densitometry. (Inset) Immunoblot of PLD1b(wt) (i.e., no Cys→Ala mutations) and PLD1.cysØ (i.e., 13 Cys→Ala mutations) illustrates the range of expression observed. (B.) Activity of PLD1b constructs was measured in Cos7 cells in the endogenous substrate assay. Activity was stimulated by 100 nM PMA and normalized to expression levels. (C.) PLD1.cysØ was expressed in baculovirus infected insect cells and purified by immunoaffinity and nickel-chelating chromatography.

Structural Predictions

Using primary sequence databases, and crystallographic structures of related enzymes, hypothetical PLD1 structures were developed by computational methods. Secondary and tertiary structure prediction methods were used to construct models of PLD1 structure.

Secondary structure prediction

To provide secondary structural information to guide mutational studies, PLD1 sequences were analyzed by a panel of prediction algorithms. Each algorithm uses a different method to predict secondary structure for protein sequences. By comparing results from a large number of prediction algorithms, a consensus prediction was constructed (Figure 37). 13 secondary structure prediction programs were used: JUFO (Meiler et al., 2002), HNN (Guermeur, 1997), GOR4 (Garnier et al., 1996), PHD (Rost and Sander, 1993), SOPMA (Geourjon and Deleage, 1995), PROF (Ouali and King, 2000), Jpred (Cuff et al., 1998), PSIPred (Jones, 1999), SSpro (Pollastri et al., 2002), PORTER (Pollastri and McLysaght, 2005), NNpredict (Kneller et al., 1990), APSSP2 (Raghava, 2000), and SAM-T02 (Karplus et al., 2003).

The predicted PLD1 secondary structure is consistent with homology-based structural models. There are regions of low complexity (perhaps unstructured) between conserved domains and in the 'loop region.' A large, helical structure is predicted to exist at the C-terminus of PLD1. This C-terminal extension is not present in prokaryotic PLD structures, but is conserved in PLD2 and other eukaryotic PLD isoforms. The catalytic subdomains have matching secondary structure profiles, consistent with the proposed pseudodimeric structure for the catalytic domains.

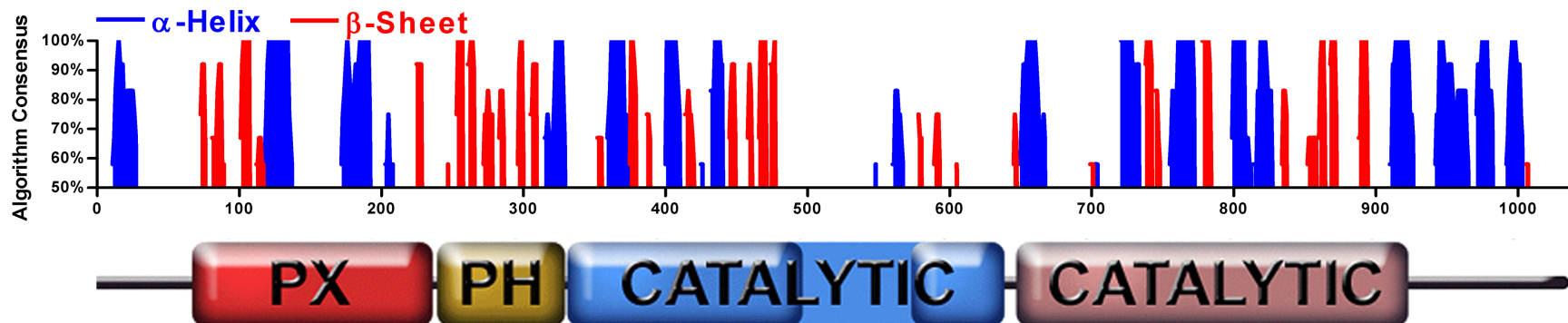


Figure 37. Secondary structure predictions for PLD1b

PLD sequences were analyzed using 13 secondary structure prediction algorithms using default settings. Sequences were scored by each method, and consensus between algorithms is depicted on the ordinate axis. A score of 100% represents unanimous agreement of the prediction algorithms for a given secondary structure element. Secondary structure was predicted by APSSP2, GOR4, HNN, Jpred, JUFO, NNpredict, PHD, PORTER, PROF, PSIPred, SAM-T02, SOPMA, SSpro. The conserved domain structure of PLD1b is illustrated below the secondary structure predictions.

Tertiary structure prediction

Existing structural information permits the construction of predictive molecular models of PLD1. A homology-based modeling approach was employed to assign three-dimensional coordinates to PLD1 sequences. Multiple structures, solved by x-ray crystallography, were used as references. Preliminary models assign structure to more than 60% of PLD1 sequence, and predict structure for isolated catalytic, PX, and PH domains. Intervening sequences could not be predicted, so it is not possible to predict the relative orientation of PLD domains without additional biophysical data.

Reference structures were aligned by CE (Combinatorial Extension of the optimal path, (Shindyalov and Bourne, 1998)) or by VAST (Vector Alignment Search Tool, (Gibrat et al., 1996)). Consensus sequences were derived from these structural alignments. Sequences conserved across the PLD superfamily were identified by multiple sequence alignments using the ClustalW algorithm (Thompson et al., 1994). PLD1 sequences were mapped to structurally conserved regions of reference proteins and structural models were built manually. Poorly conserved loops were constructed *de novo* using the HOMOLOGY software package within INSIGHTII (Accelrys). Models were refined by a modified simulated-annealing approach, with molecular dynamics simulations used in relaxation and energy-minimization steps.

These structural models give the first insight into the mechanics of intermolecular interactions that regulate PLD1 functions. However, because the reference structures are from PLD superfamily members that are only distantly related to PLD1, many interesting binding sites and critical regions are not present in the predictive models. Despite being incomplete, these models are useful for directing preliminary structural studies.

PX domain

Several PX domain structures have been solved by x-ray crystallography. Grd19p (sorting nexin-3) (Zhou et al., 2003), Vam7p (Lu et al., 2002), p40^{phox} (Bravo et al., 2001), p47^{phox} (Karathanassis et al., 2002), and cytokine-independent survival kinase (Cisk) (Xing et al., 2004) demonstrate a conserved fold for PX domains. These structures have high structural homology (RMSD < 2 Å), and conserved sequences (mean pairwise identity 26 %).

PLD1 molecular models were constructed from these PX domain coordinates and molecular dynamics simulations. The predicted structure contains an elongated β -sheet and opposed α -helices (Figures 38 and 39). Putative lipid-binding sites are present at either end of helix at the membrane interface. These binding sites and several conserved aromatic residues contribute to the membrane association of the domain.

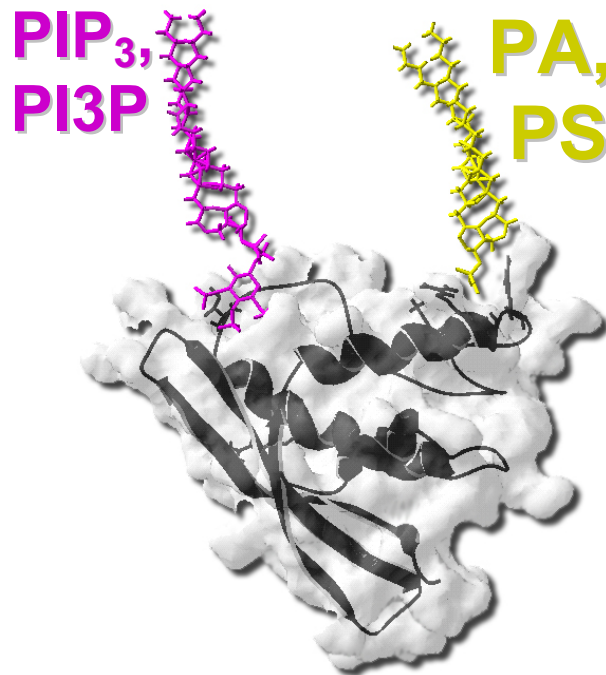


Figure 38. Structural predictions for the PLD1 PX domain

PH domain

To identify structurally conserved regions, PH domain reference structures were: 3-phosphoinositide-dependent protein kinase-1 (Pdk1) (Komander et al., 2004), Dapp1/PHISH (Ferguson et al., 2000), Tapp1 (Thomas et al., 2001), Pepp1, protein kinase B α (PKB α) (Milburn et al., 2003), Dynamin (Timm et al., 1994), Arno/Grp1 (Cronin et al., 2004b), son of sevenless (Sos) (Sondermann et al., 2004), Bruton's tyrosine kinase (Btk) (Jin et al., 2005), Skap55, and exocyst 84 (Jin et al., 2005). These structures have high structural homology (RMSD < 1.8 Å), and conserved sequences (mean pairwise identity 28 %).

PLD1 molecular models were constructed from these PH domain coordinates and molecular dynamics simulations. A compact β -structure formed the core of the domain, and a single α -helix is present at the C-terminal boundary of the domain (Figures 40 and 41). A loop is inserted between the first and second β -strands, containing a palmitoylation site. These two strands fold together to produce a conserved anionic binding cleft that binds phosphoinositides.

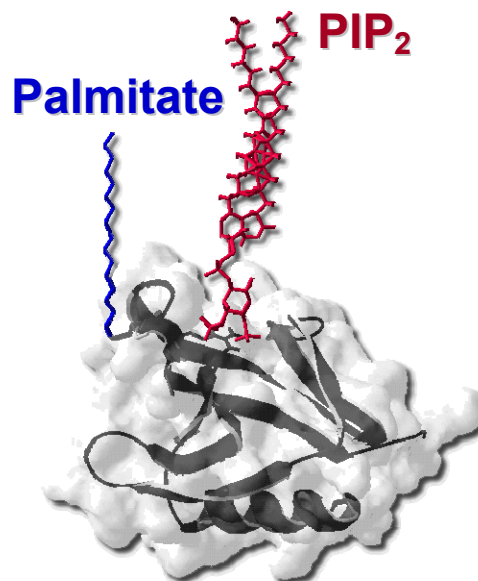


Figure 40. Structural predictions for the PLD1 PH domain

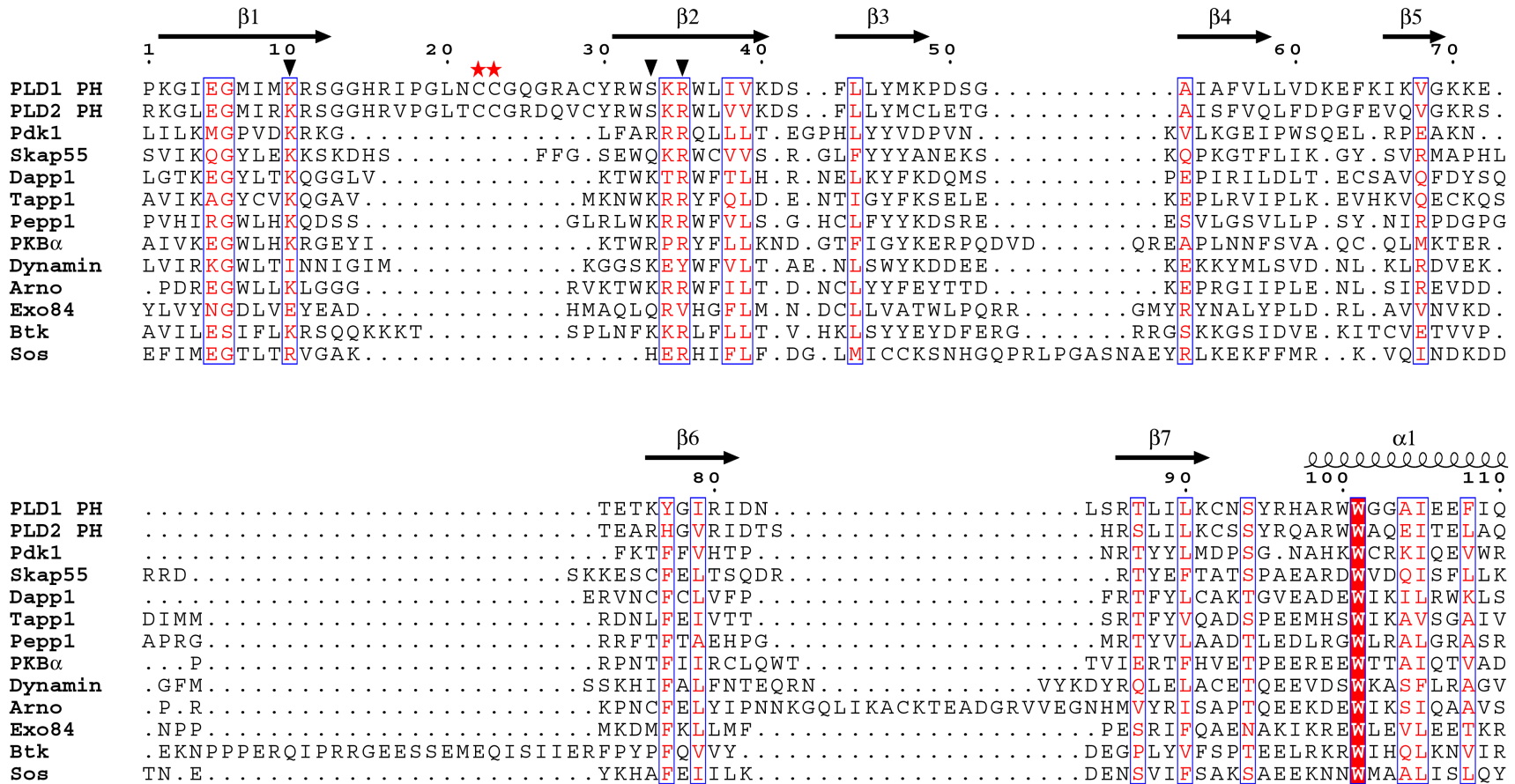


Figure 41. Alignment of PH domain sequences

Structural alignments of PH domains were aligned to PLD1 and PLD2 using ClustalW. Secondary structure elements are depicted above the aligned sequences, ▲ denotes positions that form a phosphoinositide binding site, and ★ denotes palmitoylation sites.

PLD catalytic domains

Three-dimensional structures have been obtained for a single PLD enzyme, an exotoxin from *Streptomyces sp.* PMF. Structurally conserved regions were defined by comparing the structures of catalytic domains from several superfamily members. Large alignments were constructed, containing up to 150 PLD sequences identified in public databases. These alignments were used to identify conserved sequences and to map PLD1 sequence to the *Streptomyces* structure. The bacterial PLD is a minimal structure, just over 50 kDa in molecular weight. PLD1 is considerably larger, ~ 80 kDa for the catalytic domains (124 kDa in total). C-terminal sequences are absent in the molecular models, as are the divergent sequences in the ‘loop region.’

The overall structure is predicted to have an α,β -hydrolase fold, with alternating α - and β -structural elements. An active site is formed by absolutely conserved residues at the interface between two opposed β -sheets. *Streptomyces sp.* PMF and human PLD catalytic domains share only 15 to 16% sequence identity. Residues that form the hydrophobic core and active site are highly conserved while surface residues are not conserved at all.

Published reports identified a discrete binding-site for PIP₂ within the C-terminal catalytic subdomain. This sequence maps to an amphipathic helix on a solvent exposed surface of structural models (Figure 42). Basic (i.e., arginine and lysine) residues are found on the cytoplasmic surface of the helix. Curiously, the enzyme would have to penetrate the bilayer surface, or rotate the catalytic site away from the membrane to present the proposed PIP₂-binding residues to the membrane surface.

| | | | |
|--------|-----|--|-----|
| mPLD2 | 553 | A <u>A</u> R <u>D</u> L <u>A</u> R <u>H</u> F <u>I</u> Q <u>R</u> W <u>N</u> F | 570 |
| rPLD1b | 651 | A <u>A</u> R <u>D</u> V <u>A</u> R <u>H</u> F <u>I</u> Q <u>R</u> W <u>N</u> F | 668 |



Figure 42. A putative PIP₂ binding site

(*Top*) Sequence of putative PIP₂ binding site. Underscored residues in mouse PLD2 were identified in (Sciorra et al., 1999). Highlighted residues will be mutated in a proposed cysteine-scanning mutagenesis study. (*Bottom*) Homology-based molecular model of rat PLD1 catalytic domains is displayed using DeepView 3.7 (Guex and Peitsch, 1997).

In recent years, numerous studies have identified residues that selectively disrupt RhoA regulation of PLD1 activity (Du *et al.*, 2000; Walker *et al.*, 2000b; Cai *et al.*, 2001; Walker *et al.*, 2002). Together, these residues comprise a complex binding site spanning the C-terminal fifth of the enzyme (Figure 43). Preliminary homology-based structural models of PLD1 predict a continuous binding surface composed of the disparate residues identified in mutational studies.

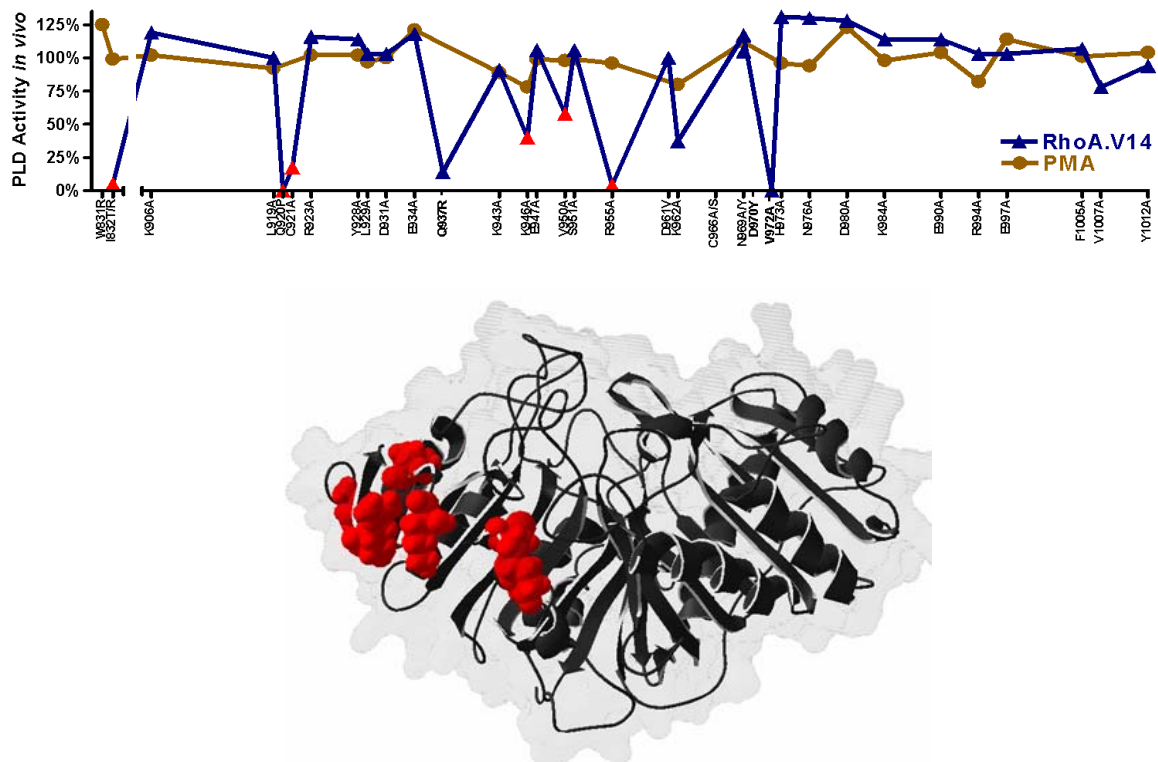


Figure 43. A putative Rho GTPase-binding site.

(*Top*) A summary of mutational studies that identify amino-acid sequences involved specifically in RhoA activation of mammalian PLD1. Data were compiled from unpublished experiments, from (Cai *et al.*, 2001), and from (Du *et al.*, 2000). Residues highlighted in red map to a continuous exposed surface on a homology-based molecular model of PLD1 catalytic domains (*Bottom*).

Monomeric G-protein activators

Many Ras superfamily G-proteins are thought to directly activate PLD1. This allosteric activation requires that both PLD1 and its activators associate with the membrane. To effectively activate PLD1, Rho, Rac, Cdc41, and Arf GTPases must be acylated. Rho GTPases are isoprenylated and Arf GTPases are N-myristoylated. Residues in switch I and II regions (Su et al.,;Bae et al., 1998), including the Rho-insert loop (Walker *et al.*, 2000b;Walker *et al.*, 2002) required for RhoA-dependent activation of PLD1. Other Rho GTPases (e.g., Rac1, Cdc42) are much less studied.

Arf GTPases require certain residues in their N-termini α 2-helix and, β 2 strand to mediate their effects on PLD1. (Jones et al., 1999;Jovanovic et al., 2005). A mechanistic understanding of PLD1 activation by monomeric GTPases awaits structural analyses of these protein complexes at the membrane. Structures of Arf-1 and RhoA GTPases suggest steric and electrostatic determinants of PLD1 activation (Figure 44).

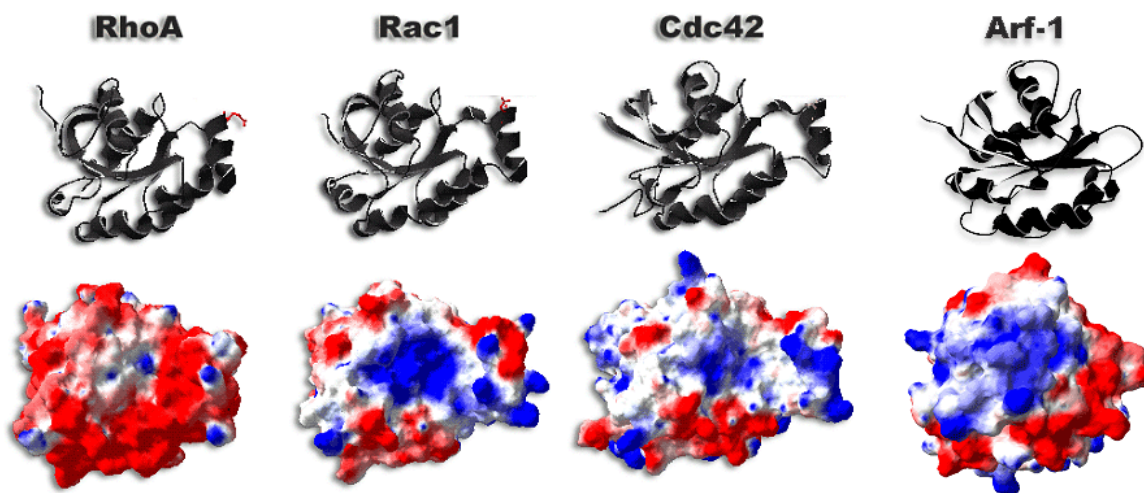


Figure 44. Comparison of PLD1 activator structures

Crystallographic structures of activated RhoA, Rac1, Cdc42, and Arf-1 (Protein Data Bank accessions 1CXZ_A, 1HE1_A, 2NGR_A, and 1O3Y_A, respectively). (*Top*) Ribbon diagrams demonstrate conserved structure among Ras-family GTPases. Residues are highlighted on ‘Rho-insert loops’ thought to directly contact PLD1. (*Bottom*) Activator structures suggest both steric and electrostatic determinants for PLD binding and activation. Surface electrostatic potentials are calculated using the GROMOS96 43A1 forcefield.

APPENDIX A

DISTRIBUTION OF PLD ISOFORMS IN HUMAN TISSUES

| Tissue Type | <i>PLD1</i> | | <i>PLD2</i> | | Ref. |
|----------------------------|---------------------|-----------------|---------------------|-----------------|---|
| | Relative expression | Splice variants | Relative expression | Splice variants | |
| Heart | +++ | b>a | ++ | a>b,c | (Saqib and Wakelam, 1997;Lopez et al., 1998;Steed et al., 1998) |
| Ovary | +++ | b | +++ | | (Saqib et al., 1997;Steed et al., 1998) |
| Spinal cord | +++ | b | +++ | | (Steed et al., 1998) |
| Pancreas | +++ | b>a | ++ | a>b>c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Small intestine | +++ | b | ++ | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| <i>high expression</i> | | | | | |
| Spleen | ++ | b>a | ++ | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Placenta | ++ | b>>a | +++ | a>b>c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Uterus | ++ | b | +++ | | (Saqib et al., 1997) |
| Brain | ++ | b>>a | + | a | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Liver | ++ | b>>a | + | a>b>c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| <i>moderate expression</i> | | | | | |
| Adrenal gland | + | b | + | | (Steed et al., 1998) |
| Bone marrow | + | b | + | | (Steed et al., 1998) |
| Lymph node | + | b | + | | (Steed et al., 1998) |
| Muscle | + | b>a | + | a | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Prostate | + | b | +++ | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Stomach | + | b | + | | (Steed et al., 1998) |
| Testis | + | b | + | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Thymus | + | b | +++ | | (Saqib et al., 1997;Lopez et al., 1998) |
| Thyroid | + | b | +++ | | (Steed et al., 1998) |
| Trachea | + | b | +++ | | (Steed et al., 1998) |
| Lung | +/- | b>a | ++ | a,b>c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Colon | +/- | b | ++ | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Kidney | +/- | b>>a | ++ | a>b>c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Leukocytes | -/+ | | + | | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| Neutrophils | + | b>a | + | a | (Di Fulvio and Gomez-Cambronero, 2005) |
| T-cells | + | a | n.d. | | (Chien et al., 2004) |
| Heart | +++ | b>a | ++ | a>b,c | (Saqib et al., 1997;Lopez et al., 1998;Steed et al., 1998) |
| <i>Lower expression</i> | | | | | |

APPENDIX B

PLD EXPRESSION IN IMMORTALIZED CELL LINES

| Cell line | Type | Species | PLD1 | PLD2 | References |
|-----------|------------------------|---------|------|------|---|
| 1321N1 | astrocytoma | human | + | + | personal communication, Lane Coffee |
| A549 | lung carcinoma | human | + | + | (Meier <i>et al.</i> , 1999;Kim <i>et al.</i> , 1999a) |
| A7r5 | vascular smooth muscle | rat | + | + | (Gibbs and Meier, 2000) |
| BALB 3T3 | fibroblast | mouse | + | n.d. | (Luo <i>et al.</i> , 1998) |
| C6 | glioma | rat | + | + | (Yoshimura <i>et al.</i> , 1996;Park <i>et al.</i> , 1997) |
| CHO-K1 | ovary | hamster | + | + | personal communication, Masami Hiroyama |
| COS-7 | fibroblast | monkey | + | + | personal communication, Masami Hiroyama |
| DLD | colon carcinoma | human | + | + | (Meier <i>et al.</i> , 1999) |
| EL4 | thyoma | mouse | - | - | (Gibbs <i>et al.</i> , 2000) |
| G361 | melanoma | human | + | + | (Saqib <i>et al.</i> , 1997) |
| HaCat | keratinocyte | human | + | + | (Muller-Wieprecht <i>et al.</i> , 1998) |
| HEK 293 | fibroblast | human | + | + | (Meier <i>et al.</i> , 1999) |
| HeLa | cervical carcinoma | human | + | + | (Hammond <i>et al.</i> , 1995;Yoshimura <i>et al.</i> , 1996;Saqib <i>et al.</i> , 1997;Colley <i>et al.</i> , 1997a;Steed <i>et al.</i> , 1998) |
| HepG2 | liver carcinoma | human | - | + | (Meier <i>et al.</i> , 1999) |
| HL-60 | promyelocytic | human | + | + | (Yoshimura <i>et al.</i> , 1996;Saqib <i>et al.</i> , 1997;Colley <i>et al.</i> , 1997a;Nakashima <i>et al.</i> , 1998;Gibbs <i>et al.</i> , 2000;Di Fulvio <i>et al.</i> , 2005) |
| HT1080 | fibrosarcoma | human | + | + | (Meier <i>et al.</i> , 1999) |
| Jurkat | leukemia | human | - | + | (Gibbs <i>et al.</i> , 2000) |
| K562 | leukemia | human | + | + | (Saqib <i>et al.</i> , 1997) |
| MB-231 | breast carcinoma | human | + | + | (Meier <i>et al.</i> , 1999) |
| MCF-7 | breast carcinoma | human | + | + | (Saqib <i>et al.</i> , 1997;Meier <i>et al.</i> , 1999) |
| MDCK | kidney | dog | + | + | personal communication, Masami Hiroyama |
| NIH-3T3 | fibroblast | mouse | + | + | (Yoshimura <i>et al.</i> , 1996;Colley <i>et al.</i> , 1997a;Lee <i>et al.</i> , 1997b;Luo <i>et al.</i> , 1998) |
| MOLT-4 | leukemia | human | - | - | (Saqib <i>et al.</i> , 1997) |
| PC12 | pheochromocytoma | rat | +/- | + | (Park <i>et al.</i> , 1997;Gibbs <i>et al.</i> , 2000) |
| PC-3 | prostate carcinoma | human | + | + | (Park <i>et al.</i> , 1997;Gibbs <i>et al.</i> , 2000) |
| RAJ1 | B-lymphoma | human | - | - | (Saqib <i>et al.</i> , 1997) |
| Rat1 | fibroblast | rat | + | + | (Park <i>et al.</i> , 1997;Min <i>et al.</i> , 1998;Gibbs <i>et al.</i> , 2000) |
| Rat2 | fibroblast | rat | + | + | personal communication, Masami Hiroyama |
| SW480 | colon carcinoma | human | + | + | (Saqib <i>et al.</i> , 1997;Meier <i>et al.</i> , 1999) |

APPENDIX C

CELLULAR STIMULI THAT REGULATE PLD IN PRIMARY CELLS

| Primary Cell Type | Stimulus |
|-----------------------------|---|
| adrenal gland | angiotensin-II, sphingosine 1-P |
| adrenal glomerulosa cells | angiotensin, carbachol |
| amnion | bradykinin, phorbol ester |
| astrocytes | norepinephrine, glutamate, carbachol |
| calvarial osteoblasts | epidermal growth factor |
| cardiac myocytes | norepinephrine, endothelin |
| cerebral cortex | epinephrine, norepinephrine (α_1), carbachol, acetylcholine, phorbol ester, ionophore |
| choroid plexus | serotonin |
| endometrial stroma | 8-Br-cAMP, phorbol ester |
| endothelial cells | bradkinin, lysophosphatidic acid, ATP, bFGF, H ₂ O ₂ , interferon, stem cell factor, phorbol ester |
| eosinophils | C5a, LTB ₄ , phorbol ester, ionophore, platelet activating factor |
| epidermal keratinocytes | phorbol ester |
| heart | carbachol, norepinephrine, acetylcholine, phorbol ester, angiotensin-II, endothelin-1, cardiotrophin-1 |
| hippocampus | glutamine, norepinephrine, ionophore |
| kidney podocytes | mechanical force |
| kidney papillary tissue | PGD ₂ |
| Leydig cells | vasopressin, bradykinin, phorbol ester |
| lymphocytes | PGE ₂ , PGF _{2α} , phorbol ester, ATP |
| macrophages | LPS, LPA, Fc-Rs, ATP, UDP, PAF, CSF-1, MCF, phorbol ester, ionophore, sphingosine 1-P |
| mast cells, basophils | IgE (Fc ϵ -RI), antigen, carbachol |
| monocytes | interleukin-1, phorbol ester |
| myometrium | endothelin, phorbol ester, ionophore |
| neural stem cells | Phorbol ester |
| neutrophils | fMLP, C5a, IL-8, leukotriene B ₄ (LTB ₄), ATP, platelet activating factor, granulocyte-macrophage colony-stimulating factor, phorbol ester, ionophore, monosodium urate, lysophosphatidic acid, arachidonic acid |
| ovarian granulosa | Gonadotrophin-releasing hormone, phorbol ester |
| pancreas (exocrine) | cholecystokinin, carbachol, bombesin, phorbol ester |
| pancreas (islets) | phorbol ester |
| parotid acini | carbachol, epinephrine (α_1), phorbol ester |
| placental trophoblasts | P2 purinergic agonists |
| platelets | thrombin, thromboxane A ₂ , collagen, , ionophore, phorbol ester |
| pneumocytes | ATP, phorbol ester |
| retina | light |
| skeletal muscle | mechanical force, lysophosphatidic acid |
| smooth muscle (airway) | bradykinin, ionophore, lysophosphatidic acid, endothelin-1 |
| smooth muscle (vascular) | norepinephrine, PDGF, vasopressin, phorbol ester, endothelin, high glucose, angiotensin II |
| smooth muscle (preglomerul) | angiotensin II |
| striatum | phorbol ester |
| sympathetic neurons | depolarization, acetylcholine, serotonin |
| T-cells | LPS, allergens |
| uterine decidua | phorbol ester |

APPENDIX D

CELLULAR STIMULI THAT REGULATE PLD IN IMMORTALIZED CELL LINES

| Cell Type | Cell Line | Stimulus |
|--------------------------|--|---|
| adenocarcinoma | LM3 | collagen, laminin, fibronectin, bradykinin, sphingosine 1-P |
| pre-adipocyte | 3T3-L1, OB1771 | growth hormone, PDGF, interleukin-11 (IL-11) |
| adrenocortical carcinoma | NCI H295R | angiotensin II, phorbol ester |
| amnion | WISH | lysophosphatidic acid |
| astrocytoma | 1321N1 | carbachol, thrombin, ATP, phorbol ester, histamine, bradykinin |
| breast cancer | MCF-7, MBA-MB-231 | phorbol ester |
| B-cell lymphoma | A20 | tumor necrosis factor- α |
| cardiac fibroblast | | angiotensin |
| cervical | HeLa | interferon- α , EGF, phorbol ester, angiotensin II |
| epidermal | A431, HEL-37 | EGF, phorbol ester |
| erythroleukemia | HEL | thrombin, PGE |
| fibroblasts | Rat1, Rat2, Rat6, Swiss 3T3, NIH 3T3, REF52, IMR-90, IIC9, SF3155, BHK, CHL, BABL _c -3T3, HIRcB, 3T3-L1 | PDGF, EGF, bFGF, LPA, REF52 vasopressin, bombesin, endothelin, bradykinin, thrombin, PGF _{2α} , H ₂ O ₂ , epinephrine, phorbol ester, sphingosine, apolipoprotein A-1, insulin, angiotensin II, growth hormone |
| glioma | C6 | endothelin, neuromedin B, sphingosine, phorbol ester |
| gonadotrophs | α T3-1 | gonatrophin releasing hormone, phorbol ester |
| hepatocytes | HepG2 | vasopressin, epinephrine (α_1), angiotensin, ATP, phorbol ester, ionophore |
| kidney | MDCK, HEK293 | epinephrine, bradykinin, phorbol ester, ATP, ionophore, mechanical force, insulin, angiotensin II |
| macrophage | BAC1-2F5 | ATP, PAF, colony-stimulating factor-1, phorbol ester, ionophore |
| mast cell | RBL-2H3 R6-XE4 | IgE (Fc ϵ -RI), antigen, carbachol stem cell factor, interleukin-3, phorbol ester |
| mesangia | A7r5, A10 | endothelin, vasopressin, angiotensin, PDGF, phorbol ester, ionophore |
| neuroblastoma, | LA-N-2, SK-N-MC, SK-N-BE, N4TG-1, NIE-115, LA-N-1, NG-108-15 | endothelin, carbachol, phorbol ester, retinoic acid, sphingosine |
| myoblasts | L6, C2C12 | vasopressin, insulin, phorbol ester, vitamin D ₃ , sphingosine 1-P |
| neutrophil, promyelocyte | HL60 | fMLP, ATP, (P2 purinergic), H ₂ O ₂ , phorbol ester, Fas ligand |
| oligodendrogloma | HOG | carbachol, histamine |
| osteoblast | MC3T3-E1, UMR-106 | thrombin, PGF _{2α} , PGD ₂ , phorbol ester, parathyroid hormone |
| ovarian | CHO | phorbol ester |
| pancreatic beta-cells | MIN6, HIT-T15 | glucose, carbamoylcholine, somatostatin |
| pheochromocytoma | PC12 | carbachol, bradykinin, ATP, phorbol ester, H ₂ O ₂ , depolarization |
| pituitary | GH3 | thyrotrophin-releasing hormone |
| promonocytes | U937 | PAF, TNF, ATP, phorbol esters, complement/C5a, fMLP |
| prostate-cancer | PC-3 | lysophosphatidic acid |
| squamous-carcinoma | SQ-20B | γ -radiation |
| T-cells | Jurkat | antigen, anti-CD3, phorbol ester, RANTES, thapsigargin |
| thyroid | FRTL-5 | thyroid-stimulating hormone |
| uterine smooth muscle | ELT-3 | endothelin-1 |

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