MECHANISTIC CHARACTERIZATION OF ISOFORM SELECTIVE INHIBITORS OF MAMMALIAN PHOSPHOLIPASE D

By

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TO MY DEAR FAMILY FOR THEIR CONTINUED SUPPORT AND LOVE, AND TO MY WONDERFUL HUSBAND WHO IS MY BEST FRIEND AND CONSTANT SOURCE OF ENCOURAGEMENT

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CHAPTER I

INTRODUCTION AND OVERVIEW OF PHOSPHOLIPASE D SUPERFAMILY

Phosphatidic acid (PA) is a critical phospholipid constituent in eukaryotic cell membranes, that accounts for 1-4 % of the total lipid [1]. This lipophilic glycerophospholipid has a phosphate head group, and as such serves not only a structural capacity in lipid bilayers, but also participates both as an intermediate in lipid metabolism and as a signaling molecule. Because of the small head group, PA facilitates changes in lipid bilayer curvature that are important for membrane fusion events, such as vesicular trafficking and endocytosis [2]. PA is also a precursor to other lipid signaling molecules including diacylglycerol (DAG) and lysophosphatidic acid (LPA). As a lipid second messenger, PA activates signaling proteins and acts as a node within the membrane to which signaling proteins translocate. Several signaling proteins, including Raf-1 [3], [4] and mTOR [5], directly bind PA to mediate translocation or activation, respectively. PA has been implicated in signaling cascades involving cell growth, proliferation, and survival. Aberrant PA signaling has been identified in multiple cancers [6], neurodegeneration [7], and platelet aggregation [8], which makes proteins that mediate cellular levels of PA attractive as potential therapeutic targets.

PA can be generated *de novo* [9], [10], [11] by sequential enzyme-catalyzed acylations of glycerol-3-phosphate, or in response to cell signaling pathways (Figure 1). Every glycerophospholipid generated in eukaryotic membranes transitions through PA, a pathway characterized by Eugene Kennedy and his colleagues more than half a century ago [11], [12]. Signal generated PA is formed by enzymes that modify existing lipids. These enzymes include lysophosphatidic acid acyltransferase (LPAAT) which acylates LPA, DAG kinase which phosphorylates DAG at the *sn*-3 position, and phospholipase D (PLD) which hydrolyzes the headgroup of a phospholipid, generally phosphatidylcholine (PC), triggering the release of choline.

PLD activity, enzyme catalyzed hydrolysis of a phosphodiester bond, was first described in plants [13], [14], [15], [16] and subsequently many enzymes from a range of viral, prokaryotic and eukaryotic organisms have been described as possessing PLD activity. To date, more than 4000 PLD enzymes have been entered in NCBI GenBank. The majority of these enzymes hydrolyze phosphodiester bonds within phospholipids such as PC (classified as EC 3.1.4.4 [17]), but there are other enzymes ascribed to having PLD activity that hydrolyze neutral lipids and even polynucleotide backbone. A large subset of enzymes with PLD activity share a conserved HxKxxxxDx$_6$GSxN motif (HKD motif) [18], or a variation thereof, that is responsible for catalytic activity. These enzymes are members of the PLD superfamily, and are proposed to follow a similar reaction mechanism in which a nucleophilic histidine residue initiates the reaction and generates a covalent intermediate, and a water or short alcohol completes the

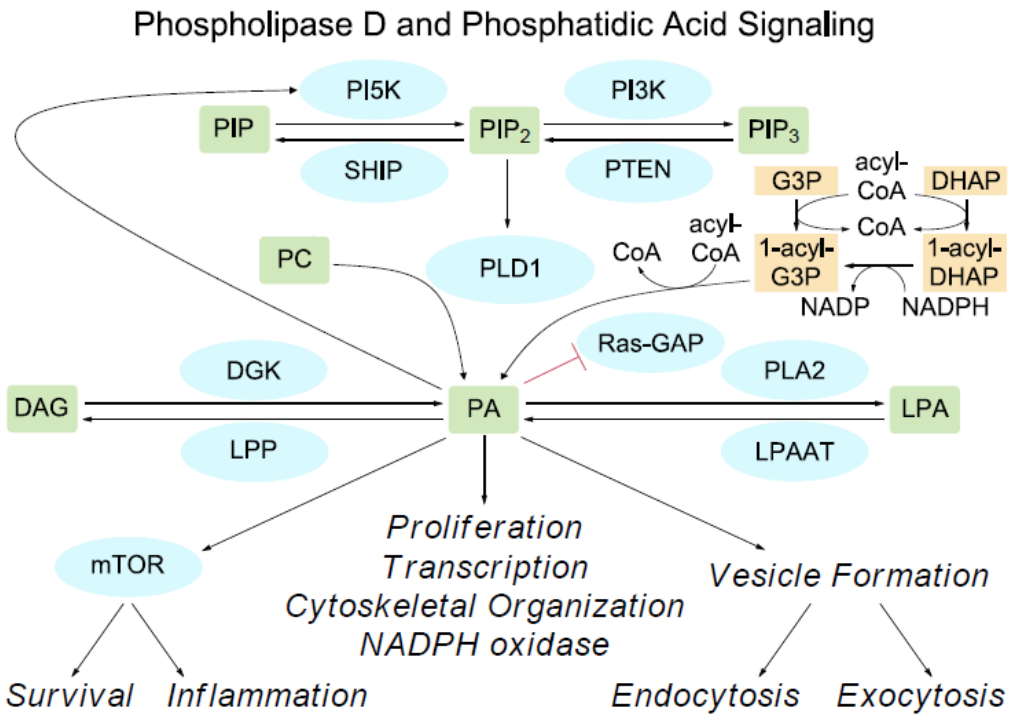


Figure 1. A schematic of the various enzyme-catalyzed reactions that results in the formation of phosphatidic acid (PA) and some of the cellular functions mediated by PA (figure from [19]).

hydrolytic or transphosphatidylation reaction, respectively (pg. 18). Non-HKD enzymes exhibiting PLD activity are predicted to have divergent structures or have divergent sequences and catalytic mechanisms. Two non-HKD enzymes, scPLD and autotoxin, are discussed in this chapter as a means of comparison to enzymes in the PLD superfamily.

This introduction serves as a brief survey of PLD enzymology with specific emphasis on the PLD superfamily and mammalian family members (a more in depth review was recently published ([19]). This chapter concludes with a brief discussion of the history and recent advances in pharmacological intervention of mammalian PLD, and possible functional consequences of such an approach. This section provides the background necassary for interpretation of the dissertation research discussed herein in which a novel class of isoform-selective small molecules was identified (Chapter II) and mechanistically characterized (Chapter III).

Enzymes with phospholipase D activity

Prior to sequencing technology or cloning of genes, enzymes were purified from the host organism and biochemically characterized. Enzymes with similar activities were described with similar nomenclature. Such is the case with PLD enzymes. Historically, many bacterial virulence factors that demonstrated the release of a choline headgroup were named PLDs for this function. Subsequent cloning and sequence analysis of these enzymes demonstrated that not all of these enzymes bear the conserved HxKxxxxD(x_6GSxN) motif first

described by Ponting and Kerr [18] and Koonin [20]. Therefore these enzymes named as PLDs are not classified as members of the PLD superfamily. At the same time, superfamily classification based on a conserved HKD motif characterized some enzymes as PLDs that were not previously considered as such solely based on biochemical analysis (e.g. some endonucleases). The PLD superfamily classification based on the conserved HKD catalytic motif is useful since these enzymes are proposed to hydrolyze phosphodiester bonds via a similar reaction mechanism.

Non-HKD enzymes

Enzymes lacking a conserved HKD motif are referred to here as non-HKD PLDs. These enzymes exhibit PLD-like activity and are no less physiologically relevant than members of the PLD superfamily. Detailed description of this class is not the focus of this chapter. However, brief mention of a couple of these enzymes is necessary to clarify their distinction in mechanism and enzymology from the PLD superfamily (Table 1).

Streptomyces chromofuscus PLD

S. chromofuscus secretes a 57 kDa phospholipase D, scPLD. This enzyme, first purified in the 1970's [21] and cloned in the early 1990's [22], is the best characterized non-HKD PLD [23]. scPLD exhibits both phosphodiesterase as well as phosphatase activities [24], and is proposed to be secreted by bacteria

Table 1: NON-HKD PLDs (table from [19])				
SPECIES	**ENZYME**	**ACTIVITY**	**FUNCTION**	**LOCALIZATION**
Streptomyces chromofuscius	scPLD	PLD (transphosphatidyl-ation w/ M alcohol)	virulence factor	secreted into extracellular milieu
Corynebacterium	PLD	sphingomyelinase (releases C1P)	membrane remodeling	secreted
	Sphingo-myelinase D	LPC → LPA (in plasma)	vascular permeabilization	
Arcanobacterium	PLD	sphingomyelinase (releases Ceramide-1-phosphate)	bacterial adhesion	secreted
	Sphingo-myelinase D	LPC → LPA	escape from vacuole	
			host cell necrosis	
Loxosceles reclusa	lysoPLD	SM → C1P	hemolysis	venom
	Sphingo-myelinase D	LPC → LPA (in plasma)	platelet aggregation	
			inflammatory responses	
Mammalian	Autotaxin	LPC → LPA, cyclic LPA	production of lysolipids in blood	secreted into blood
Mammalian	cyp1A2	monooxygenase → drug metabolism	hepatic	microsomal, membrane-bound
	cyp2E1	PLD (PC → PA) → unknown	microsomes/ER	
Mammalian	GPI-PLD	GPI → IPG + PA, GPI-protein → protein + PA	signaling and membrane-associated protein release	secreted into serum
Mammalian	NAPE-PLD	NAPE → NAE + PA	endocannabinoid signaling	microsomal, membrane-associated

to scavenge for phosphate in the microenvironment [23]. Biochemical and mutagenesis analyses of scPLD demonstrate that this enzymeutilizes a metal-coordinated reaction mechanism similar to the purple-acid phosphatase family (PAP) [24]. A Fe^{3+} cation is essential for the one-step classic acid-base catalyzed reaction mechanism, whereas a Mn^{2+} cation is thought to be necessary for proper substrate binding.

scPLD is also able to perform transphosphatidylation, but less efficiently than HKD PLD enzymes (8-10 M primary alcohol is necessary for scPLD, compared to >95 % transphosphatidylation with 1-2 M alcohol for HKD PLD) [25]. scPLD also does not exhibit interfacial activation. Known as the surface dilution effect, HKD enzyme activity is affected (pg. 24), whereas scPLD activity is not dependent on the surface mole fraction of substrate within a lipid micelle or vesicle, hence substrate presentation does not impact scPLD activity [26]. This is also referred to as the “hopping” versus “scooting” mode of activity (Figure 2). scPLD activity is dependent on whether the substrate is readily accessible, and therefore exhibits greater activity towards monomer and mixed micelle than substrate present in a lipid vesicle [27].

scPLD is also the only PLD known to be activated by PA, most likely allosterically [26], [28]. Calcium can activate PLD by two mechanisms: calcium can directly bind the enzyme with biphasic affinity (K_{d1} and K_{d2}), but is also able to bind to PA and make the lipid more rigid triggering product release from the active site to allow new substrate to bind [26]. The allosteric PA binding domain is predicted to be in the *C*-terminal domain, as proteolytically cleaved $scPLD_{42/20}$

does not exhibit PA activation to the extent that uncleaved $scPLD_{57}$ responds [29]. This activation is believed to be elicited via an allosteric site secondary to the catalytic site because soluble PA can increase V_{max} towards substrate present at an interface.

Despite the fact that scPLD is not a member of the PLD superfamily, many studies have used, and some still use, exogenous application of recombinant scPLD to rescue the deleterious effects of deletion of a HKD PLD. This is a legitimate approach as long as the results are clearly understood with regards to substrate-product relationships. Supplemental application of scPLD will hydrolyze a range of phospholipids generating PA and possibly perform phosphatase activities. Observation that scPLD rescues a phenotype following deletion of a HKD PLD enzyme suggests that PA may in fact be the functional consequence of that particular HKD PLD. However, this result or the possible lack of a “rescue” effect should not be over-interpreted. Recent studies of viral, prokaryotic, and eukaryotic PLD superfamily members demonstrate that the function of these enzymes stretches beyond generation of PA or classic catalytic product. New descriptions of protein-protein interactions and alternate catalytic products are only recently gaining an appreciation in the literature.

Autotaxin

Lysophospholipase D activity has been described in human blood. Autotaxin (ATX or NPP2) was determined to be responsible for this lysoPLD activity and is the main source of LPA in human blood [30], [31]. ATX, a member

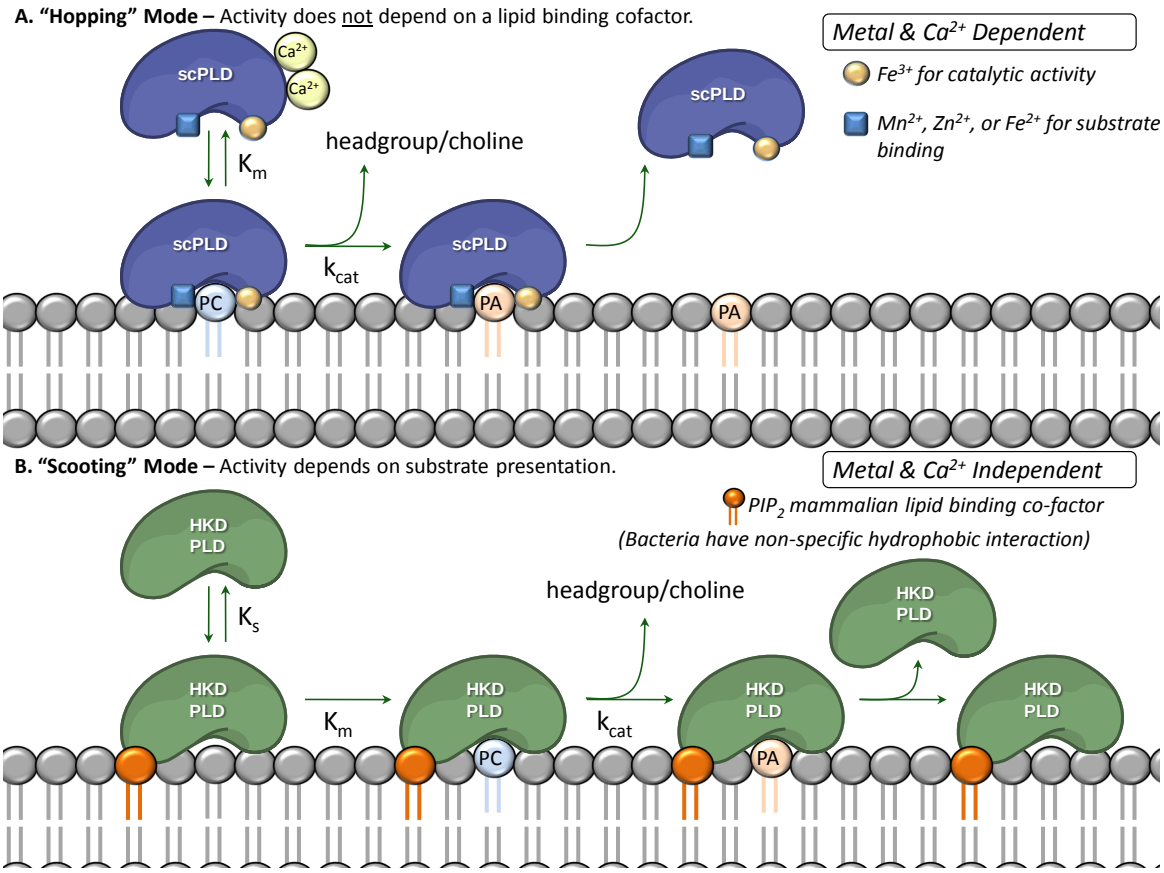


Figure 2. Mechanisms of phospholipase D enzyme activities. Many bacterial PLD enzyme activities proceed in a hopping mode and are dependent on the presence of metal ions, whereas mammalian PLD activity proceeds in a scooting mode and is largely dependent on the interfacial lipid environment (figure from [19]).

of the nucleotide pyrophosphatase/phosphodiesterase family, is expressed as a preproenzyme and secreted into the extracellular milieu and serum via an *N*-terminal secretion signal. This enzyme does not include a conserved HKD motif and is not related to scPLD or the PLD superfamily. *In vitro* characterization of ATX demonstrates it has a range of activities, including phospholipase (to produce LPA and S1P) [32], [33], [34], and nucleotide pyrophosphate hydrolysis. Lysophospholipids, including lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and LPS are high affinity substrates and predicted to be the physiologically-relevant target [35]. ATX uses two Zn^{2+} ions in the active site for coordination and intermediate stabilization. However, unlike the scPLD described above, ATX can perform both hydrolysis and transphosphatidylation [36]. Depending on the divalent cation identity and salt concentration in the microenvironment, ATX will either hydrolyze LPC to form LPA, or transphosphatidylate LPC, similar to scPLD, and use the free hydroxyl group in the *sn*-2 position to generate cyclic LPA (cLPA) [31]. This difference in reactions is critical since the physiological function of LPA is distinct from cLPA. LPA is important in chemotactic cell migration and platelet aggregation, whereas cLPA inhibits cell proliferation, tumor cell invasion and metastasis. Three splice variants of ATX have been identified, ATXα, ATXβ, and ATXγ [37]. ATXα and ATXβ both perform transphosphatidylation and generate cLPA. The transphosphatidylation activity of ATXγ has yet to be characterized, but is expressed in the brain where it is proposed to be responsible for the high concentrations of cLPA [37].

The crystal structures of rat [38] and mouse [39] ATX were recently determined. Careful analysis of the structures in tandem with further biochemical characterization will be necessary to understand hydrolytic versus transphosphatidylation mechanisms and the role of divalent cations in serving as a switch between the two divergent reactions. Because of the stark contrast in signaling function of LPA versus cLPA it will be necessary to identify pharmacological agents that can be used to elicit one reaction over the other.

ATX knockout mice exhibit severe phenotypic deficiencies and die around embryonic day 9.5-10.5 [40], [41]. Much of this phenotypic response is thought to be due to the absence of ATX catalytic activity, since knock-in of a catalytic mutant elicits similar phenotypic deficiencies. However, analysis of ATX crystal structures shows two predicted LPA binding sites, and suggests that ATX may also serve as a lipid-protein carrier and deliver LPA directly to LPA receptors at the membrane via a hydrophobic tunnel [39]. Recent studies also suggest that via a *C*-terminal MORFO (modulator of oligodendrocyte remodeling and focal adhesion organization) domain, ATX may be important for eliciting focal adhesions during oligodendrocyte maturation and myelination [42], [43]. Two groups have implicated ATX in regulating lymphocyte trafficking [44], [45]. Further structural and biochemical characterization of this enzyme is necessary, but due to its role in generating both LPA and cLPA, autotaxin appears to be a novel therapeutic target. A recent study has identified ATX as a potential therapeutic target for atherosclerosis [46].

HKD enzymes

In contrast to the varied sequence, catalytic, and biochemical characteristics found in non-HKD PLDs, HKD enzymes share a conserved catalytic domain. While these enzymes do not share significant sequence identity outside of this catalytic domain, conservation of this domain means these enzymes do share a similar structural core that hydrolyzes phosphodiester bonds with a similar reaction mechanism for a range of substrates. Historically there has been some dispute as to the classification of some or all of these HKD enzymes as members of the PLD superfamily. Differences in substrate (DNA backbone versus lipid) and function (endonuclease versus lipase) amongst HKD PLD enzymes have lead to discrepancies in definition of requirements for classification in the PLD superfamily. Here it is proposed that all phosphodiesterases with a conserved HKD or HKD-like motif are members of this diverse superfamily. Conservation of the HKD motif permits inclusion in PLD superfamily because, regardless of substrate identity, these enzymes share an S_N2 ping-pong reaction mechanism that proceeds through a covalent phospho-protein intermediate in phosphodiester hydrolysis (pg. 18). Members of the superfamily also perform transphosphatidylation in parallel with hydrolysis in the presence of alcohol versus water, respectively. Further subclassifications in the superfamily delineate differences in sequence, substrate and function, but superfamily classification based on the conserved HKD motif is a useful descriptor in characterizing the enzymological and mechanistic identity of an HKD enzyme. With this definition of the PLD superfamily described, the following

sections briefly highlight members possessing a variety of functional and biochemical characteristics.[47]

Sequence

PLD enzymes have been identified in viruses, bacteria, plants, fungi and mammals and were historically classified based on biochemical activity. However, following cloning and sequencing of several PLD genes a common set of conserved motifs (I-IV) were observed [18]. Conserved motifs II and IV comprise the duplicate catalytic sequence, $HxKxxxxDx_6G(G/S)xN$ (referred to here as HKD). In fact, there is significant homology between motifs I & II and III & IV. Based on this internal homology and the presence of 1-HKD motif enzymes in viruses and lower prokaryotic species, there is considerable evidence for a gene duplication event (Table 2), resulting in many PLD superfamily enzymes containing two putative HKD motifs [20] (Figure 3). As discussed in a following section (pg. 18), the histidine residue of the HKD motif has been demonstrated to be the nucleophilic residue responsible initiating phosphodiesterase activity. Motif III is comprised of the highly conserved sequence of unknown function 'IYIENQFF.' In between the catalytic HKD motifs, and N-terminal to motif III, a putative polybasic phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) binding domain has been described in higher eukaryotes. The *C*-terminus of all PLD superfamily members, despite the fact that it is not homologous, must be integral for catalysis, since activity decreases upon mutation in or truncation of this region.

Table 2. Alignment of Catalytic motifs for PLD superfamily (table from [19])			
ENZYME	**SOURCE**	**CATALYTIC MOTIF**	**ACTIVITY**
p37	*Vaccinia* Virus	QNNTKLLIVDDE	lipase towards phospholipids, DAG, & Lysolipids
K4	*Vaccinia* Virus	VLHTKFWISDNT	endonuclease
BfiI	*E.coli* / *B.Firmus*	ILHAKLYGTSNN	site-specific endonuclease
Nuc	*E.coli* / *S.typhimurium*	IQHDKVVIVDNV	endonuclease
PLD	*S. antibioticus*	WMHSKLLVVDGK	lipid phosphodiesterase towards PC, PE, PS, PG
PLDα1	*A. thaliana*	YVHTKMMIVDDE	lipid phosphodiesterase towards PC & PE
PLDζ	*A. thaliana*	YVHSKMMIVDDE	lipid phosphodiesterase
Spo14	*S. cerevisciae*	AHHEKFVVIDET	lipid phosphodiesterase towards PC

Ponting and Kerr suggested that enzymes with these four conserved motifs were members of the PLD superfamily as described above [18]. Within this superfamily, further classification was proposed based on sequence homologies. Class I comprises HKD PLDs from fungi and higher eukaryotes. Many of these enzymes have divergent *N*-terminal sequences that include lipid- or calcium-binding regulatory domains to allow tailored control of PLD activity in response to signaling cascades. Class II enzymes include bacterial PLDs, such as *Yersinia* murine toxin (YMT) and *Streptomyces sp*. PMF PLD (pg. 35) with known lipase activities. Classes III and IV include enzymes involved in lipid biosynthesis, bacterial cardiolipin synthase and phosphatidylserine synthase, respectively. The remaining classifications describe enzymes with significantly divergent functions. Class V enzymes include viral p37 and K4. Class VII and VIII comprise endonucleases Nuc and *BfiI*, respectively.

Structure

Protein crystals of PLD superfamily members have been reported, including endonucleases and several bacterial enzymes [Nuc, *BfiI*, tyrosyl-DNA phosphodiesterase (tdp-1), YMT [48], cowpea [49], *Streptomyces sp*. PMF PLD [50], and *Streptomyces antibioticus* PLD, entered in PDB, (unpublished)], and tertiary crystal structures have been reported for Nuc [51], *BfiI*, tdp-1 [52], [53] *Streptomyces sp*. PMF PLD [54] and *S. antibioticus* PLD. Structures for YMT and cowpea PLD were never reported. It is apparent

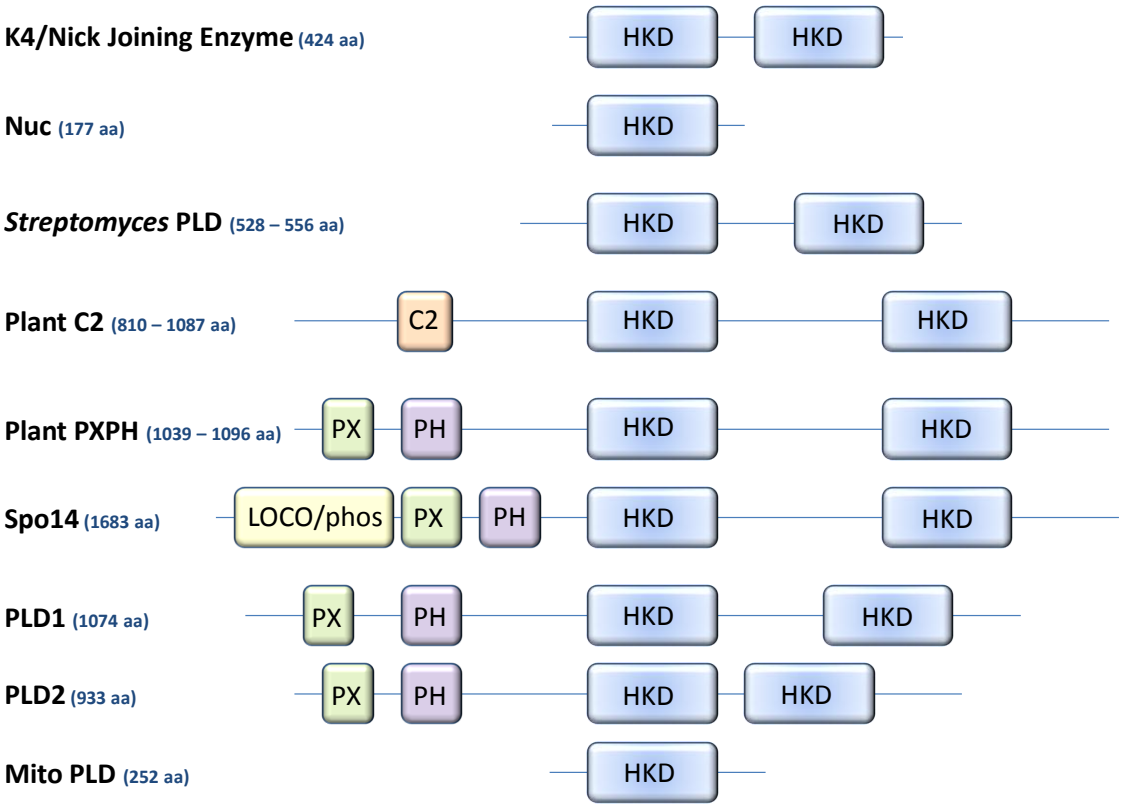


Figure 3. Comparison and domain alignment for different PLD superfamily enzymes. The HKD motif responsible for catalytic activity is conserved among all superfamily members. Higher order PLD enzymes are composed of nonconserved regulatory domains (figure from [19]).

from the available structures that a conserved fold exists for the catalytic domains of PLD superfamily members.

Nuc endonuclease from *Salmonella typhimurium*, a 1-HKD PLD, crystallized as a homodimer with each monomer arranged around a crystallographic two-fold axis of symmetry [51]. Conserved HKD residues emanate from β-strands at the interface of the dimer and lie adjacent to one another to form the active site. Within each monomer, the eight β-strands form two β-sheets that are sandwiched by five α-helices.

Streptomyces sp. PMF PLD was the first reported crystal structure of a 2-HKD PLD.[54] PMF PLD consists of 35 secondary structural elements situated in repeated α-β-α-β orientation (Figure 4). In the tertiary structure, similar to the Nuc endonuclease, a common β-sandwich fold is observed, with each of the two β-sheets comprised of 8 β-strands sandwiched between 18 α-helices. This enzyme is bilobal with a pseudo two-fold axis of symmetry. Conserved HKD residues lie adjacent to one another along this axis, and at the interface exists the active site with a 30 Å aperture to allow substrate entrance. Biochemical studies with *Streptomyces* PLD point mutants have attributed function to specific structural elements (pg. 35, also reviewed [55]). Two flexible loops extend over the entrance to the active site and are thought to modulate interfacial lipid interactions and substrate specificity [56], [57]. The duplicate histidine and lysine residues exist on β-strands that line the active site and directly interact with substrate as it enters the active site. The aspartate residues do not directly interact with substrate, but do shuttle protons to the deprotonated histidine

residue during the reaction. The GG/GS residues line the base of the catalytic pocket and accommodate large substrate headgroups during transphosphatidylation headgroup exchange [58].

In contrast to bacterial PLDs, *in vitro* studies of eukaryotic PLD structure and mechanism are lacking due to difficulties in expression and purification of recombinant enzyme. In the absence of a crystal structure for a higher eukaryotic PLD, much of our enzymological understanding of the PLD mechanism is based on characterization of bacterial PLDs.

Mechanism: hydrolysis versus transphosphatidylation

In nature, phosphodiester hydrolysis does not commonly occur in the absence of metals [59]. When it does, the mechanism must proceed through a nucleophilic attack of the substrate phosphate group, which facilitates breakage of the phosphodiester bond, and protonation via acid catalysis to enable release of the leaving group. Depending on the source of the initial nucleophile, phosphodiester hydrolysis can proceed in a single step, or in two steps, with a covalent phospho-protein intermediate. Decades of biochemical [48], structural [60], and biophysical [59] research support the latter mechanism for PLD superfamily enzymes, in which a nucleophilic protein residue forms a covalent linkage to the phosphate group of the substrate (Figure 5). This covalent intermediate is subsequently destroyed via nucleophilic attack of a water molecule or alcohol, releasing the hydrolytic or transphosphatidylation product, respectively.

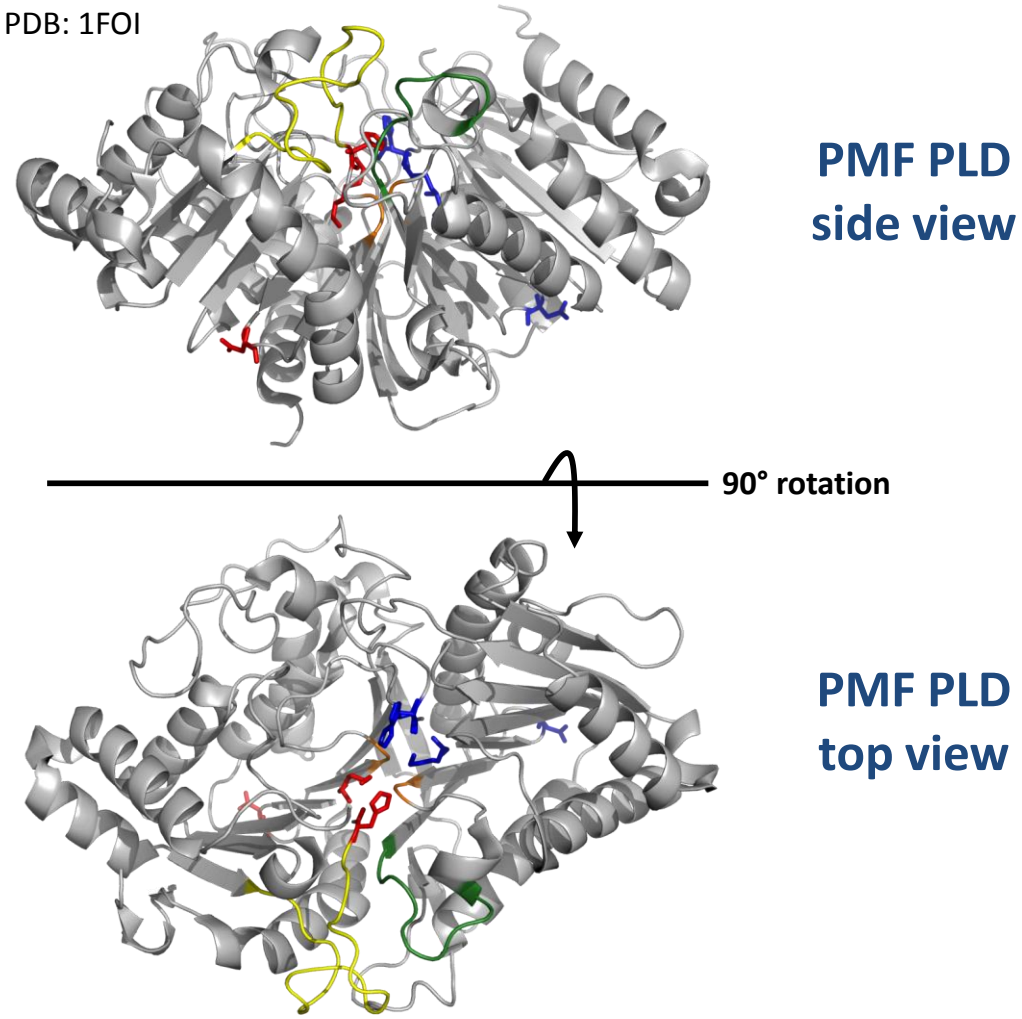


Figure 4. Crystal structure of *Streptomyces sp.* PMF PLD (PDB ID: 1FOI), 2-HKD enzyme. The conserved HKD motifs are highlighted in blue (N-terminal motif) and red (C-terminal motif), and the loops characterized in mutagenic studies are shown in green (N-terminal loop) and yellow (C-terminal loop) (figure from [19]).

More than four decades ago, Yang *et al.*[61] and Stanacev and Stuhne-Sekalec *et al.*[62] proposed that PLD catalysis proceeds through a two-step ping-pong reaction mechanism with a covalent phospho-protein intermediate. This postulation was based on analyses of cabbage PLD-induced product formation in the presence of primary alcohol. Subsequent hydrolysis and transphosphatidylation then proceed in parallel dependent on the presence of water or primary alcohol. Early studies suggested that the sulfhydryl group of a cysteine residue may serve as the nucleophilic residue.[61] This was proposed because *p*-chloromercuribenzoate (PCMB) treatment modified free sulfhydryl groups and disrupted catalysis, in the seven cysteine residue containing cabbage PLD enzyme.[61]

In the 1990's other studies to characterize the PLD superfamily reaction mechanism attempted to identify the nucleophilic protein residue that might catalyze phosphodiesterase activity. Following Ponting & Kerr[18] and Koonin's[20] observations of duplicate $HxKxxxxDx_6G(G/S)xN$ motifs in PLD superfamily members, it was suggested that the nucleophilic residue might exist in this sequence. Sung *et al.* proposed the conserved serine residue in the second HKD motif of yeast Spo14/PLD1 was the nucleophile.[63] This conclusion was based on studies with recombinant Ser911Ala mutant. Subsequent studies using a 1-HKD bacterial enzyme, Nuc endonuclease [64], and a 2-HKD bacterial PLD, YMT [48], demonstrated the serine mutation resulted in a significant drop in catalytic activity. However, it was ultimately determined

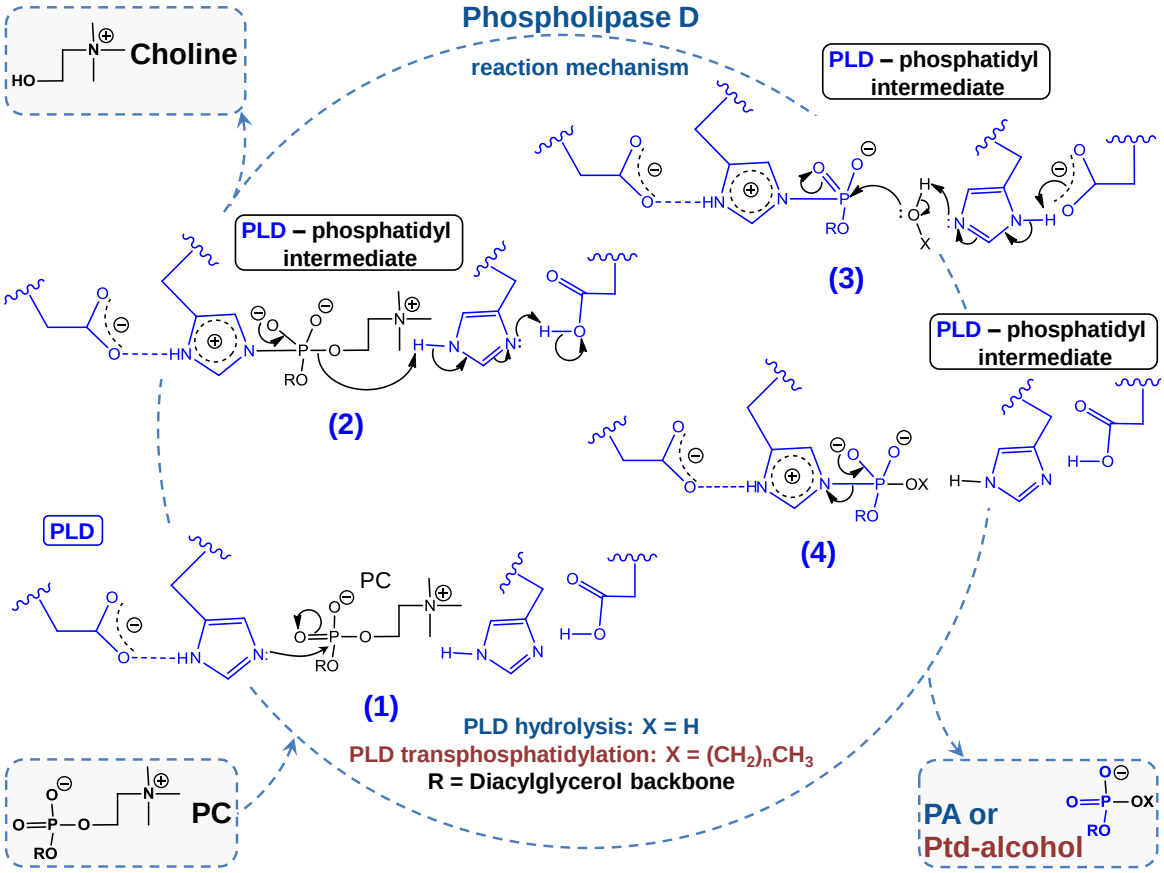


Figure 5. Proposed PLD superfamily reaction mechanism based on biochemical studies of bacterial PLD enzymes. The histidine of the conserved HKD motif mediates a nucleophilic attack on the phosphate group of the lipid substrate, yielding a covalent intermediate. A water molecule or a primary alcohol completes the hydrolysis or transphosphatidylation, respectively (figure from [19]).

that histidine residues, and not serine, are integral for catalysis. These studies used recombinant point mutants and varied pH or chemical treatments to isolate ^{32}P-phospho-histidine intermediates. These studies proposed the reaction mechanism that is currently favored within the field, where the *N*-terminal histidine residue within the HKD motif nucleophilically attacks the phosphate group of the substrate, (step 1, Figure 5) and forms a covalent phospho-histidine intermediate. The histidine residue of the *C*-terminal HKD motif serves as a general acid, and donates a proton to the leaving group (step 2, Figure 5). For PLD enzymes with lipase activity, this leaving group is generally choline, and the intermediate a covalent phosphatidyl-histidine. Formation of this phospho-histidine intermediate has been proposed to be the rate limiting step, and subsequent nucleophilic attack of the hydroxyl group from either a water or a primary alcohol (steps 3 and 4, Figure 5) followed by PA or phosphatidylalcohol product release rapidly occurs in parallel.[25] For most HKD enzymes, including mammalian PLDs, short chain primary alcohols are the preferred nucleophile over water (in some cases more than 1000-fold preference), allowing transphosphatidylation to occur at very low concentrations of alcohol.[62] This is in contrast to the non-HKD PLD enzyme scPLD, which requires molar concentrations of alcohol to generate significant transphosphatidylation product. Some HKD enzymes, including certain bacterial, plant, and fungal PLD, are able to utilize methanol or branched alcohols in addition to other primary alcohols.[25],[65],[66]

These mechanistic conclusions were further validated when structural evidence was found to support the *N*-terminal histidine as the nucleophilic protein residue that forms a phospho-histidine intermediate. Histidine residues in the duplicate HKD motifs are adjacent to one another at the interface of the *Salmonella typhimurium* Nuc homodimer. This is also observed for the histidine residues on the duplicate HKD motifs in the crystal structure of PMF PLD. As a follow up to the first crystal structure of a 2-HKD PLD, Leiros *et al.* soaked PMF PLD crystals with short chain soluble PC substrate (dibutyrylphosphatidylcholine) to capture crystal structures of reaction intermediates [60]. PMF PLD complexed with this substrate demonstrates that the *N*-terminal histidine (H170) forms a phospho-histidine intermediate Another study describes the *C*-terminal HKD histidine as the initial nucleophile and this may differ amongst PLD species [67]. In this structure a water molecule is positioned near the *C*-terminal HKD histidine (H448) and 4.02 Å from the phosphate group, an easy distance to serve as a nucleophile for completion of the hydrolytic reaction.[60] Structural data lend credit to the proposed S_N2 reaction mechanism, and as the catalytic cores of PLD superfamily enzymes are predicted to share a similar bilobal structure with the conserved HKD residues oriented adjacent to one another in the active site, this reaction mechanism is thought to extend to all PLD superfamily enzymes.

Finally, biophysical data also support the two-step reaction mechanism for PLD superfamily enzymes. Measurement of the changes in enthalpy and Gibbs free energy of a one-step versus a two-step mechanism demonstrates significant thermodynamic favorability for a two-step reaction proceeding through a

phospho-histidine intermediate.[59] In addition to the thermodynamic likelihood of the S_N2 mechanism, Orth *et al.* used sensitive electrospray ionization mass spectrometry (ESI-MS) analysis to capture the highly unstable covalent phospho-histidine intermediate, demonstrating that it does indeed form in solution.[59] Build up of covalent intermediate to levels detectable by ESI-MS was suggested to occur because the second nucleophilic reaction is the rate limiting step. This contradicts earlier studies with bacterial PLD that proposed the formation of the phospho-histidine intermediate is the rate limiting step, and hydrolysis or transphosphatidylation occur rapidly in parallel.[25] Discrepancies in reaction rates require further characterization, and it is important to observe that specific activities vary depending on the biochemical reaction conditions used, including concentrations of divalent cation and substrate presentation. Such differences for *in vitro* activity assays are further discussed in the following section.

Interfacial kinetics

Phospholipases act on substrate present in an insoluble aggregate (i.e. the membrane). Many phospholipases therefore demonstrate interfacial kinetics, and do not follow classic Michaelis-Menten kinetic assumptions because the substrate is not freely diffusible in solution and is not randomly encountered dependent on soluble substrate concentration [68], [69]. Therefore, phospholipase activities can be described as one of two modes: “hopping” and “scooting” (figure 2) [70]. In “hopping” mode surface dilution of substrate does not impact specific activity, and the interfacial component is contained in the

equilibrium dissociation constant, K_m. Enzymes that exhibit “hopping” mode dissociate from the interface in between hydrolytic events. In contrast, enzymes that exhibit “scooting” mode first interact with the lipid interface independent of substrate interaction, in an event described by the equilibrium dissociation constant, K_s. Following interfacial binding, the enzyme laterally diffuses along the interface (in two dimensions) to encounter substrate. This is described by the equilibrium dissociation constant, K_m. “Scooting” enzymes exhibit processive activity, and do not dissociate from the interface between hydrolytic reactions.

The non-HKD enzyme, scPLD, does not demonstrate protein-lipid interfacial binding independent of substrate interaction [25]. This enzyme functions in “hopping” mode, and directly binds substrate headgroup present at the interface [23]. Following hydrolysis, scPLD falls off the substrate aggregate and the cycle recommences. scPLD activity is dependent on substrate presentation, accessibility, divalent cation concentration and cofactor binding, and positive feedback through allosteric binding of product to enhance activity [25] (pg. 5).

HKD enzymes demonstrate a scooting kinetic mechanism. A lipid cofactor binds to a hydrophobic patch on the surface of the protein, at regulatory domains or within the catalytic domain, to enhance protein recruitment to the lipid interface. For many eukaryotic PLD superfamily enzymes, $PI(4,5)P_2$ is a lipid cofactor that binds at the putative polybasic binding domain present between the catalytic HKD motifs. $PI(4,5)P_2$ significantly enhances protein-lipid binding and decreases K_s. Once at the membrane, catalysis is controlled by multiple factors

including lipid interface charge, membrane fluidity, substrate presentation or accessibility, and substrate molar fraction [69], [71] (i.e. concentration of substrate present at the interfacial surface). Because of the significant impact of interfacial environment on PLD catalysis, the format of *in vitro* activity measurement is essential to consider (pg. 26, and Figure 6).

In order to study kinetic parameters for “scooting” mode enzymes, interfacial binding, K_s, must be measured separately from substrate affinity and reaction velocity. Bulk lipid binding, K_s, can be measured as described by Buser and McLaughlin [72]. Following determination of K_s, Michaelis-Menten kinetic assumptions can be applied for “scooting" mode enzymes if bulk lipid concentration $>>>K_s$, and interfacial binding is saturated. Molar fraction of substrate can then be varied while holding bulk lipid concentration constant by compensating for substrate molar fraction with a neutral lipid, called a neutral diluent. This format for studying kinetic parameters of an interfacial enzyme is referred to as surface dilution kinetics [71]. Beyond bulk lipid composition and substrate presentation, other regulatory mechanisms control eukaryotic catalysis, including binding of calcium to the C2-domain in plant PLDs, or small GTPase and PKC protein-protein interaction for mammalian PLD. Elegant kinetic analyses of plant [73] and mammalian PLD [74] have been reported.

In vitro activity assays

Initial characterization of PLD activity monitored substrate depletion and product formation using thin layer chromatography (TLC), and co-migration of

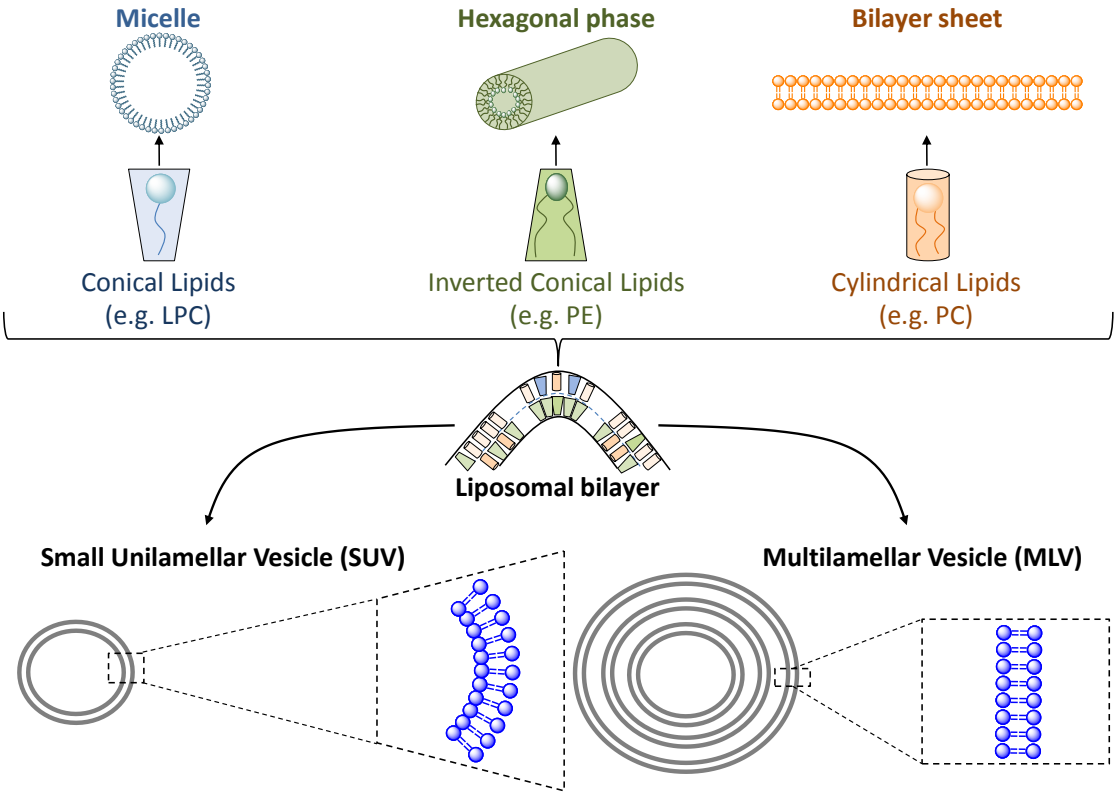


Figure 6. Substrate presentation in the liposome is highly dependent on the interfacial lipid composition due to biophysical properties of the lipid and headgroup exposure for lipid binding cofactors and substrate (figure from [19]).

specific lipid species with purified lipid standards. Next, *in vitro* assays with increased precision and sensitivity have been developed that use head group release or product formation as readouts of enzyme activity. It is important to keep in mind the specific readout being measured when drawing conclusions from *in vitro* assays. Commercial kits are available for measuring *in vitro* PLD activity. However, these kits indirectly measure choline release via two subsequent enzyme-catalyzed reactions, and this method is not uniformly suitable for activity measurement. Other *in vitro* assays have been developed that directly measure PLD activity, and can be used to directly measure kinetic parameters.

Early studies of bacterial PLD enzymes utilized soluble small molecules with phosphodiesterase bonds to serve as substrate analogs. These small molecules have a detectable shift in light absorbance following hydrolysis, and some are capable of differentiating phosphodiester versus phosphatase activities. Soluble monomeric substrates with short acyl chains can also be used. Despite the fact that affinity for these soluble substrates is often poor, requiring higher concentrations to detect product formation, the benefit of these two options are that Michaelis-Menten kinetics can readily be performed since K_s component is omitted.

Mixed micelle and micelle assays can also be performed. Use of this format allows simple surface dilution experiments, since detergent readily compensates to adjust molar fraction of substrate (titration of increasing amounts of detergent, that will insert into mixed micelle to dilute substrate) [75]. In the

micelle format, phospholipids and lysophospholipids are of a conical shape.[76] However, many eukaryotic PLDs exhibit low activity in the absence of lipid cofactor(s) and in the presence of detergents, especially anionic detergents such as Triton-X100. Therefore use of pure substrate lipid micelles or mixed detergent-lipid micelles is not practical for biochemical study of eukaryotic PLD superfamily members.

Liposome assays are more complex, but liposomes more closely mimic natural, physiologically relevant membranes [77], [78]. Higher eukaryotes demonstrate increased specific activity in the presence of the lipid cofactor, $PI(4,5)P_2$. HKD-PLD enzyme will perform processive activity if bulk lipid binding is held saturated. Separate lipid compositions can be made to vary substrate molar fraction by changing ratio of substrate to neutral diluents. Sonication is frequently used for simple liposome generation, but this makes multilamellar vesicles [(MLV), Figure 6]. These are adequate for both simple measurements of activity, and for comparisons of different reaction conditions within an assay. However, surface concentration of substrate is not controlled in MLV, making them imprecise for measurement of kinetic parameters. Extrusion is the preferred method for generating more uniform, unilamellar vesicles. The biophysical properties of the lipids in phospholipid liposomes have a significant impact on the PLD activity of scooting enzymes (Figure 6).

Cellular Activity Assays

It has long been appreciated that PLD enzymes perform transphosphatidylation [61], [65]. Stanacev and Stuhne-Sekalec demonstrated that transphosphatidylation preferentially occurs in very low concentrations of alcohol [62]. This characteristic of PLD has been exploited in cellular studies of the enzyme [78]. Phosphatidylalcohols are metabolically more stable than PA, which fluxes quickly. Historically, thin layer chromatography (TLC) has been used to visualize phosphatidylalcohols by monitoring co-migration of radioisotopically labeled lipids (on the fatty acids) with phosphatidylalcohol standards. Recently, a non-radioisotope-based cellular assay was developed [78]. This assay uses ESI-MS to monitor formation of deuterated-phosphatidylbutanol following incubation of cells with low concentrations of deuterated-butanol. However, results from alcohol-treated cell preparations to identify and parse the signaling functions of PLD may have been incorrectly interpreted. Some recent characterizations of PLD functions using RNAi and small molecule PLD inhibitors have not been able to recapitulate some of the earlier findings obtained through the use of alcohols [79], [80]. Small molecule inhibitors in combination with alkyne-modified lipids are powerful tools, and are being used to measure flux of specific pools of metabolic and signaling lipid [81].

Prokaryotic PLD

Prokaryotes express PLD superfamily genes that range in function from hydrolysis of the DNA backbone, to protein-protein interactions with host signaling pathways, to the more classic lipase function. While PLD enzymes with lipase activity are not commonly expressed among bacteria compared to other phospholipases, bacterial PLDs with lipase activity have been identified in many pathogenic bacteria.[82] Even though most bacterial PLD enzymes have different activity, because of the ease of expressing and purifying these bacterial PLDs recombinantly, much of our structural and biochemical understanding of PLD enzymology stems from studies of bacterial PLD.

Bacterial endonucleases

Evidence that the PLD superfamily arose from a gene duplication event stems from studies of EDTA-resistant bacterial endonucleases with a single HxKxxxxD. In fact, initial characterization of the PLD superfamily was performed using Nuc, an ATP-independent, nonspecific endonuclease encoded on plasmid DNA found in *Salmonella typhimurium* and *Escherichia coli*. The crystal structure of Nuc was determined to 2.0 Å (PDB accession codes 1BYR and 1BYS, native and complexed with tungstate inhibitor, respectively), and found to contain a single HxKxxxxD motif that forms a homodimer with a crystallographic two-fold axis [51]. The HKD motif within each enzyme exists on two loops held at the interface of the dimeric subunits via hydrogen bonds to form a single active site. Structural and biochemical characterization of Nuc reveals a ping- pong-like

Table 3: BACTERIAL & VIRAL PLDs (table from [19])				
SPECIES	**ENZYME**	**ACTIVITY**	**FUNCTION**	**LOCALIZATION**
Orthopox virus	p37	PLC, PLA, PLA2	IMV wrapping	TGN & inner membrane wrapping of EEV
(Vaccinia, variola)		TAG lipase	IEV fusion & release	
		transphosphatidylation		
Orthopox virus	K4	endonuclease	single strand (ss)/double strand (ds) DNA torsion release	within IMV
(Vaccinia, variola)	Nick-joining enzyme			
Salmonella typhimurium, Escherichia coli	Nuc	nonspecific endonuclease	ssRNA	periplasm
			breakage during DNA conjugation	
Escherichia coli	Bfil	site-specific endonuclease	Degrades dsDNA during DNA conjugation	periplasm
Neisseria gonorrhoeae	NgPLD	PC hydrolysis	combination of lipase & protein-protein interaction elicits bacterial invasion	host cell cytoplasm
		transphosphatidylation	binds AKT to trigger membrane ruffling	extracellular milieu
Yersinia pestis	YMT	PLD (PC/PE lipase)	*in vivo* facilitates *Y. pestis* colonization of flea gut	bacterial cytosol
(formerly Pasteurella pestis)		transphosphatidylation	protects against murine plasma component	
Chlamydiae	chromosomal pz PLDs	PLD	unknown	reticulate bodies
		unknown lipase activity	lipid acquisition from LD	
		transphosphatidylation		
Acinetobacter baumanii	Act bau PLD	unknown	unknown function	secreted
			enhances serum survival/host cell invasion	
Pseudomonas aeruginosa	PLDa gene	PLD (PC → PA)	increases long term infectivity/bacterial homeostasis	periplasm
		transphosphatidylation		
Streptomyces sp	PMF PLD	PLD	unknown	periplasm
		transphosphatidylation		secreted

S_N2 reaction mechanism that utilizes both HKD motifs within the active site. The imidazole group of one 'HKD' histidine residues nucleophilically attacks the phosphate atom on the substrate, breaking the phosphodiester bond within the DNA backbone and generates a covalent phospho-histidine intermediate. The histidine of the second subunit's HKD donates a proton to the leaving group, which, in the case of an endonuclease, is the 3' end of the DNA backbone. Hydrolysis is complete upon a water molecule nucleophilically attacking the phosphate, breaking the phospho-histidine bond, and leaving a phosphorylated 5' terminus [64]. This two-step, water-exchange reaction mechanism that proceeds through a covalent phospho-histidine intermediate is consistent with other HKD PLD enzymes, as previously described (pg. 18).

Nuc endonuclease is encoded for on the 35.4 kilobase pKM101 plasmid, a member of the broad-host range IncN plasmid classification [83]. This plasmid is responsible for conjugal DNA transfer between bacterial cells via thin rigid sex pilli [84]. pKM101 plasmid renders bacterial drug resistance by encoding for 15 genes that trigger spontaneous mutagenesis and error-prone DNA repair to facilitate survival [85]. Nuc is expressed as a 177 amino acid (19 kDa) protein in the bacterial cytosol, but is processed to 155 amino acids (17 kDa) when the 22 amino acid signal sequence is cleaved upon secretion into the periplasmic space [86], where it is constitutively localized and never secreted into extracellular growth media. Nuc endonuclease nonspecifically hydrolyzes internal phosphodiester bonds within the backbone of single and double stranded duplex DNA and RNA (*in vitro*), but does not elicit exonuclease activity at terminal

phosphodiester bonds. Maximal activity is observed in the presence of divalent cations, but unlike other bacterial endonucleases, Nuc remains catalytically acitve in the presence of EDTA. This unique characteristic allowed characterization of Nuc endonuclease activity in the bacterial cell background [83]. Despite rigorous biochemical characterization of Nuc, its functional role remains unclear. Similar to the viral endonuclease, Nuc is nonessential for bacterial survival and does not degrade plasmid or phage DNA as it crosses the periplasmic membrane. Rather, Nuc is proposed to provide an ancillary role in DNA conjugation.

Bacterial PLD as virulence factors

Phospholipases are common toxins and virulence factors for pathogenic bacteria. These enzymes facilitate bacterial infection and replication through several functions, including penetration of basal cell membranes (e.g. mucus layer or blood vessel wall), triggering engulfment of the bacterium by the host cell, or cytolysis to release intracellular bacteria from host cells such as macrophages. Phospholipase C and Phospholipase A are the most common class of bacterial phospholipases that serve as virulence factors. These enzymes are capable of destabilizing or destroying host cell membranes directly, through lipid hydrolysis or indirectly, through upregulation of host cell signaling pathways via lipid product formation [87], [88], [89], [90]. Although less common, some bacterial PLDs have also been identified as virulence factors. The localization and functions of these enzymes in eliciting virulence are divergent, and the

unifying theme amongst these enzymes is the conserved HKD motif responsible for catalytic activity.

Bacterial PLDs that function as virulence factors are generally expressed by Gram-negative pathogenic bacteria that are obligately intracellular, and require plant or mammalian host cell invasion in order to replicate (extensively reviewed [19]). These enzymes are often secreted by the bacteria into the extracellular milieu or directly injected into the host cell cytosol via one of several known secretion mechanisms. Several of these PLD genes have been proposed to be acquired by lateral gene transfer from other bacteria or host cells.[91] Acquisition of these bacterial PLDs can enable immune evasion, expand potential host colonization, and can provide pathogenic advantage.

***Streptomyces* PLD**

Gram-positive *Streptomyces* encompass the largest genus within the *Actinomycetes* class of bacteria that includes *Corynebacterium* and *Mycobacterium*. *Streptomyces* bacteria flourish in soil and secrete secondary metabolites and enzymes, including phospholipases, able to scavenge the environment for nutrients. *Streptomyces* are rarely pathogenic to humans [92]. In fact, many *Streptomyces* species are of immense commercial and industrial value for several reasons. More than two thirds of all clinically relevant natural antibiotics are derived from these bacteria, including vancomycin, chloramphenicol, and rapamycin [92], [93]. Also, enzymes secreted by

Streptomyces species are used as biocatalysts in industrial manufacturing of foods, cosmetics, and pharmaceuticals [55], [94].

Enzymes belonging to the PLD superfamily have been isolated in secretions from *Streptomyces* species including *S. antibioticus*, *S. cinnanoneus*, *S. halstedii*, and *S. septatus*. These enzymes share significant sequence homology (>70 %) and are some of the most rigorously biochemically and structurally characterized members of the PLD superfamily [55]. In contrast to scPLD from *Streptomyces chromofuscus*, these *Streptomyces* enzymes maintain the conserved domains I-IV and are class II members of the PLD superfamily, similar to YMT, as characterized by Ponting and Kerr [18]. These enzymes are robustly expressed and secreted into the extracellular milieu, but their exact function is unknown.

Robust expression and secretion of *Streptomyces* PLD, coupled with the observation that many of these enzymes display the highest transphosphatidylation activity of any bacterial PLD make these enzymes useful tools for industrial production of natural and synthetic phospholipids [94]. These enzymes exhibit broad substrate specificity that is exploited to facilitate headgroup exchange with natural and unnatural nucleophiles. In fact, use of these enzymes in industry has spurred rigorous enzymological characterization in order to engineer *Streptomyces* PLD with enhanced activities or altered substrate specificities for tailored use [95], [96].

The crystal structures of *Streptomyces sp.* PMF PLD [54], [60] and *Streptomyces antibioticus* (deposited in PDB, unpublished) have been

determined without substrate (PDB code: 1F0I, 1V0S, 2ZE4), in complex with short acyl chain substrate (1V0W, 2ZE9) or complexed with phosphate analog, tungstate (1V0R). These structures are reviewed in detail (pg. 15). These structures, in addition to biochemical data, further validate the proposed two-step S_N2 reaction mechanism. The *Streptomyces* PLD structures share a common fold to that of the Nuc endonuclease homodimer [51] and the endonuclease domain of *BfiI* [97]. The bilobal structure has an apparent crystallographic two-fold axis of symmetry that cuts through the cone-shaped active site at the interface of the two lobes. The conserved HKD motifs exist on loops that lie adjacent to one another within the active site pocket. Crystal structures with complexed substrate or phosphate analogs demonstrate there is significant hydrophobic bonding between residues of the active site holding the substrate in place. The reaction mechanism proceeds via a covalent intermediate that is formed following *N*-terminal histidine, of the HKD motif, nucleophilic attack on the phosphate of the substrate headgroup. Biochemical analysis suggests formation of this covalent intermediate is the rate limiting step of catalysis, and that subsequent nucleophilic attack of the lone pair of electrons on the oxygen from either the water or primary alcohol molecule, for hydrolysis or transphosphatidylation reaction, respectively, can proceed in parallel with similar rates [25]. Crystal structures of PMF PLD suggest that *Streptomyces* PLD can also perform a second round of hydrolysis of PA, to release DAG and a covalently-bound phosphate to active site, referred to as the dead end reaction [55], [60]. although this reaction appears to be much slower (product reportedly

observed for crystals soaked with substrate for a week, this was reproduced using an *in vitro* biochemical assay, Selvy and Brown, unpublished observation).

Because these enzymes are stably secreted into the extracellular growth medium, rigorous *in vitro* biochemical characterization of *Streptomyces* PLD has been possible. These enzymes possess a signal sequence that facilitates secretion from the bacterial cytosol into the non-reducing environment of the periplasmic domain. Some *Streptomyces* PLD have been reported to possess a critical disulfide bond that is thought to form in conjunction with proper folding only in the non-reducing environment of the periplasm [98]. Historically, much of the biochemical and structural studies have used secreted enzyme purified from the growth media of native *Streptomyces* cultures. Efforts to recombinantly express these enzymes in Gram-negative *E. coli* has proven difficult, and required use of secretion signal sequences [99] (to elicit periplasmic localization and secretion), or vectors with thioredoxin tags [100] (to enhance cytosolic disulfide bond formation).

Following structural characterization, biochemical studies of *Streptomyces* PLD homologs with significant sequence identity have subsequently been performed to further probe the function of different components of the *Strepotmyces* PLD structure. *S. septatus* TH-2PLD has the highest specific activity and transphosphaditylation rates of any bacterial PLD identified to date [101], while *pldp* exhibits quite low activity. These differences in PLD activity between these two enzymes exist, despite the fact that these enzymes share significant sequence identity. This suggests that critical differences in a small

number of residues elicit major differences in PLD enzymatic activity. Uesugi *et al.* used these two PLD genes to generate a series of chimeric constructs [102]. Using a random repeat-length independent and broad spectrum, RIBS, *in vivo* DNA shuffle technique chimeric mutants were generated composed of stretches of TH-2PLD and *pldp* [102], [103]. Biochemical characterization of these constructs identified residues that were critical in modulating substrate specificity, interfacial activity, transphosphatidylation, and thermostability. The tertiary locations of these residues were then mapped in the *Streptomyces* PLD structure (models of TH-2PLD based on the PMF PLD structure) to further clarify their mechanistic function.

The *Streptomyces* PLD structures show two flexible loops that gate the 30 Å wide entrance to the active site cleft. The *N*-terminal loop is located between beta-strand 7 and alpha-helix 7. The *C*-terminal loop is between beta-strand 13 and beta-strand 14. Chimeric analysis identified two residues in the *N*-terminal loop, (Gly188 and Asp191 for TH-2PLD) that dictate interfacial activity and sensitivity to substrate presentation [102]. *Streptomyces* PLD prefer substrate presented as monomer or mixed micelles, and demonstrate lower activity towards phospholipid vesicles [25]. Computer modeled docking of phospholipids into the *Streptomyces* PLD structure suggests these residues in the *N*-terminal loop might serve as a second phospholipid binding site for PA, PE, or PS [104]. The *C*-terminal loop, specifically residues Ala426 and Lys438 of TH-2PLD, are involved in enhancing the specific hydrolase and transphosphatidylation activity, regardless of substrate presentation. These residues also participate in

phospholipid head group specificity, and enhance thermostability of the enzyme [57]. Uesugi *et al.* used surface plasmon resonance and inactive mutants to measure substrate binding affinities [56]. The specificity for zwitterionic phospholipids over anionic phospholipids was narrowed down to the same residues, Ala426 and Lys438, in the *C*-terminal loop that are proposed to act as a gate at the entrance to the active site cleft [56]. Substrate specificity can be altered by point mutation of residues in this loop. Masayama *et al.* have exploited this characteristic by mutating residues in the *C*-terminal loop to facilitate production of phosphatidylinositols via head group exchange, an activity that is not observed with the wildtype enzyme [95].

Other studies have characterized the function of the conserved GG and GS residues that lie downstream of the HKD motifs, *N*-terminal and *C*-terminal motifs, respectively, in most PLD superfamily enzymes. Ogino *et al.* showed that the GG/GS residues, specifically the serine residue, downstream of the putative HKD motifs are critical for dictating the transphosphatidylation activity of the enzyme [58]. These residues line the base of the active site and are proposed to control active site conformation and stability, and subsequently modulate substrate specificity and ability to transphosphatidylate. Deletion of the serine residue decreases overall activity by a third compared to wildtype enzyme [58].

Plant PLD

Plant PLDs make up the largest family of HKD enzymes, with more than 80 genes identified and several dozen cloned. These enzymes are more complex

than bacterial PLD, because they encode regulatory domains that facilitate differential activities under various signaling environments (reviewed [105], [106], [107], [108]). Plant PLD enzymes contribute to the rich history of the PLD superfamily, in that the first description of a PLD enzyme was made from carrot.[13] The PLD hydrolytic and transphosphatidylation activities were originally described in plants, in 1947 [14] and 1967 [61], [65], respectively. Also, the first PLD enzyme was cloned from the castor bean in 1994 [109]. Cloning of the castor bean PLD by the Xuemin Wang lab subsequently facilitated identification and cloning of fungi [66], [110], and animal [111] homologs. The *Arabidopsis thaliana* genome has been sequenced, making identification of PLD superfamily members and genetic manipulation of this model organism feasible. The bulk of the plant PLD literature focuses on *Arabidopsis*, therefore this model organism will be the focus of this section.

PA makes up less than 1 % total lipid in plants, but is an important second messenger [107], [112]. Several pathways have been characterized that generate PA, but in plants the two main signaling mechanisms for generating PA involve PLC-DAGK tandem activity, or PLD activity. Lipidomic analyses have been performed and characterized the major PA species in *Arabidopsis* as having long polyunsaturated fatty acids [34:2(16:0-18:2); 34:3(16:0-18:3); 36:4(18:2-18:2); 36:5(18:2-18:3); 36:6(18:3-18:3)].[112] Different PA species change in response to different stimuli and environmental conditions. Drought and soil salinity are common environmental stresses, and are a major focus of plant research because these conditions affect crop production worldwide [112].

Plant PLD enzymes have variant regulatory mechanisms to respond to extracellular stimuli such as these, and mediate intracellular responses via PA production and protein-protein interactions.

Classes of plant PLD enzymes

Plant PLD enzymes consist of two-conserved HxKxxxxD motifs separated by roughly 320 aa, which include the conserved region III (IYIENQFF). The function of this region is unknown, but is present in every PLD superfamily member with true phospholipase activity. Most plant PLD region III sequences encode 'IYIENQYF', while two enzymes more closely related to the mammalian PLDs encode for 'IYIENQFF' [113]. Plant PLD enzymes can be divided into two subdomains, C2-PLDs and PXPH-PLDs, based on the presence of amino-terminal regulatory domains upstream of the catalytic domain [108], [114]. C2-PLDs have an *N*-terminal C2 calcium binding domain that is distinct to plant PLD enzymes [112]. This domain is not found in other higher order PLDs. PXPH-PLDs are more closely related to mammalian PLDs, and have amino-terminal phox homology (PX) and pleckstrin homology (PH) domains important for specific lipid interactions [112], [113]. At least 12 *Arabidopsis* genes have been identified, of which ten are classified as C2-PLD genes and two are classified as PXPH PLD genes [115]. Within these classes specific isoforms have been identified that exhibit differential genetic architecture, sequence identity, catalytic activities, and regulatory requirements [105], [113].

In contrast to the multiple crystal structures available for bacterial enzymes, a crystal structure for the more complex plant PLD does not exist, despite reported crystallization of cowpea PLD over a decade ago [49]. Therefore, the current model of proposed tertiary structure of the catalytic domain and reaction mechanism are based on the structure and characterization of the bacterial PLDs (pg. 15). The limited structural analysis of plant PLD that does exist has used non-crystallographic analytical tools. One such study used mass spectrometry analysis to characterize the sulfhydryl groups on cabbage PLD [116]. Increasing numbers of plant PLDs of both C2 and PXPH subfamilies have been cloned and recombinantly expressed in bacteria [73], [113], [117], [118], which has lead to a greater understanding of the individual biochemical characteristics of different plant PLD isoforms.

C2-PLD

In the mid to late 1990's following cloning of the castor bean PLD [109], a surge of plant PLD enzymes were identified, sequenced, and characterized by genetic and biochemical approaches [108]. Comparisons within this growing pool of plant PLDs led to observations of clusters of similar enzymes based on genetic architectures, sequences, and biochemical characteristics. Members of the C2-PLD subdomain were subsequently categorized as PLDα, PLDβ, PLDγ, PLDδ, PLDε. It is important to note that as sequence and biochemical characterization

Table 4. Plant PLD Enzymes (table from [19])				
ENZYME	**REGULTORY DOMAIN**	**CATALYTIC REQUIREMENTS**	**SUBSTRATE**	**SIGNALING**
PLDα	C2-domain	mM Ca^{2+}	PC>PE	hormone/stress response, senescence, nutrient sensing
PLDβ	C2-domain	μM Ca^{2+}, $PI(4,5)P_2$	PC=PE=PS=NAPE	actin polymerization
PLDγ	C2-domain	μM Ca^{2+}, $PI(4,5)P_2$	PE=NAPE>PC	hormone/stress response (?)
PLDδ	C2-domain	μM Ca^{2+}, oleate, $PI(4,5)P_2$	PE>PC	cell viability, ROS response, binds microtubules
PLDε	C2-domain	μM Ca^{2+}, oleate, $PI(4,5)P_2$	PE>PC	root growth, elongation
PLDζ	PX-PH	$PI(4,5)P_2$	PC	root growth, elongation

improved, some initial cluster designations have changed (PLDα4 is no longer included as a PLDα isoform, and PLDδ1 was reclassified PLDβ2)[113].

Regardless of cluster classification, all members of the C2-PLD subfamily encompass a conserved 130 aa C2 domain at the amino terminus that is important in calcium sensing and phospholipid binding [106], [108]. More than 4000 consensus sequences have been reported for the C2 domain and are commonly present in proteins involved in lipid metabolism, signal transduction, and membrane trafficking [119]. The crystal structure for several C2 domains has been determined and a common antiparallel 8-β strand sandwich fold is conserved [120], [121], [122], [123]. Two or three calcium ions are known to bind at 4-5 acidic residues in the loops between the beta strands [119]. The β-strand sandwich fold is predicted to be conserved in plant PLD, but structural characterization of this domain from several C2-PLD isoforms demonstrates that a significant conformation change occurs upon calcium ion binding, which is not observed in C2 domains from other proteins or species [124]. This suggests plant C2 domains may be a variant of those previously characterized.

In addition to the divergent protein conformations upon calcium binding, some plant PLD isoforms have substitutions in the C2 domain acidic residues [124]. This results in isoform-selective differences in calcium binding affinities and catalytic responses. C2 domains also bind lipids dependent on calcium concentration, therefore cytoplasmic calcium levels are thought to modulate C2-domain conformation and lipid binding affinity [118]. C2-domains also demonstrate lipid binding specificity. *Arabidopsis* C2 domains bind $PI(4,5)P_2$ and

PC in a calcium-dependent manner [124], [125]. C2 truncation mutants bind lipid vesicles but with lower affinity and these PLD enzymes display decreased activity [126]. Many C2 domains elicit constitutive binding to the lipid membrane (ie. no stimulus-induced translocation) therefore these enzymes are proposed to function in the scooting mode with processive catalytic activity. All C2-PLD enzymes characterized to date require some level of calcium for catalysis and can perform transphosphatidylation [126]. This plant PLD subfamily is responsible for the majority of the PA produced in response to environmental stress signaling.

PXPH-PLD

In contrast to C2-PLD enzymes, two plant PLDs have been identified that encode for phox homology (PX) and pleckstrin homology (PH) lipid binding domains at the amino-terminus, PLDζ1 and PLDζ2 [113], [127]. These genes are both located on chromosome III. PLDζ1 and PLDζ2 do not require calcium for catalysis, rather these enzymes selectively cleave PC in a $PI(4,5)P_2$ dependent manner [113]. As such, these plant PLDs are more closely related to the mammalian PLD enzymes PLD1 and PLD2. In mammalian PLDs, the PX domain has been shown to bind $PI(3,4,5)P_3$, and anionic lipids, while the PH domain binds PI and PIP_n species. PLDζ1 and PLDζ2 also retain four of the five basic residues in the conserved $PI(4,5)P_2$ binding motifs that flank the 2^{nd} HKD [113]. These polybasic motifs may serve to regulate PLDζ catalysis in response to $PI(4,5)P_2$, similar to mammalian PLDs.

Cellular characterization of this subfamily of plant PLD enzymes remains sparse, but some recent studies have shown PLDζ enzymes are involved in environmental stress responses. PLDζ2 is transcriptionally regulated in response to phosphate starvation and auxin levels [127], [128]. Exogenous auxin supplementation can stimulate PLDζ2 transcription. Plant PXPH PLDs have also been shown to mediate vesicular trafficking, phosphate recycling and root gravitropism [129].

Signaling

Plant PLD enzymes are structurally more diverse and complex than bacterial homologs. As in other higher eukaryotes, PA is largely involved in stress-mediated signaling pathways in plants (detailed review recently published [130], including response to environmental conditions such as dehydration, high salinity, pathogenic defense and wound healing [107], As such, plant PLD enzymes have evolved diverse regulatory mechanisms to respond to specific extracellular stimuli. Plant PLD enzymes can be regulated at the level of transcription or translation, via post-translational modification (lipidation or phosphorylation), or via cytosolic and membrane cofactors and conditions (calcium, $PI(4,5)P_2$, substrate presentation/membrane fluidity, and pH). PA signaling can also be regulated and attenuated post production by phosphorylation to generate DAG pyrophosphate (DGPP) [131]. It is currently unknown whether DGPP is itself also a signaling molecule.

Despite the historical precedent in plant studies of PLD, development of pharmacological tools to modulate the activities of these enzymes has lagged behind that of other eukaryotes. To this day, the use of knockout models and primary alcohols remain the only known tools with which plant PLD can be studied [132]. Using a primary alcohol, product formation can be diverted to the transphosphatidylation product phosphatidylalcohol (Figure 5). However, alcohols are imprecise tools because of their lack of specificity and potency. While only primary alcohols are able to serve as nucleophiles in the PLD reaction mechanism, both primary and secondary alcohols activate plant PLD activity [133]. Off target activation of heterotrimeric G proteins also occurs in response to alcohols, making it difficult to delineate the specific role of PLD in receptor-mediated stress induced signaling pathways. A few plant stress response cascades are briefly described here to demonstrate a few of the numerous roles in which plant PLD enzymes have been implicated.

Fungal PLD

Fungal PLD, identified in yeast and slime mold, regulate critical developmental functions. Similar to plants, PLD activity was first described in yeast using biochemical methods. Nearly four decades ago glucose-stimulated PLD activity was measured for a species of budding yeast, *Saccharomyces cerevisiae*, grown in low (1 %) glucose content [134], [135]. These growth conditions induce glucose repression that triggers low oxygen uptake. Yeast harvested from these growth conditions demonstrated ^{14}C-lecithin hydrolysis and

PA production in mitochondrial fractions [135]. This activity was increased in response to glucose repression during aerobic growth and decreased oxygen uptake. The increased activity was determined to be due to induction of an unknown cytosolic enzyme rather than a protein of mitochondrial origin since cyclohexamide blockage of cytosolic protein synthesis perturbed the PLD activity, and chloramphenicol inhibition of mitochondrial protein synthesis did not [135]. This observation was largely ignored until a series of parallel studies decades later identified specific PLD enzymes in different yeast species. Spo14, a PLD superfamily member also known as PLD1, was identified in the budding yeast *Saccharomyces cerevisiae* [66], [110], [136], [137], [138]. Other groups have identified similar Spo14-like enzymes in pathogenic budding yeast [139], *Candida albicans* [140], and in fission yeast, *Schizosaccharomyces pombe* [136]. In addition a biochemically distinct enzyme, PLD2 [139], has been described in *Saccharomyces cerevisiae*. Subsequent studies have demonstrated that the PLD activity initially observed in budding yeast (in the 1970's) is distinct from the PLD superfamily, and this activity has been attributed to PLD2 [141], [142]. Yeast PLD1 enzymes, including Spo14, share sequence and biochemical similarities to plant and other eukaryotic PLDs. These enzymes have been shown to function in yeast sporulation [110], [137], vesicular trafficking [143], mating [144], and virulence for the pathogenic species [145], [146].

Budding yeast Spo14

Spo14 was originally identified during phenotypic studies of fission and budding yeast deficient in meiosis and sporulation [136], [137]. The most extensive follow up studies of this gene and gene product have been performed using the budding yeast *Saccharomyces cerevisiae*. *S. cerevisae* have distinct regulatory pathways for mitosis separate from those observed for meiosis I and meiosis II during sporulation. Early studies observed sporulation defects in mutagenized yeast as a means of identifying genes that might be involved in meiotic signaling pathways [137]. In the first meiotic step, parental cells replicate genomic DNA and homologous chromosomes perform recombination as they align near the spindle pole bodies (SPB) in preparation for meiosis I. During meiosis I, similar chromosomes move to opposite poles of the nucleus and two diploid daughter nuclei are generated by separation of the chromosomes with the SPB. Reversal of meiosis is possible through meiosis I. In fact, cells with fully formed SPB are able to instead perform mitosis in response to changes in extracellular conditions and remain diploid. However, upon entry into meiosis II, the cell is committed to meiosis and unable to reverse to mitosis despite changes in extracellular growth conditions. During meiosis II, sister chromatids move to opposite poles of the nucleus to generate four haploid nuclei. These haploid nuclei are packaged into spores with prespore membrane (PSM), double layer membrane generated *de novo*, within the mother cell. This packaging is akin to acrosomes generated during spermatogenesis [141].

Spo14 was identified as a gene involved in *S. cerevisiae* sporulation by Honigberg *et al.* [137]. In this study, mutagenized *S. cerevisiae* were subjected to various growth conditions, including changes in temperature, in order to observe phenotypic sporulation deficiencies. Cells with disrupted Spo14 genes showed 1.5-fold less yeast transition through meiosis I, and 10-fold fewer cells complete meiosis II [110], [137]. The cells that did complete meiosis I and meiosis II had degraded nuclei and were not viable. It was also observed that cells with disrupted Spo14 did not commit to meiosis at meiosis II, a phenomenon in wildtype yeast referred to as “commitment to meiosis [147]. Rather, cells in later stages of sporulation with irregular nuclear composition were observed to reverse and mitotically divide [147].

In parallel, Ella *et al.* subjected *S. cerevisiae* to different growth medium and measured changes in PLD activity [66]. This group demonstrated that PLD activity is induced under nitrogen deprivation when yeast are grown in a medium containing a non-fermentable carbon source, ie. acetate [66]. Supplemental application of glucose to these growth conditions decreased PLD activity. Sporulation, more specifically meiosis I, is triggered under nutrient deprivation conditions but cells can be reversed and induced to mitotically divide if nutrients are supplemented prior to transition into meiosis II [137], [147]. These studies suggest PLD activity is increased during sporulation, and the activity measured by this group is the same as that characterized by Rose *et al.* [110]. Spo14, called PLD1 by this group [138] and others [148], is the enzyme responsible for the observed sporulation-induced activity. Spo14 is capable of PC hydrolysis and

can perform transphosphatidylation with primary alcohols [66]. These activities suggested that this newly-identified enzyme was indeed a PLD similar to PLDs identified in plants.

Sequence, catalysis, and regulation

Earlier cloning of castor bean PLD sequence facilitated cloning of Spo14 [137], also known as PLD1 [138], [148], which later lead to cloning of the human PLD homolog [111]. Genomic sequencing of *Saccharomyces cerevisiae* identified Spo14 on chromosome XI. Spo14 is predicted to be the only HKD PLD in this organism, and is a member of the PLD superfamily. The gene for PEL1/PGS1, a phosphatidylglycerol phosphate synthase, is the only other gene encoding for an HKD enzyme in *S. cerevisiae* [141].

Spo14 protein sequence is 1683 amino acids, with a molecular weight of 195.2 kDa. A stretch of 440 amino acids in the middle of the sequence are 21 % identical to castor bean PLD, demonstrating conservation of the catalytic domain observed for members of the PLD superfamily [110]. Separate groups cloned this enzyme, naming it either Spo14 [110], based on function in the initial sporulation defects study, or PLD1 [138], [148] to delineate this activity from an apparently separate PLD activity described in the 1970's. Spo14 sequence analysis shows this enzyme retains two conserved HKD catalytic motifs, present in the majority of eukaryotic PLD superfamily members. A putative polybasic $PI(4,5)P_2$ binding domain, found in other PLD superfamily members and originally described in Spo14, exists between these HKD motifs [149].

Unique to yeast PLD, the amino-terminus contains a regulatory LOCO/phos domain encompassing residues 1-313 [150]. This region is hyperphosphorylated at serine and threonine residues upon meiotic initiation [150]. Hyperphosphorylation shifts the molecular weight of Spo14 from 195 kDa to roughly 220 kDa. Hyperphosphorylation is a necessary regulatory mechanism for Spo14 function in meiosis, but not for other cellular functions of Spo14 or *in vitro* catalytic activity. Downstream of the LOCO/phos domain, the amino terminus also possesses PX and PH domains. The PH domain binds $PI(4,5)P_2$ to facilitate basal protein-membrane localization as well as protein translocation within the cell [151], [152], [153]. As such, amino-terminal LOCO/phos and lipid binding domains are not integral to *in vitro* catalytic activity.

In vitro biochemical characterization of Spo14 has been performed using recombinant protein heterologously expressed in either insect [110] or bacterial [138] systems. Similar to other eukaryotic PLD enzymes, $PI(4,5)P_2$ binding at the putative polybasic motif, but not the PH domain, is requisite for catalytic activity [149]. Similar to some eukaryotic PLD enzymes, oleate (5mM) was shown to stimulate activity seven-fold [139]. However, Spo14 is unique from plant or mammalian PLD in that it is insensitive to calcium, and inhibited by magnesium.

Spo14 catalytic activity is substrate-specific to PC, and little to no PI or PE is hydrolyzed [66]. Spo14 can catalyze transphosphatidylation reactions with a broader range of alcohols than other eukaryotic PLDs. Although preference is given for primary alcohols, such as *n*-butanol, branched-chain alcohols, such as 3-methyl-1-butanol can also be used as nucleophilic substrates [66]. Spo14

appears to be less effective at transphosphatidylation than mammalian homologs. This is postulated to be due to Spo14 potentially hydrolyzing phosphatidylalcohols [141] shortly after production, but this remains to be demonstrated. Also, *in vitro* catalytic activity is stimulated in the presence of alcohol [66].

In vitro, Spo14 catalytic activity is regulated by access to lipid cofactor $PI(4,5)P_2$ and substrate, PC. In contrast to other eukaryotic PLD enzymes, Spo14 activity is not modulated by small GTPases, such as ADP-ribosylation factor (Arf) [154]. In contrast to *in vitro* regulation, cellular regulation of Spo14 is more complex and is dependent on the specific functional pathway, such as sporulation or mating. Cellular Spo14 is not directly regulated by Arf, but Arf GTP/GDP cycling via Arf GAP, Gcs1, does modulate Spo14 activity during sporulation [155]. Arf cycling is also is critical for sporulation [154]. In general, cellular Spo14 is transcriptionally and translationally regulated in most functional pathways in which it has been implicated. Induction of Spo14 RNA and protein is observed, 7-fold and 3-fold, respectively, in late meiosis. Post-translational modification such as phosphorylation has been shown to regulate Spo14 localization. Finally, access to the lipid cofactor $PI(4,5)P_2$ regulates both localization via the PH domain as well as activity via polybasic binding domain. Vegetative cells demonstrate PLD activity in both soluble and particulate fractions, likely localized to intracellular endosomal membranes, while Spo14 translocation to specific membranes, such as the PSM, has been demonstrated for specific functional responses [139].

In the 1970s, Dharmalingam *et al.* [134] and Grossman *et al.* [135] described glucose-stimulated PLD activity in *S. cerevisiae*. More recent characterization of yeast PLD activities suggests this observed PLD activity is due to a separate class of enzyme, likely that of PLD2 [141], [142], [156], [157]. PLD2 was described, but not cloned, as a calcium-dependent enzyme and does not require $PI(4,5)P_2$ for activity. This activity was observed in Spo14 deletion mutants in the absence of EGTA or EDTA. This enzyme does not perform transphosphatidylation and preferentially hydrolyzes PE and PS rather than PC. This demonstrates PLD2 activity is distinct from that of Spo14. The fact that Spo14 is the only HKD PLD present in the *S. cerevisiae* genome, and that PLD2 does not perform transphosphatidylation suggests this enzyme is likely a PLD-like enzyme distinct from the PLD superfamily with a unique reaction mechanism.

Function

Spo14 deletion mutants do not demonstrate any phenotypic disruption in vegetative growth. Similar to the exocytic and vesicular function of HKD PLD enzymes in other higher eukaryotes, Spo14 appears to be integral for specific functional processes involving membrane formation, fusion, and secretion. In response to nitrogen deprivation and non-fermentable carbon sources, Spo14 responds by translocating in preparation for sporulation [151]. Spo14 activity is integral for rescuing vesicular trafficking in a mechanism that responds to loss of PI-transfer protein Sec14 [152], [158]. Finally, Spo14 has recently been shown to

participate in mating and pheromone signaling pathways [144], and is a virulence factor integral for pathogenic yeast *Candida albicans* [145], [146].

Dictyostelium PLD

Another type of fungi that is extensively studied is the unique slime mold *Dictyostelium discoidium*. This model organism possesses PLD activity, and similar to budding yeast Spo14, this activity has proven integral for critical developmental processes. This slime mold is found in soil of Eastern North America and Eastern China, and is studied as a model organism because it exhibits several distinct life cycles dependent on environmental growth conditions [159]. Also, *Dictyostelium* bears many similar signaling pathways and mechanisms to eukaryotes in which PLD participates.

Slime mold grows in monolayer or suspension cultures and feeds on bacteria. In the presence of ample nutrients, *Dictyostelium* exists as a haploid unicellular form that mitotically replicates. In response to low nutrients or high density, unicellular cells replicate in one of two cycles: sexual or assexual. These replication cycles are the reason that *Dictyostelium* are so intensely studied. *Dictyostelium* are referred to as social amoeba that exhibit social cooperation or altruism by sacrificing some individual cells for the benefit of the species [159]. Sexual replication occurs upon contact with a haploid cell of opposite mating type, and the cells and nuclei fuse to form a diploid zygote [160]. The zygote secretes cAMP and other chemoattractant molecules to coercively draw other cells near, whereupon the zygote cannibalizes them to harvest nutrients and form

the cellulose-bound macrocyst structure [159]. The macrocyst replicates via meiosis and then germinates. Signaling pathways and mechanisms in the sexual reproduction pathways have not been characterized, but this has been because the emphasis has been on the asexual cycle.

In the absence of fusion with opposite mating type cells, *Dictyostelium* respond to low nutrient and high cell density by secreting chemoattractant molecules. This facilitates quorum sensing and triggers cell signaling responses in neighboring cells. Unicellular forms constitutively express a glycoprotein, conditioned medium factor (CMF), which is only secreted in response to low nutrient starvation conditions. In response to quorum sensing molecule CMF, heterotrimeric G-protein signaling pathways ensue. Under low nutrient and high density conditions *Dictyostelium* secrete waves of cAMP [160]. The waves of cAMP bind cyclin AMP-receptors (cAR), GPCRs at the surface of neighboring cells. Binding elicits signaling pathways that trigger cell migration and aggregation towards one another. A mound of cells forms, and continued waves of cAMP and bioactive molecule secretion, such as differentiation inducing factor (DIF-1), generate a molecular gradient of small molecules. This gradient elicits polarization of the cooperative cells into anterior and posterior regions, and DIF-1 induces non-uniform cell differentiation into one of two types of pre-cells that ultimately generate either a stalk or spore-harboring fruiting body. The polarized cell aggregate, called a slug, is able to migrate greater distances than unicellular forms and is protective from predatory consumption (e.g., *C. elegans*). Once a new location is selected, the pre-stalk and pre-spore forms further differentiate

into mature stalk and spore, destined for cell death, or dispersal and germination, respectively.

PLD activity has been described in *Dictyostelium* for the different growth stages of the three distinct reproductive cycles. Three PLD transcripts were identified, plda, pldb, and pldc [161], [162]. The plda is constitutively expressed at unaltered levels in vegetative and reproductive cell types, whereas pldb mRNA message and protein levels fluctuate with changes in growth or reproductive cycle. As such, pldb is the most extensively studied isoform, and has been shown to participate in quorum sensing and facilitate polarized cell migration. This 867 aa enzyme is 32 % similar and 21 % identical to human PLD1, with conserved PH and CRI-IV domains, and loop and tail regions. The pldb is $PI(4,5)P_2$-dependent and performs transphosphatidylation with primary alcohols. However, in contrast to human PLD, *Dictyostelium* pldb preferentially hydrolyzes ether-containing PE species [163].

The pldb negatively regulates quorum sensing in two ways. First, PLD-generated PA counteracts cell responses to CMF by modulating heterotrimeric G-protein signaling responses and RGS (regulatory of G-protein signaling) regulation [161], [164]. Also, pldb-generated PA is suggested to facilitate cAR receptor internalization and recycling [161], [162], [164]. Unicellular *Dictyostelium* treated with primary alcohol, or pldb deletion mutants aggregate at lower densities independently of CMF. This is likely due to enhanced cAR levels at the plasma membrane (lack of receptor internalization or recycling) and increased G-protein signaling in the absence of RGS modulation. The pldb overexpression

mutants increase the density and CMF signaling threshold necessary to trigger unicellular aggregation [161].

The pldb is also necessary for actin localization and actin-based motility in two ways. Pldb localizes to the leading pseudopodia extensions of the slug, and PLD-generated PA levels are highest at the leading edge, with a decreasing gradient towards the posterior [165]. PA facilitates membrane curvature necessary for pseudopodia formation, but PA also activates PI4P5K, which generates $PI(4,5)P_2$ in a positive feedback loop on pldb [163]. In addition to activating PLD, $PI(4,5)P_2$ localizes actin nucleating factors (Arp2/3 complex) to the leading edge of pseudopodia for F-actin polymerization [163]. As a result of primary alcohol treatment, actin assembles in the nucleus and results in aberrant morphologies. In light of the importance of PA in specific signaling and structural capacities, pldb activity is integral to asexual reproduction in *Dictyostelium*. Further study will determine the role of PLD in vegetative or sexual reproduction cycles.

Zebrafish PLD

Seminal work in characterizing the function of PLD in the context of a whole vertebrate was recently performed using zebrafish, *Danio rerio*. In 2003, Ghosh *et al.* partially cloned a PLD enzyme (aa 380-916) from zebrafish embryos and determined it was expressed during gastrulation [166]. Zeng *et al.* followed up this study with cloning the complete zPld1 sequence [167]. This 1042 aa enzyme contains the two HKD motifs present in most eukaryotic PLDs, and is 64-

68 % and 50 % homologous to mammalian PLD1 and PLD2, respectively. zPld1 regulation is also similar to mammalian PLD, with conserved PKC (1-314 aa) and Rho (859-1010 aa) binding domains. *In vitro* characterization shows this enzyme is activated by Arf1 and PKCα. A second zebrafish PLD isoform, 927 aa zPld2, has been partially cloned.

Zebrafish are uniquely suited to whole organism studies of PLD activity and function because, as Zeng *et al.* demonstrated, PLD activity can be stimulated and measured with whole organism treatment of phorbol ester (PMA) and deuterated *n*-butanol. zPld1 activity was monitored using MS by monitoring deuterated phosphatidylbutanol formation [78]. Similar to other zebrafish phospholipases, in whole animal studies, zPld11 was determined to be involved in vascular development. This was determined using two parallel methods: (1) zPld11 was either knocked down using targeted morpholinos to disrupt zPld11 translation and mRNA splicing, or (2) zebrafish were treated with *n*-butanol to divert zPld11 activity to transphosphatidylation. Unlike the development of other systems including motor neuron organization, there was a severe deficiency in intersegmental blood vessel formation. More recent studies have observed zPld11 mediates Golgi secretory vesicle formation [168]. Aberrant zPld11 activity due to unregulated Arf-stimulation results in decreased lipid absorption in the intestine. Utility of zebrafish in measuring PLD activity and monitoring substrate and product localization in a whole vertebrate animal will facilitate determination of the function of PLD with respect to the whole organism.

Mammalian PLD

While PLD was first identified in plants in 1947 [14], PLD activity was not described in mammalian tissues until 1973 by Kanfer and colleagues [169]. Subsequently, multiple mammalian PLD enzymes and isoforms have been cloned, rigorous biochemical characterization performed, and extensive cell signaling studies undertaken. From this, mammalian PLD enzymes have been implicated in critical cell signaling pathways involved in development and cell death. These pathways modulate cell growth, proliferation, survival, and migration. As such, aberrant PLD activity has been detected in disease states, including cancer, inflammation, pathogenic infection, and neurodegeneration**.**

Isoforms

Cloning of plant and yeast PLD enzymes facilitated cloning of a full length PLD enzyme from HeLa cell cDNA [111] and rat [170] PLD1. Shortly thereafter, a second mammalian PLD enzyme, PLD2, was cloned [171], [172], [173]. These two isoforms share 50 % sequence homology, mostly at the catalytic domain that includes two conserved HxKxxxxDxxxxxxG(G/S)xN catalytic motifs separated by variable length of sequence predicted to form a thermolabile loop. *N*-terminal to a conserved polybasic PI(4,5)P_2 binding domain [149], the loop region differs for these two PLD isoforms. PLD1 harbors an extended thermolabile loop prone to proteolytic cleavage [174]. The length of this loop region is variable dependent on the splice variant [175] (PLD1a = 116 aa versus PLD1b = 78 aa), while PLD2 does not possess a significant loop region (4 aa predicted loop). Shortened

splice variants of both PLD1 and PLD2 have been identified that compose catalytically inactive enzyme [173]. Expression of these inactive enzymes is observed in different tissues, including the brain, but their function is unknown.

At the amino-terminus, PLD1 and PLD2 share similar regulatory domains to PLDζ and Spo14, including PX and PH lipid binding domains. The PX domain binds polyphosphoinositides with high specificity, and anionic lipids with lower specificity [176], but this domain has also been implicated in protein interactions with regulatory proteins, including Dynamin and Grb2. Tyrosine residues in the PLD2 PX domain can be phosphorylated. The PH domain binds anionic phospholipids with low specificity. This domain is palmitoylated at two conserved cysteine residues that facilitate protein localization and do not impact catalytic activity (Figure 7).

Despite similarities between the regulatory domain architecture of the classic PLD isoforms, the majority of the sequence divergence between these two mammalian PLD isoforms exists at the amino-terminus. Deletion of the PX domain enhances PLD1 activity. Truncation of the PLD1 PX domain and a portion of the PH domain further increases activity. However, conserved residues in a predicted α-helix at the *C*-terminal end of the PH domain are necessary for catalysis in the liposome activity assay [177] (unpublished data, Henage, Selvy and Brown). Cell-based studies demonstrate that *N*-terminally truncated PLD1 enzymes maintain high activity levels upon cellular stimulation. This suggests, similar to the extended loop region of PLD1, the amino terminus of PLD1 is autoinhibitory, whereas deletion of the amino-terminus of PLD2 decreases

activity and suggests PLD2 amino-terminus might facilitate increased basal activity.

PLD1 and PLD2 share homologous *C*-terminal sequences. The specific identity of the residues in this sequence must be maintained for mammalian PLD activity. Non-conserved point mutation or deletion impairs catalytic activity [178]. The *C*-terminal residues are suggested to interact with the catalytic core [178]. Studies by Steed *et al.* support this with identification of naturally occurring PLD2 splice variants with truncated *C*-termini that result in significantly decreased activity [173].

The bulk of mammalian PLD activity is attributed to these classical PLD isoforms. These two isoforms, and subsequent splice variants, hydrolyze phospholipids to generate phosphatidic acid, and readily perform transphosphatidylation in the presence of low concentrations of alcohol to perform headgroup exchange and phosphatidylalcohol formation. Both isoforms are capable of hydrolyzing PC, PE, PS, LPC, and LPS, but are not capable of hydrolyzing PI, PG or cardiolipin. Although PA is the major hydrolytic product, hydrolysis of a lyso-lipid generates LPA. Recently, mammalian PLD was proposed to generate cLPA from lyso-lipids [179]. cLPA could be formed similar to the transphosphatidylation of LPC observed with autotaxin, where the internal *sn*-2 hydroxyl group serves as the secondary nucleophile to cyclize the product (pg. 8).

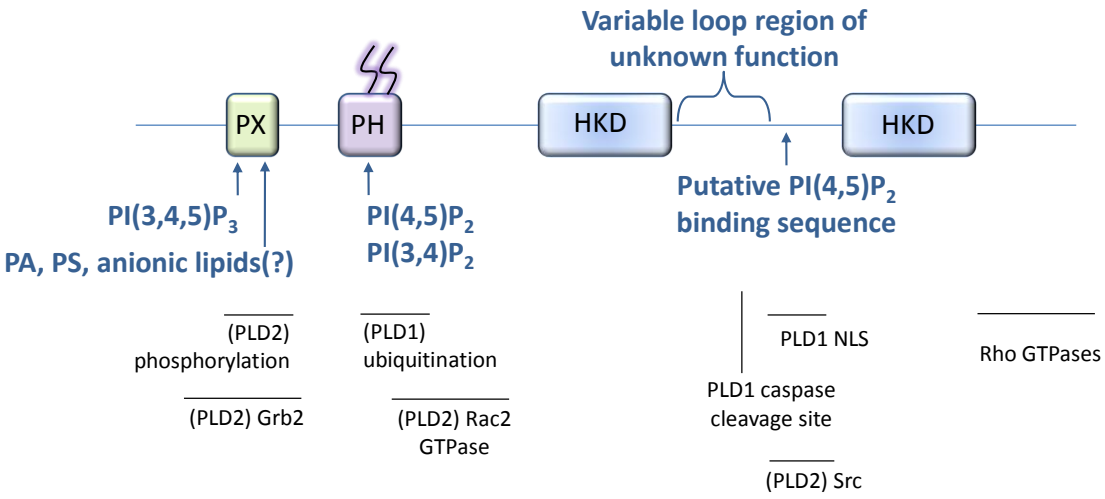


Figure 7. Conserved domains in mammalian PLD include HKD motifs responsible for catalytic activity, and phox homology (PX) and pleckstrin homology (PH) lipid binding domains thought to be involved in regulation of the enzyme. Other known sites of protein interaction are illustrated as well (figure from [19]).

In addition to PLD1 and PLD2, two mammalian enzymes have been identified with significant sequence homology to viral and prokaryotic PLD. PLD3, also called Hu-K4, bears significant sequence homology to viral PLD enzymes K4 (48 %) and p37 (25-30 %) [180]. This enzyme has two HxKxxxxD/E motifs (in one motif the aspartate is mutated to glutamate) and was recently shown to harbor a predicted *N*-terminal type II transmembrane domain [181]. This facilitates protein insertion into the ER, with 38 aa exposed to the cytosol, and the large *C*-terminus, including the HKD motifs and multiple glycosylation motifs, exposed to the ER lumen. Catalytic activity has not been detected for this PLD isoform, but it has been postulated that this enzyme might hydrolyze lipids at the lumenal phase of the ER, or may not bear lipase activity, similar to the endonuclease activity of viral K4 [181]. The murine homolog of this enzyme, Sam9, is expressed in the forebrain during late neural development [182]. Catalytic activity has yet to be defined for this enzyme as well.

A single-HKD enzyme with homology to Nuc endonuclease, called mitoPLD, was described [183]. This enzyme bears an *N*-terminal mitochondrial localization sequence (MLS) in place of PX or PH lipid binding domains. However, this localization sequence is not processed, and instead may facilitate insertion or anchoring into the outer mitochondrial membrane. This enzyme is predicted to homodimerize, similar to Nuc. This is not the first description of PLD activity localized at the mitochondria [184], but previous reports suggested mitochondrial PLD hydrolyzed PE to generate PA. Instead, mitoPLD hydrolyzes cardiolipin, an abundant mitochondrial lipid, to generate PA. This product

facilitates mitochondrial fusion events, since overexpression of mitoPLD results in formation of a single large perinuclear mitochondrion, whereas expression of a catalytically inactive mutant resulted in fragmented mitochondria [185].

Tissue expression and subcellular localization

The classic PLD isoforms, PLD1 and PLD2 are expressed in nearly all mammalian tissues. Due to the lack of clean, specific antibodies northern blot analysis has routinely been used to characterize PLD expression patterns. PLD1 and PLD2 are both robustly expressed in heart, brain, and spleen. PLD1 exhibits low expression in peripheral blood leukocytes and PLD2 is poorly expressed in liver and skeletal muscle.

While classic PLD isoforms, PLD1 and PLD2, catalyze the same reaction, and utilize similar substrates to generate PA or transphosphatidylation species, these enzymes are differentially localized within the cell. There has been some discrepancies in reports of subcellular localization of each PLD isoform, but this could be due to differences in the cellular systems, growth conditions, or the methods of detection (ie. subcellular fractionation or immunofluroescence of native versus tagged proteins; note that tags can impact localization).

PLD1 subcellular localization

It is generally accepted that PLD1 is localized to perinuclear membranes, including early endosomes, and Golgi under basal conditions [171], [186], with no reported difference in localization for splice variants PLD1a and PLD1b [186].

Different regions of the protein contribute to basal subcellular localization. Truncation and point mutations have been used to identify the contribution of these different regions. Sugars *et al.* determined PLD1 basal localization is dependent on the PH domain, specifically an acidic region (residues 252 and 253) thought to be important for IP_3 binding, and conserved tryptophan residues in a predicted α-helix that is critical for catalytic activity [187]. PLD1 is palmitoylated on two cysteine residues in the PH domain and this lipid modification supports basal protein localization at intracellular membranes [177]. Point mutation of these cysteine residues impairs palmitoylation and results in aberrant protein localization. In the presence of serum protein basally localizes to the plasma membrane [187], whereas in the absence of serum these palmitoylation mutants are dispersed in the cytosol and translocation is triggered to the plasma membrane only upon serum stimulation [188]. Hughes and Parker suggested the *C*-terminal residues of PLD1 might also be necessary for endosomal localization [186]. This region of the enzyme is certainly necessary for catalytic activity, and native splice variants of PLD1a and PLD1b that lack these *C*-terminal residues do not basally localize to endosomes. However, it has been suggested that the *C*-terminus is integral for catalysis because it supports the structure of the active site [178]. Therefore enzymes lacking this region may not in fact be folded properly, and this could result in aberrant localization rather than the *C*-terminus itself directly participating in protein localization. Catalytic activity is not requisite for protein localization. Catalytically inactive point mutants (PLD1b K466E and K860E) localize to perinuclear endosomes similar to wildtype enzyme

[186]. It should be noted that individual domains of PLD1 expressed in isolation do not localize similar to the full enzyme [187], [188], [189]. This suggests that multiple components and regions of the enzyme participate in basal localization.

Upon cell stimulation, PLD1 translocates to the plasma membrane or late endosomes. However, the type of stimulation results in differences in translocation, for example serum stimulation in Cos7 cells results in translocation to late endosomes and plasma membrane, whereas PMA stimulation triggers translocation to the plasma membrane [188] (unpublished data Selvy and Brown). PLD1 translocation to the plasma membrane in response to cell stimulation is thought to be due to $PI(4,5)P_2$ binding at the polybasic binding region between the HKD motifs [188]. Point mutations in this polybasic region, including mutation of highly conserved arginine residues 691 and 695, impair PLD1 translocation to the plasma membrane upon stimulation. These data are supported by evidence that production of $PI(4,5)P_2$ positively increases PLD activity. Finally, *N*-terminal PX and PH domains facilitate recycling to specific intracellular membranes [188].

Nuclear PLD activity that responds to GTPγS via Rho GTPase, but not Arf activation, has been described [190]. A recent report suggests this activity is due to nuclear import of PLD1 via direct protein interaction with importin-β [191]. A highly conserved putative nuclear localization sequence (NLS) was identified between residues 553 and 564 for PLDb (KxRKxxKxxxxK). Importin-β binds the NLS and facilitates active transport into the nucleus. The NLS sequence exists in the loop region between the catalytic HKD motifs, and is not present in PLD2.

Mutation of any or all of the conserved residues in this NLS sequence impairs nuclear localization. Similar to the plasma membrane, PC is the major phospholipid present in nuclear membrane. Nuclear PLD activity generates PA that is rapidly metabolized to DAG. Jang *et al.* report this PLD activity stimulates nuclear PKCα and ERK phosphorylation and activation [191]. Catalytically-inactive PLD1 point mutants and PLD-selective small molecule inhibitors disrupt nuclear PKCα and ERK activation, supporting the lipase-dependent activation mechanism. Immunofluoresence microscopy and subcellular fractionation analysis have also identified a nuclear PLD2 population, however a putative NLS has not been identified in PLD2 sequence [191], and further study is necessary to determine the mechanism for PLD2 nuclear import. The intriguing report of a PLD1 nuclear import mechanism begs further investigation to determine the potential signaling pathways modulated by nuclear PA production.

PLD2 subcellular localization

In contrast to the intricate regulation of PLD1 via protein translocation, PLD2 is generally observed to be constitutively localized to the plasma membrane under basal conditions and translocates to recycled vesicles with agonist-stimulated and desensitized receptors [171]. PLD2 also binds to and localizes with β-actin [192], and in response to EGF-stimulation localizes at membrane ruffles [193]. Instead of translocation upon cell stimulation, as is the case for PLD1, PLD2 activity and protein interactions are modulated via phosphorylation at multiple residues.

PLD activity has also been described for crude preparations of mitochondria. Biochemically, this PLD activity differs from that attributed to MitoPLD. In these mitochondrial fractions, calcium-stimulation of an unknown enzyme hydrolyzes PE to generate PA. This enzyme may not be a member of the PLD superfamily since it is unable to perform transphosphatidylation [194].

In some studies, PLD1 and PLD2 were observed to colocalize at perinuclear and plasma membrane under basal conditions. The finding that PLD isoforms may form intracellular complexes might explain why introducing catalytic point mutants results in dominant negative effects and reduces basal PLD activity [63], [195].

Regulation

PA is a critical lipid second messenger for a range of signaling cascades, but makes up 1-4 % total lipid in the cell [196]. PLD contributes to signaling pools of PA, and therefore this enzyme is under tight regulation by elaborate mechanisms including cofactor availability, signal induced subcellular translocation, post-translational modifications, and protein-protein interactions.

Divalent cations

Similar to other PLD superfamily members, mammalian PLD catalysis responds to divalent cation concentrations. However, in contrast to other superfamily members, including many plant enzymes, mammalian PLD catalysis is largely unresponsive to calcium concentration *in vitro* [175]. *In vivo*, however,

PLD activity is mediated by cellular calcium fluctuations, which suggests calcium facilitates protein-activator activation, such as PKC, and indirectly modulates PLD activity. In contrast, optimal catalysis levels require the presence of magnesium. *In vitro* PLD activity responds to changes in magnesium concentration, with half maximal Arf-activated PLD activity at 100 μM magnesium. This concentration of magnesium may facilitate catalysis directly, because this divalent cation does not impact *in vitro* protein-lipid binding (unpublished data Selvy and Brown).

Post-translational modification

Shortly after cloning of the first mammalian PLD enzymes, reports emerged that these enzymes were post-translationally modified in response to specific signaling pathways. Further characterization highlights lipid modification, phosphorylation, ubiquitination, and proteolytic mechanisms of PLD regulation.

Lipid modification

PLD1 [177] and PLD2 [197] are post-translationally palmitoylated at two cysteine residues in the PH domain. *In vitro*, this modification does not significantly impact catalytic activity, suggesting palmitoylation serves to regulate protein localization [177], [187]. In the cell, however, this lipid modification facilitates protein sorting into specific intracellular and plasma membrane domains including lipid rafts. In PLD1, Cys240 and Cys241 are palmitoylated, with Cys241 the dominant modification site. As determined by modeling the

PLD1 PH domain onto the crystal structure of PLCδ PH domain, these residues exist in a region predicted to be an extended loop of the PH domain [177], [187]. Lipid modification requires expression of full length, catalytically-competent PLD1. Expression of the PH domain in isolation, or of severely truncated constructs of the enzyme do not result in modification [187]. ΔPX PLD1 construct, lacking first 210 amino acids, is the shortest truncation that can be expressed that yields similar localization and catalytic activity to wildtype PLD1, and this truncation construct is lipid modified.

Phosphorylation

Mammalian PLD isoforms PLD1 and PLD2 are phosphorylated in response to signal transduction as a regulatory mechanism. PLD was originally determined to be phosphorylated when it was immunoprecipitated with polyclonal phospho-tyrosine antibodies [198]. Since this initial discovery, rigorous biochemical and molecular biology techniques have been employed to determine specific residues that are modified and the resulting impact on PLD activity and signal transduction.

PLD1 regulatory mechanisms reported to date largely center on protein translocation, while multiple PLD2 phosphorylation sites have been described. Therefore, few reports of PLD1 phosphorylation exist. Early studies used sequence analysis to identify two putative tyrosine phosphorylation sites $[RK](x)_{2/3}[DE](x)_{2/3}Y$ in PLD1 (aa 288-295 and aa 807-815). Evidence of phosphorylation at these residues does not exist. PLD1 phosphorylation occurs

in response to H_2O_2 stimulation, and increased phosphorylation has been shown to correlate to increased lipase activity [199]. c-Src has also been reported to phosphorylate PLD1, but this does not modulate lipase activity, rather modulates c-Src activity for downstream protein substrate [200]. PKC isoforms are also known to modulate PLD1 activity [201]. Despite the evidence that PKC activation of PLD1 is phosphorylation-independent, three residues are phosphorylated by PKC (Ser2, Thr147, Ser 561) [202]. *In vitro* catalytic analysis demonstrates that PKC phosphorylation of PLD1 likely serves as an inhibitory mechanism [203]. Maximal PKC-stimulated PLD activity is observed roughly one minute following PLD-PKC mixing. The timecourse of this activation suggests protein-protein interactions induce PLD activation. Maximal PLD1 phosphorylation at threonine 147, however, occurs nearly 60 minutes after PLD-PKC mixing [203]. Maximal PLD1 localization to the membrane also occurs at 60 minutes.

Multiple PLD2 residues are reportedly capable of being phosphorylated by numerous kinases. Gomez-Cambronero and colleagues have characterized tyrosine residues in the PLD2 PX domain that mediate lipase activity and binding with SH2 domains. Tyr169 is highly conserved in all eukaryotic PLD and is proposed to be important for high PLD2 basal activity [204]. Tyr179 is present only in mammalian PLD and has been proposed to negatively regulate Ras signaling [204]. (Ras/MAPK signaling is increased nearly two-fold with Y179F mutation). Phosphorylation at these residues recruits the SH2 domain of Grb2, which binds the Ras GEF, Sos, via its SH3 domain, to activate MAPK pathway [204]. The kinase responsible for phosphorylation of these residues has not been

identified. However, kinases responsible for phosphorylation at other PLD2 residues have been identified. Tyr175 exists in a consensus Akt phosphorylation site, and was identified using a polyclonal antibody for tyrosine phosphorylation at these consensus sequences [205]. Phosphorylation at Tyr175 reportedly increases DNA synthesis via MEK activation.

Recently, a better understanding of the regulation of PLD2 activity via phosphorylation was reported [206]. Cycling of phosphorylation and dephosphorylation of PLD2 results in differences in lipase activity and downstream signaling consequences. PLD2 binding Grb2 via phosphorylated tyrosine residues in the PX domain results in increased lipase activity, while dephosphorylation of these residues by tyrosine phosphatase, CD45, increases cell proliferation [206]. Further studies have used MS-based proteomic analysis to identify other modified residues [207]. Epidermal growth factor receptor (EGFR) negatively regulates PLD2 lipase activity via phosphorylation at Tyr296. In contrast, JAK3 increases PLD2 lipase activity via Tyr415 phosphorylation. Finally, Src, also shown to modify PLD1, phosphorylates Tyr511 on PLD2. The latter modification does not directly modulate lipase activity, instead likely impacts protein interaction with Src and facilitates downstream events, similar to Src interaction with PLD1. Multiple phosphorylation modifications can be integrated to finely tune the activity level of PLD2 dependent on signaling requirements.

Ubiquitination

A recent report demonstrated a previously uncharacterized post-translational modification of PLD1, but not PLD2, important for modulating both protein localization and curbing lipase activity [208]. PLD1 is multi-monoubiquitinated at the PH domain in a catalytic and palmitoylation-dependent manner. Catalytically-inactive point mutants are not ubiquitinated, and treatment with PLD-selective pharmacological inhibitors (chapter II) but not primary alcohol, disrupts PLD1 ubiquitination. Also, disruption of PLD1 palmitoylation impairs ubiquitination. Taken together, this suggests that properly localized and catalytically-competent PLD1 allows ubiquitination, and this modification is not a substrate-product feedback mechanism. The precise E3 ubiquitin ligase responsible for this modification is unknown, but following ubiquitination PLD1 is shuttled to the proteasome for degradation rather than the lysosome. Also, this modification translocates protein from endosomal membranes to an enlarged vesicle structure present in all cells transfected with stably ubiquitinated PLD. These stably-ubiquitinated constructs are not processed by de-ubiquitinating enzymes. As this modification results in changes in PLD1 localization and marks PLD1 for proteosomal degradation, ubiquitination of PLD1 is likely an important regulatory mechanism to change or curb lipase activity [208].

Proteolysis

Classic mammalian PLD isoforms PLD1 and PLD2 have been implicated in pro- and anti-apoptotic signaling mechanisms, and were recently reported to

be substrates for proteolytic caspase cleavage. Caspase cleavage of the PLD isoforms appears to divergently regulate these enzymes during apoptotic signaling. *In vitro* [209] and *in vivo* [174], [210] studies demonstrate PLD1 is cleaved in multiple locations by activated caspase 3, 7, and 8, while PLD2 is cleaved at several sites by caspase 3, and 8. During apoptosis initiation, caspase 8 cleaves pro-caspase 3 to generate active caspase 3. Caspase 3 cleaves amyloid β4a precursor protein, making this enzyme the dominant caspase in neuronal cell death mechanisms in Alzheimer's disease. Caspase-3 cleavage of PLD2 occurs at two or three sites near the *N*-terminus (aa13-28, a region *N*-terminal to the PX domain) and does not result in significant changes to molecular weight, catalytic activity, localization, or apoptotic signaling.[174],[209] PLD2 renders an anti-apoptotic response, likely via induction of anti-apoptotic protein expression (Bcl-2 and Bcl-XI) and down-regulation of pro-apoptotic proteins (Egr-1 and PTEN). Inhibition or RNAi knockdown of PLD2 increases apoptotic signaling.

In contrast, caspase proteolysis appears to be a significant regulatory mechanism for PLD1. *In vitro*, PLD1 is cleaved by caspase 3 in three positions (Asp41, Asp545, Asp581) [209]. *In vivo*, position 545 is the dominant cleavage site [174]. This residue lies in the PLD1 loop region that separates the two catalytic HKD motifs. Cleavage at this position produces a 56 kDa *C*-terminal fragment (CF-PLD1) which localizes to the nucleus via an exposed nuclear localization sequence, and a 60 kDa *N*-terminal fragment (NF-PLD1) that remains in the cytosol [174]. Full length PLD1 activity is protective against

apoptosis by suppressing p53 signaling. NF-PLD1 acts as a dominant negative for full length PLD1 (via hydrophobic interactions), inhibiting PLD1 activity, and resulting in de-repression of p53 [210]. Therefore, caspase cleavage of PLD1 decreases in vivo activity, and induces p53-dependent apoptotic signaling. Steed *et al.* identified a PLD1 splice variant, PLD1c, that expresses a PLD1 enzyme with an early stop codon at residue 513 [173]. This protein is expressed in human brain, and may function in a pro-apoptotic mechanism, similar to NF-PLD1. Further study of this truncated splice-variant and NF-PLD1 induced signaling is necessary. Jang *et al.* demonstrated PLD1 proteolytic processing is pathologically relevant [174]. Analysis of post-mortem brain tissue from Alzheimers patients demonstrated increased active caspase 3 and evidence for caspase-proteolyzed PLD1 fragments, compared to age-matched brain tissue.

Lipid cofactors

PLD localization and subsequent post-translational modification have a significant impact on lipase activity. In cells, lipid cofactors are thought to mediate subcellular localization through directly interacting with lipid binding domains of the enzyme as deletion or mutation of these domains changes subcellular localization. In some cases the mutant constructs change the ability of the enzyme to interact with membranes basally or change translocation of the enzyme to membranes upon cell stimulation. Recruitment of PLD upon PIP_2, or PIP_3 production allows upstream lipid kinases or phosphatases to mediate PLD lipase activity. It has been observed that when PLD fails to localize properly or be

recruited to the proper membrane substrate upon stimulation total lipase activity is impaired.

In vitro, phospholipids directly and indirectly modulate lipase activity. Many of the observed *in vitro* effects of specific lipid species must be rigorously confirmed, because the properties of the lipid substrate presentation can modulate PLD activity in ways that may or may not be physiologically relevant. For example, inclusion of high concentrations of negatively charged phospholipid may impair the ability of the enzyme to interact with the lipid interface or with substrate head group. Also, lipase activity on lysolipid substrates is significantly enhanced when presented in a lyso-lipid micelle when compared to more complex presentations, such as lyso-lipids in a diacyl phospholipid liposome (unpublished observations Scott and Brown). As previously discussed, this is likely due to headgroup access, rather than a direct allosteric modulatory affect on the enzyme.

The presence of some lipid species can directly affect protein-interface binding (K_s) by directly binding the enzyme. Separate from the active site, three other allosteric lipid binding sites have been described for PLD1 and PLD2, including the PX, PH, and polybasic PI(4,5)P_2 lipid binding motif. The PX domain binds polyphosphoinositides [PI(3,4,5)P_3>>PI(3)P>PI(5)P>other PIs] with high specificity at the putative primary binding pocket composed of conserved lysine and arginine residues [176]. At a secondary site, likely in the form of an exposed protein surface rather than a binding pocket including a conserved arginine (present in PLD1 and not PLD2), anionic lipids including PA and PS also bind.

However, in comparison to the other two lipid binding domains, the PLD PX domain binds lipids with poor affinity. This suggests the PX domain likely acts as a tertiary regulatory domain, to fine tune protein-lipid interactions initiated by another lipid binding site.

The PH lipid binding domain binds $PI(3,4)P_2$ and $PI(4,5)P_2$ with specificity over other phosphoinositides [153], [211]. However, as discussed, this domain is lipid modified, and many of the observed effects of deletion of this domain may be due to the absence of this palmitoylation. *In vitro*, the entire PH domain is not requisite for PLD activity, although deletion of a conserved alpha-helix at the *C*-terminus of the PH domain does impair lipase activity towards substrate present at an interface.

Finally, the polybasic $PI(4,5)P_2$ binding motif binds $PI(4,5)P_2$ with high specificity and affinity [149]. Lipid binding at this motif facilitates interfacial lipid interaction and enhances catalytic activity. Human PLD1 bulk lipid binding constant (K_s = 10 μM) for PE:PC:$PI(4,5)P_2$ lipid vesicles (87:8:5 mol %) is more than 7-fold higher than bulk lipid binding constant for PE:PC vesicles (unpublished data Selvy and Brown). Optimal $PI(4,5)P_2$ mol %, 5-8 %, in a phospholipid vesicle enhances stimulation by regulatory proteins including Arf GTPase [74] (pg.). Some reports of *in vitro* lipase activity can be measured for full length PLD in the absence of $PI(4,5)P_2$ with the addition of molar concentrations of ammonium sulfate (optimal activity at 1-1.6 M) [212], [213].

Other reports of modulatory phospholipids are scattered in early PLD literature, but have not been followed up on. An intriguing observation by

Nakayama *et al.* suggested that PE, including dioleoyl and plasmalogen-rich species but not dipalmitoyl-containing species, enhances PC hydrolytic activity of PLD isolated from bovine kidney [212]. Another report suggested that that PI, LPI, and LPS, but not PS, negatively impact PLD activity [214]. It is unknown whether these effects are direct or indirect and whether they were specific to the *in vitro* assay format.

Regulatory proteins

With increased ease of recombinant PLD expression and measurement of *in vitro* PLD activity, a growing number of proteins have been reported to modulate PLD activity. Some of these proteins have been shown to directly modulate mammalian PLD activity through a protein-protein interaction; those are described here, whereas others may indirectly regulate PLD and participate in PLD signaling pathways, these proteins are mentioned in Table 5.

Small GTPases

Small GTPases were the first proteins demonstrated to directly modulate PLD activity through allosterically binding PLD. These enzymes are conformationally-activated upon binding GTP in place of constitutively-bound GDP, sometimes with the aid of guanine exchange factors (GEF) proteins, in response to signal transduction. GTPase activating proteins (GAPs) functionally inactivate the GTPases through facilitating intrinsic GTP hydrolysis. Subfamily

members of the Ras GTPase superfamily, including Arf [77], [215], and Rho family of GTPases [201], [216], [217] stimulate PLD activity *in vitro*.

Arf GTPases, including Arf1 and Arf6, stimulate PLD activity [77]. These were the first proteins demonstrated to activate mammalian PLD in an *in vitro* reconstitution system. Early *in vitro* characterization of PLD1 and PLD2 suggested that PLD1 alone was stimulated by Arf [171]. Subsequent studies have shown that PLD2, while not activated to the same extent, can be stimulated 2-fold over the already high basal activity with GTPγS-activated Arf [172], [218]. Henage *et al.* demonstrated that Arf1 increases total maximal activity (k_{cat}) in a concentration dependent manner. At 150 nM Arf1, PLD1 activity increased 4 to 6-fold over basal levels [74]. Arf stimulation is strongly dependent on the $PI(4,5)P_2$ mol %. This has lead some to speculate that Arf may indirectly activate PLD by rearranging the phospholipid head groups at the interface in a $PI(4,5)P_2$ dependent fashion [219]. This may be true, but we have recently demonstrated that Arf activates PLD in the absence of $PI(4,5)P_2$ [220], [221] (unpublished data Selvy and Brown), suggesting possibly a second mechanism of activation for Arf. Intriguingly, synergistic stimulation of PLD1 activity is observed when Arf is combined with PKCα or Rho family GTPases [74]. This demonstrates that Arf acts in concert with other modulatory enzymes to titrate the PLD response, and this finding could be of immense consequence *in vivo*. Some groups have attempted to identify the precise PLD binding site for Arf, [222], but to this date the site has not been unambiguously determined. *In vitro*, Arf activates *N*-

Table 5. Mammalian PLD regulatory proteins (table from [19])			
CLASS	**ACTIVATOR**	**PLD ISOFORM**	**CONSEQUENCE**
small GTPase	Arf	PLD1, PLD2	activate (k_{cat})
	RhoA	PLD1	activate (K_m)
	Rac1	PLD1	activate (K_m)
	Rac2	PLD2	activate
	Cdc42	PLD1	activate (K_m)
Kinase	PKC	PLD1 (PLD2)	activate (k_{cat} & K_m)
	Src	PLD2	phosphorylate
Other	Gβγ		inhibit
	Grb2	PLD2	activate
	F-actin		activate
	G-actin		inhibit
	Amphyphysin II		inhibit
	AP3/AP180		inhibit

terminally truncated PLD1 [74] and PLD2 [218], therefore the site likely exists somewhere in the catalytic domain. Arf is myristoylated at its amino-terminus. Arf-activation of PLD does not require this lipid modification, but stimulation is enhanced with *N*-myristolated Arf. In fact, the *N*-terminus Arf is the specific region implicated in PLD interactions [223].

The Rho family of GTPases, including RhoA, Cdc42, Rac1 and Rac2 directly activate mammalian PLD. Rho, Cdc42, and Rac1 are binding activators of PLD1, and stimulate substrate binding affinity ($1/K_m$) [74]. Arf and Rho family GTPases synergize to significantly increase PLD1 activity beyond an additive response. PLD1 does not have a putative CRIB (Cdc42 and Rac-interactive binding) motif, but using truncation deletions, the Rho family-PLD1 binding site was mapped to a region in conserved domain IV in the carboxy-terminus of PLD1 [224]. In a GTP-dependent mechanism, the Rho family GTPases bind PLD through the switch I region [225]. However, binding occurs independently from activation. Geranylgeranylation of Cdc42 is not required for PLD binding, but is required for PLD activation [225]. Cdc42 activation of PLD1 is mediated through the Rho-insert region, an alpha helix conserved in all Rho GTPases. However, this insert is not necessary for RhoA or Rac activation of PLD1 [226]. Rho, Cdc42, and Rac1 selectively activate PLD1. However, a recent report suggests that Rac2 may activate PLD2 via previously uncharacterized mechanism [227]. This report identifies two poorly conserved CRIB motifs (CRIB1 aa 255-270, and CRIB2 aa 306-326) in or near the PH domain of PLD2. Rac2 co-

immunoprecipitates with PLD2, and mutation within these regions disrupts this interaction [227].

Two other Ras GTPases have been proposed to directly modulate PLD. RalA, a Ras-like GTPase implicated in cancer cell transformation, co-immunoprecipitated with PLD1 but not PLD2 [228]. In another study, identification of the RalA binding site on PLD1 was attempted [222]. This study suggested that RalA binds at a site independent of Arf, allowing Arf and RalA to synergistically activate PLD1 [222]. In *vitro*, RalA enhanced PLD1 activity in a GTP-dependent mechanism [222], [228]. Rheb, a member of the Ras GTPase family, has also been reported to directly activate PLD1 *in vitro* [229].

Kinases

As mentioned above, PLD is phosphorylated post-translationally as a regulatory mechanism. Therefore, it is not surprising that kinases directly interact with PLD to regulate activity. Protein kinase C (PKC) isoforms are the most well studied kinases that directly interact with PLD. Classic PKC isoforms α, β, and γ are stimulated by calcium and DAG, and are therefore responsive to PMA-stimulation. In cells, these classic isoforms stimulate PLD1 and PLD2 activity downstream of PLC activation. PKCα phosphorylates PLD1 [202] and PLD2 [230] at serine and threonine residues, but activation is not phosphorylation-dependent. In timecourse studies, PMA-induced PLD activity occurs immediately, and phosphorylation only occurs later with a concomitant decrease in lipase activity, suggesting phosphorylation decreases PLD activity [203], [230]. The

PKC binding domain was mapped to the amino-terminus of PLD1 [231], however, PKC is able to activate *N*-terminally truncated PLD1 in a phosphorylation-independent mechanism [74]. PKC modulates PLD activity in a bimodal fashion. PKC enhances k_{cat} as well as substrate binding (K_m), and therefore synergistically activates PLD1 in combination with catalytic activator Arf GTPase [74]. However, amino-terminally truncated PLD1 constructs only show enhanced K_m in response to PKC.

Other regulatory proteins

Numerous proteins have been reported to modulate PLD activity in response to signaling pathway activation, and a number of them have been demonstrated to do so directly. PED/PEA-15 (phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes) is overexpressed in many tissues in type II diabetes patients. This protein directly binds CR IV of PLD and enhances PKC-activation of PLD [232]. This interaction impairs insulin regulation of the glucose transporter and insulin secretion, whereas competing for the PED/PEA-15 protein interaction with expression of the PLD1 CRIV domain restores insulin secretion [232]. This interaction is suggested by the authors to be a novel therapeutic target for type II diabetes. Grb2 is another protein that positively regulates PLD activity. Grb2 serves as a scaffolding protein to recruit signaling proteins including Sos, the Ras GEF, to the plasma membrane. The Grb2 SH2 domain binds PLD2 through phospho-tyrosine residues [204]. The

SH3 domains that flank the SH2 domain have been suggested to stimulate PLD activity.

Direct protein interactions that curb lipase activity have also been described. The heterotrimeric Gβγ subunits, dissociated from the Gα subunit upon GPCR stimulation, directly interact with the catalytic domain of PLD1 and PLD2 to inhibit activity [233]. PLD has been implicated in synaptic vesicle trafficking. Two synaptic vesicle-associated proteins, amphiphysin I and AP3 (also called AP180) directly bind PLD and inhibit lipase activity. Amphiphysin I heterodimerizes with Amphiphysin II in order to associate with clathrin coated vesicles. The *N*-terminus of Amphiphysin I directly binds PLD1 and PLD2 with affinities of roughly 15 nM, inhibiting catalytic activity. Assembly protein 3 (AP3, also called AP180) binds clathrin-coated vesicles and the *C*-terminus of PLD1 to inhibit lipase activity.

Cytoskeletal components directly modulate PLD activity. Monomeric G actin inhibits PLD activity. Conversely, PLD activity triggers actin polymerization, and polymerized F-actin stimulates PLD activity. This divergent signaling mechanism may enhance cytoskeletal reorganization in localized subdomains of the cell. PLD2 has also been shown to directly bind microtubules, again suggesting that these interactions sequester the protein as a means of ensuring phospholipase activity is limited to the correct locations within the cell. Other proteins originally thought to directly interact with PLD and inhibit activity include α-synuclein, which has subsequently been shown to not inhibit PLD activity *in vitro* or in cells overexpressing this protein [234].

Recombinant protein expression and purification

A limiting factor in studying the biochemical and structural character of mammalian PLD enzymes is that, to date, the enzymes have proven tremendously difficult to express and purify recombinantly. In contrast to plant and fungal enzymes, which are readily expressed and isolated from bacterial expression systems, mammalian PLD enzymes have not, to date, been expressed as catalytically active proteins in prokaryotic expression systems. Even plant and yeast enzymes with highly conserved regulatory and catalytic domains, such as PLDζ and Spo14, are catalytically active when expressed and purified from bacteria, whereas catalytically competent mammalian PLD enzymes have not been expressed or isolated from bacteria [235]. In *Escherichia coli*, mammalian PLD protein is highly proteolyzed and localizes to inclusion bodies, where insoluble, unfolded, aggregate protein is collected. Attempts to purify and refold mammalian PLD from inclusion bodies have not been reported.

However, there are multiple instances of recombinant mammalian PLD expression in eukaryotic systems, including insect cells, *Spodoptera frugiperda* [78], [111], yeast, and *Schizosaccharomyces pombe* [236]. Catalytically active mammalian PLD1 and PLD2 can be expressed and partially purified from these eukaryotic recombinant systems. Since post-translational modifications, including lipid modification and phosphorylation, are not necessary for catalysis and refolding from inclusion bodies has never been successful, this suggests eukaryotic protein chaperones may be integral for proper folding of mammalian PLD enzymes. Intriguing studies from John Exton's group support this by

demonstrating that the amino and carboxy terminal domains can be expressed on separate plasmids and co-purified as catalytically active complex [189]. However, mixing of amino and carboxy termini that were expressed and purified in isolation does not yield catalytically active protein.

When expressed in insect cells (monolayer cultures of *Sf*21 or *Sf*9 cells), the bulk of mammalian PLD1 protein is soluble or loosely membrane-associate and is easily extracted with mid ionic strength buffers and can be purified in the absence of detergent. Mammalian PLD2, however, is mostly membrane-associated, and efficient protein extraction requires high salt and detergent. Throughout purification, this enzyme is not stable without detergent, which can be used at concentrations below the critical micelle concentration (cmc).

Purification of mammalian PLD1 and PLD2 using classic chromatographic methods, such as ion exchange, heparin, and size-exclusion, yields partially pure fractions. Purity is further enhanced when mammalian PLD is expressed with affinity-tags, the best results are obtained through the use of multiple tandem affinity purification steps coupled with classic chromatographic methods. However, placement of the affinity tag at the amino-terminus is critical. Modification to the carboxy terminus significantly decreases catalytic activity, as would be expected based on PLD2 splice variants with truncated carboxy termini that yield proteins with 8-12 % of the activity of full length PLD2 enzyme [173].

Despite the increased purity afforded by tandem affinity tags, mammalian PLD, particularly PLD1, is poorly expressed in insect cells. Low expression levels may be due to the fact that expression of catalytically active PLD enzymes is

deleterious to insect cell viability. Supporting this is evidence that expression is significantly increased for catalytically-inactive mutants or amino-terminally truncated constructs that do not exhibit proper localization or catalytic activity in cells. Recent studies demonstrate that truncation of the amino terminus of PLD1 coupled with use of a large affinity tag (bacterial maltose binding protein, commonly used to enhance solubility of recombinant proteins) significantly increase expression and enable one-step affinity purification of homogenous PLD [74], [78].

Signaling pathways

More than 15 years after the cloning of the first mammalian PLD, this enzyme, its activity, and products continue to be implicated in a wide range of signaling pathways and cellular functions. These pathways include receptor-mediated responses, growth and survival pathways, and vesicular trafficking. PLD-mediated cytoskeletal reorganization in response to chemoattractants, and pathogenic infection are critical immunologic functions. Only recently have potent and isoenzyme selective small molecule inhibitors of mammalian PLD isoforms become available. Many studies continue to utilize primary alcohols to implicate PLD in different signaling pathways. In the presence of low concentrations (<3 %) of primary alcohol, mammalian PLD will perform transphosphatidylation and generate a metabolically-stable phosphatidylalcohol instead of phosphatidic acid. Discrepancies are now emerging between functions of PLD previously reported using alcohols, and those demonstrated using RNAi knockdown, small molecule

inhibitors, or those observed in knockout animals [237]. Signaling roles for PLD mentioned here include those determined using primary alcohols as well as knockdown or pharmacological inhibition. However, further characterization of PLD activity using these newer methods is necessary to clarify and validate previously-defined roles of mammalian PLD.

Receptor-mediated signaling

Extracellular stimuli trigger intracellular responses via cell receptors present at the plasma membrane. These include GPCRs, receptor tyrosine kinases (RTKs), and integrins, all of which mediate signaling through PLD activation. The specific mechanisms for receptor-mediated PLD activation differ between cell types, but the canonical pathways are described here.

GPCR signaling

G protein-coupled receptors (GPCR) trigger dissociation of Gα and Gβγ heterotrimeric G proteins upon agonist stimulation. Uncoupled heterotrimer subunits elicit signaling cascades through downstream effector proteins. Many of these pathways elicit functional responses through signaling to PLD in multiple ways (figure 8). In the canonical pathway, upon agonist stimulation, GTP-$G\alpha_q$ stimulates PLCβ hydrolysis of $PI(4,5)P_2$, producing DAG and IP_3 (see excellent reviews on PLC subtype activation [238], for review on G_q family [239], and [240]. IP_3 triggers calcium release from the ER, and this coupled with DAG synergistically activates PKCα, which in turn bimodally activates PLD. Litosch

and colleagues recently showed that this PLCβ signaling is potentiated by PLD-produced PA [241], [242]. Dissociated Gβγ also activates PLCβ, to indirectly activate PLD in a PKC-dependent manner. Additionally, Preninger *et al.* demonstrated that the Gβγ subunit of the heterotrimer can directly inhibit PLD activity via interactions through the PLD catalytic domain [233]. Gβγ interaction disrupts both basal and Arf-stimulated activity [233], [243]. As illustrated in Figure 8, levels of PLD activation are intricately titrated in response to specific agonist-mediated or intracellular circumstances.

The $G_{12/13}$ class of heterotrimers activates PLD in a small GTPase dependent manner. $G\alpha_{12}$ activates RhoA via Pyk2, a focal adhesion tyrosine kinase, which directly stimulates PLD1 activity. As shown in Figure 8C, $G\alpha_{13}$ activates the γ subtype of PI3K to generate PIP_3. Upon PIP_3 binding, ARNO and Rho GEF trigger GDP for GTP exchange on Arf and RhoA, respectively [244]. These activated small GTPases then directly activate PLD. G_q and G_{12} also stimulate Src, which tyrosine-phosphorylates both PLD at the PH domain, and the receptor tyrosine kinase, EGFR (Figure 9). PLD phosphorylation does not affect cellular phospholipase activity, but the direct interaction does enhance Src kinase activity [200]. EGFR phosphorylation results in homodimerization, autophosphorylation, and GPCR-EGFR transactivation in the absence of EGFR agonist [245], [246].

Roles for PLD in pathogenic response have been reported, many of which are GPCR-mediated and result in changes in reactive oxygen species formation, vesicular trafficking or transcription. In leukocytes, PLD1 expression is induced in

response to pathogenic and pro-inflammatory stimuli through activation of membrane receptors including the G_i-coupled f-Met-Leu-Phe receptor (fMLPR). PLD activity in macrophages and neutrophils is implicated in respiratory burst [247], engulfment of bacteria, and reorganization of cytoskeletal elements. Recently, PLD was shown to be involved in HIV replication via CCR5, an MIP-1 chemokine receptor that interacts with an HIV glycoprotein [248], In response to CCR5 agonist stimulation, PLD is activated in an ERK1/2-dependent manner to activate transcription factors, including NFκB, that facilitate replication of the latent HIV genome integrated into the host genome.

PLD is a major source of PA generated by cell surface receptor-mediated signaling pathways. Its primary substrate in mammalian cells is PC, but consistent with its catalytic mechanism it can also utilize other amine containing glycerophospholipids as substrates (e.g., PE and PS). The molecular species of PA generated by PLD are predominantly mono- and di-unsaturated species, particularly 16:0/18:1 containing fatty acyl species. Work from Michael Wakelam's laboratory provided an insightful comparison of DAG and PA species generated from PLC and PLD sources, respectively [249], [250]. The authors reported differences in cellular targets modulated by these distinct signaling pathways, such as the lack of PKC activation by molecular species of DAGs generated downstream of PLD. Activated in parallel by many of the same cell surface receptors, PLC isoenzymes generate two second messengers from the hydrolysis of $PI(4,5)P_2$, namely DAG and IP_3. The DAG generated via the PLC

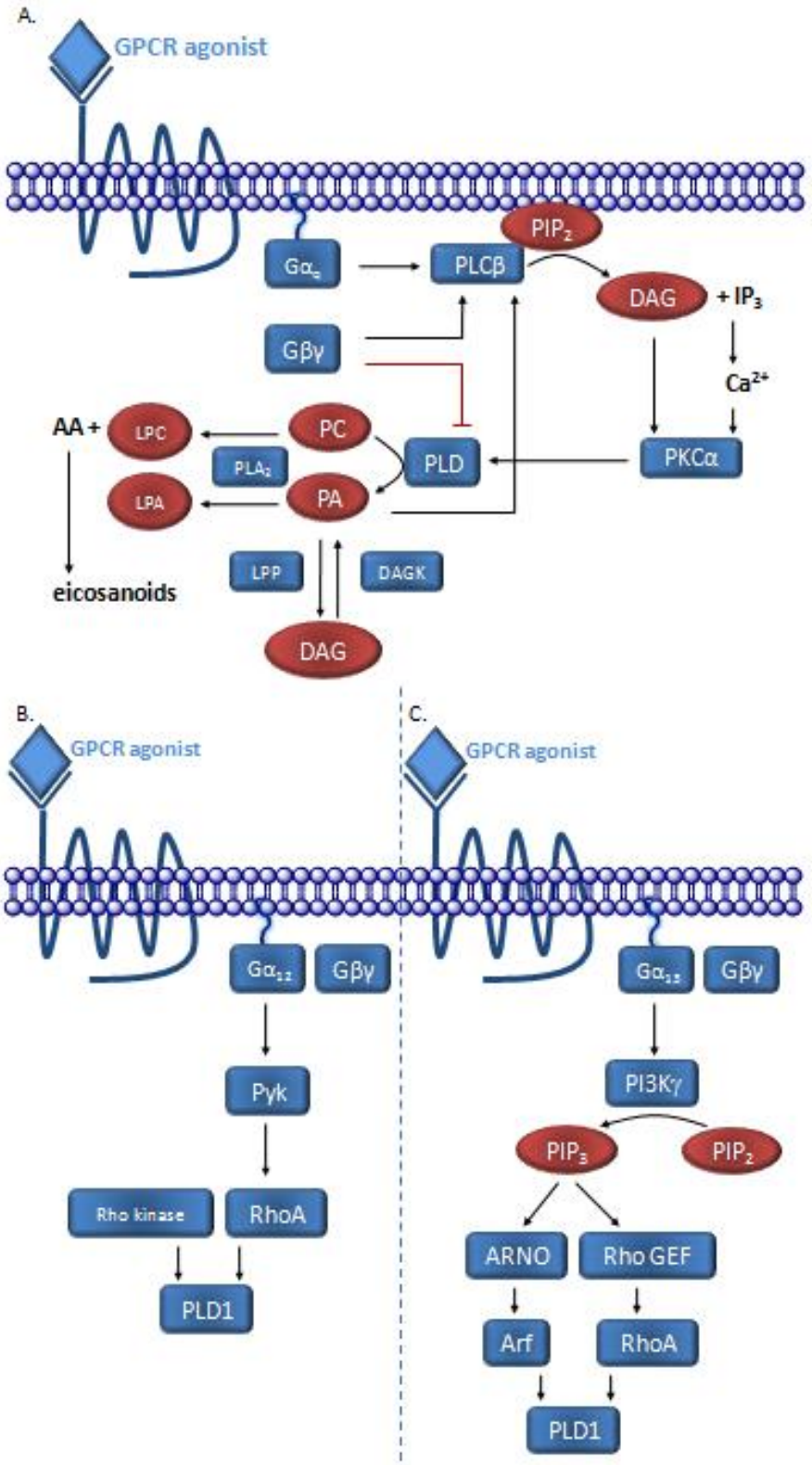


Figure 8. G protein coupled receptor activation of PLD through $G\alpha_q$, and protein kinase C (panel A), $G\alpha_{12}$ and RhoA (panel B), and $G\alpha_{13}$ and Arf (figure from [19]).

pathway is typically polyunsaturated (e.g., 38:4 DAG) reflecting the major species of the PIP_2 substrate available in mammalian cells [251]. The polyunsaturated DAG generated from PLC provides a second and distinct signaling source of cellular PA via the transfer of a phosphate from ATP to DAG through the action of a DAG kinase. An excellent review of DAG kinase isoenyzmes types and regulation was recently published [252]. Different isoenzymes of DAG kinases have distinct substrate specificities. Recent advances in electrospray ionization mass spectrometry have identified a surprising diversity of DAG molecular species that can now be resolved and quantitated using a linear regression algorithm [253]. This type of analysis has revealed that DAK kinase isoenzymes have extremely diverse functionalities and substrate preferences leading to differences in the array and relative concentrations of acyl species of DAGs in cells following perturbations, such as overexpression or genetic knockouts [254], [255]. For example, the DAG kinase epsilon shows the ability to select acyl chains on both the *sn*-1 and *sn*-2 positions of the glycerol backbone of the DAG substrate as well as on its product, PA, which modulates a feedback inhibition of this isoenzyme [256]. This PLC-DAG kinase pathway provides a distinct phase of PA that appears later in the temporal sequence of receptor-mediated PA generation. By contrast the PA molecular species generated by PLD appear rapidly after receptor activation, but are also rapidly metabolized into DAG via the actions of lipid phosphatases. The ultimate metabolic fates and functional distinctions of these two sources of signaling PA species are not as yet fully defined, but recent development of new types of lipid probes that utilize alkyne-

cobalt chemistry [81] provides opportunities to track and identify lipid metabolites even after multiple biotransformations. This will facilitate identification of distant metabolites and allow the functional consequences of different sources of PA production to be unambiguously determined.

Canonical RTK signaling via EGFR

The EGFR, is highly conserved in eukaryotic organisms, and is a representative member of the ERBb family of growth factor receptors with intrinsic tyrosine kinase activity. EGFR activates downstream signaling pathways including those responsible for growth, survival, and cytoskeletal reorganization. Aberrant EGFR signaling has been implicated in tumorigenesis.

Upon GPCR transactivation or binding epidermal growth factor (EGF), EGFR homodimerizes and tyrosine phosphorylates the adjacent receptor in the cytosolic region to generate an active receptor complex (activation mechanism reviewed [257]). These phosphotyrosine residues serve as docking sites for downstream effector proteins, including PLCγ1, Grb2, and PI3K. Even prior to cloning the mammalian PLD isoforms, PLD activity was shown to be activated by EGFR stimulation. Critical characterization of the multiple, and sometimes overlapping, mechanisms in which EGFR signaling activates PLD activity has been performed. For simplification, these are illustrated and described in separate schematics.

PLD2 can be localized to EGFR via its PX domain. In Figure 9A, the PX domain of PLD2 binds the SH3 domain of PLCγ1, which directly localizes to the

EGFR. PLCγ1 hydrolyzes $PI(4,5)P_2$ to generate DAG ad IP_3. Similar to GPCR-activation of PLCβ, PLC-derived products induce PKCα activation of PLD.

Changes in actin polymerization can occur in response to GPCR or RTK signaling. PLD has been shown to directly bind actin, resulting in mutual regulatory interactions.

In a separate mechanism of PLD activation PLD2 directly interacts with the EGFR. At the receptor, phosphotyrosine residues in the PLD2 PH domain bind the Grb2 SH2 domain [258]. This interaction enhances phospholipase activity, via Grb2 SH3 interaction, to generate PA. Recently Zhao *et al.*, demonstrated that the Ras GEF, Sos localizes the PLD2-produced PA, where it is activated by Grb2 [259]. Subsequent Ras activation elicits a host of signaling cascades. Ras activates PI3K, which generates PIP_3 and induces Akt translocation and activation. Ral GEF is also a Ras effector protein, which results in GTP-Ral activation of PLD [228], [260]. Finally, Ras activates Raf, which localizes to the plasma membrane via PLD-produced PA interactions. Ras signaling through Raf triggers activation of the MAPK pathway and via NFκB, subsequently upregulates transcription of genes involved in survival, proliferation, and differentiation.

Somewhat more controversial is the role of PLD in EGFR-stimulated mTOR signaling (reviewed [261], [262]) illustrated in Figure 9C. Several reports suggest PLD generated PA competes for rapamycin and FKBP binding in the FRB domain of mTOR [5]' [263]. These studies were performed using primary alcohols to show mTORC1 kinase activity was significantly decreased upon

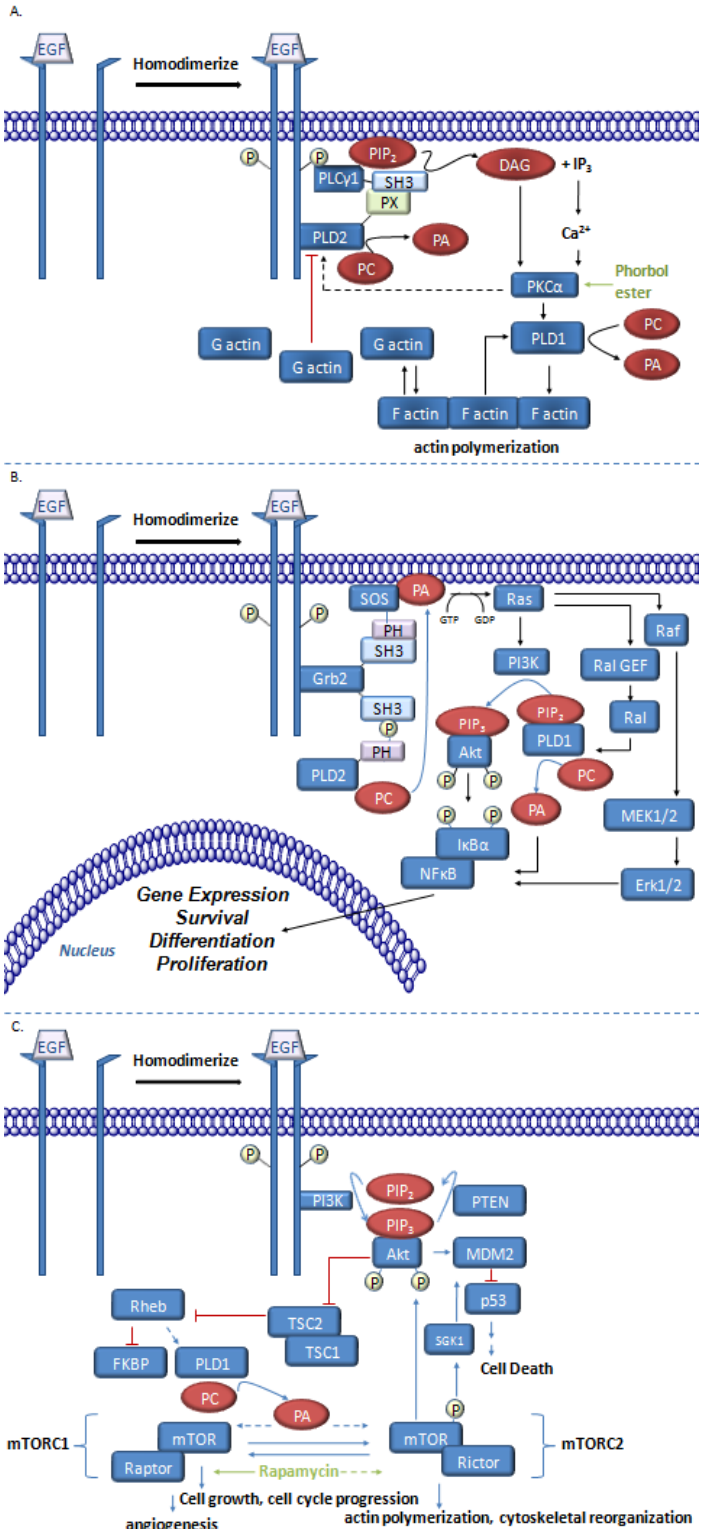


Figure 9. Activation of PLD through epidermal growth factor receptor (EGFR), a canonical receptor tyrosine kinase. Activated EGFR and PLCγ regulate PLD (panel A); EGFR activation of Grb2 and Sos induces PLD activation (panel B); and EGFR activation of PI3K regulates PLD and mTOR signaling (panel C) (figure from [19]).

diverting PLD activity to generation of transphosphatidylation product. A follow up study used NMR to map the PA binding site within the FRB domain [264]. The small GTPase Rheb was recently suggested to stimulate PLD1 as a feed forward mechanism of mTORC1 activation [229]. Again these studies relied heavily on the use of primary alcohols, RNAi knockdowns, and a somewhat incomplete biochemical analysis. Subsequent use of PLD-selective small molecule inhibitors (chapter II) and genetic knockouts may illuminate that the role of PLD in mTOR regulation is considerably more complex with both feedforward and feedback modulation.

Integrin signaling

Integrins support cell adhesion as well as growth and survival by functioning as both an anchor to the extracellular matrix (ECM) as well as a signaling receptor. Although integrins do not possess intrinsic enzymatic activity, upon ligand binding, these receptors elicit similar signaling pathways to those of growth factor receptors by heterodimerizing and binding various effector proteins at their cytosolic face. Integrins heterodimers can signal independently or complexed with growth factor receptors to trigger chemotaxis, cell differentiation, proliferation, and survival (reviewed [265]). As in EGFR signaling pathways, PLD is activated downstream of integrin receptors via multiple mechanisms.

Focal adhesion kinase (FAK) directly binds the integrin receptor to induce Ras-mediated signaling and MAPK activation. Ras activates PI3K to generate

PIP_3. In response, the Rho GTPase, Rac, undergoes guanine nucleotide exchange thereby triggering PLD activation.[266] Canonically, PLD1 is directly stimulated by *C*-terminal interaction with Rac1. However, Pang *et al.* have shown that Rac2 directly interacts with PLD2 via CRIB domains in the PLD *N*-terminal regulatory domain [227]. *In vitro* the *C*-terminus of Rac selectively binds PA. In cells, PLD-produced PA triggers Rac translocation to membrane ruffles and lamellipodia [266]. Treatment with *n*-butanol results in cytosolic localization of GTP-bound Rac, supporting the role of PLD in Rac translocation. At regions of membrane protrusion and lamellipodiae formation, Rac facilitates cytoskeletal reorganization. PLD colocalizes at these membrane microdomains and induces actin polymerization.

Integrin signaling also mediates Arf activation of PLD. Integrin effector proteins elicit Arf GAP, ASAP, localization to the leading edge of migrating cells to attenuate Arf signaling (reviewed [267]) and perturb Arf-activation of PLD (reviewed [268]). This bimodal mechanism of small GTPase regulation titrates levels of phospholipase activity during integrin-mediated membrane ruffling, cell migration, and invasion.

Similar to the role of PLD in *Dictyostelium* migration, mammalian PLD isoforms have been implicated in chemotaxis. These enzymes, stimulated by Rho GTPases downstream of integrin, chemokine, and growth factor receptors, trigger cytoskeletal rearrangement and membrane ruffling. Primary butanol and PLD-selective inhibitors disrupt these pathways, suggesting PA formation as well as protein-protein interactions participate in these signaling responses.

As discussed above, PLD-produced PA has been suggested to directly activate mTOR and facilitate mTOR complex formation and signaling, including mTORC2 and subsequent Akt phosphorylation. Akt and mTORC2 signaling not only support pro-survival signaling via MDM2 stabilization, and BAD and Bcl-XI activation, but also induce cytoskeletal reorganization. mTORC2 induces actin polymerization and triggers myosin II assembly and cell migration via PAK and myosin phosphorylation. PLD activity also induces secretion of proteolytic matrix metalloproteases that degrade surrounding ECM to facilitate cellular movement.

Vesicular trafficking

Mammalian PLD enzymes differentially localize to cellular membranes to directly and indirectly induce changes in membrane curvature and fusion that facilitate endocytosis/exocytosis and vesicular trafficking. PLD1 primarily localizes to intracellular membranes including TGN and endosomal membranes and has constitutively low basal activity. Upon cell stimulation, PLD1 translocates to plasma membrane and is activate. PLD2 is generally constitutively localized to the plasma membrane and has high basal activity.

Arf GTPases activate the otherwise low basal activity of PLD1. Arf1 stimulates Golgi-localized PLD [269], while Arf6 stimulates PLD1 at the plasma membrane [270]. In an independent mechanism, Arf present at either membrane cooperates with Arf-stimulated PA to facilitate vesicles formation [271], [272]. In contrast to the Sec14 bypass mechanism in yeast, PA accumulation, rather than DAG, facilitates vesicle budding. This may be due to several PA-related

mechanisms. PA is a cone shaped lipid, and induces changes in membrane curvature. Arf and PA also trigger recruitment of coatomer proteins, including COPI [273], [274]. PA activates PI4P5K, which generates $PI(4,5)P_2$ and induces translocation of coatomer proteins and proteins involved in vesicle budding,including dynamin (a GTPase involved in endocytosis and membrane scission) and AP180 (a clathrin assembly protein) (Figure 10). Following recruitment, AP180 directly inhibits PLD activity [275], [276]. Recently, PLD was also reported to directly interact with dynamin. This interaction occurred in a GTP-dependent manner, and it was suggested that the PX domain of PLD2 might serve as a GAP for dynamin [277].

PLD and PA-dependent mechanisms function in vesicle formation to facilitate receptor internalization and recycling (Figure 10), SNARE-mediated synaptic vesicle fusion (similar to that observed in Spo14-mediated prospore membrane formation), and exocytic mechanisms including respiratory burst [278] and degranulation [279].

PLD inhibitors

A large body of knowledge of the role of PLD in signaling pathways has been determined using small molecules as tools. A wide range of chemically diverse small molecules have been reported to be PLD inhibitors. In the 1960's short-chain primary alcohols (e.g. n-butanol) were identified as compounds that could modulate PLD product formation [61], whereas secondary and tertiary alcohols do not. Primary alcohols function as a preferred nucleophile to water in

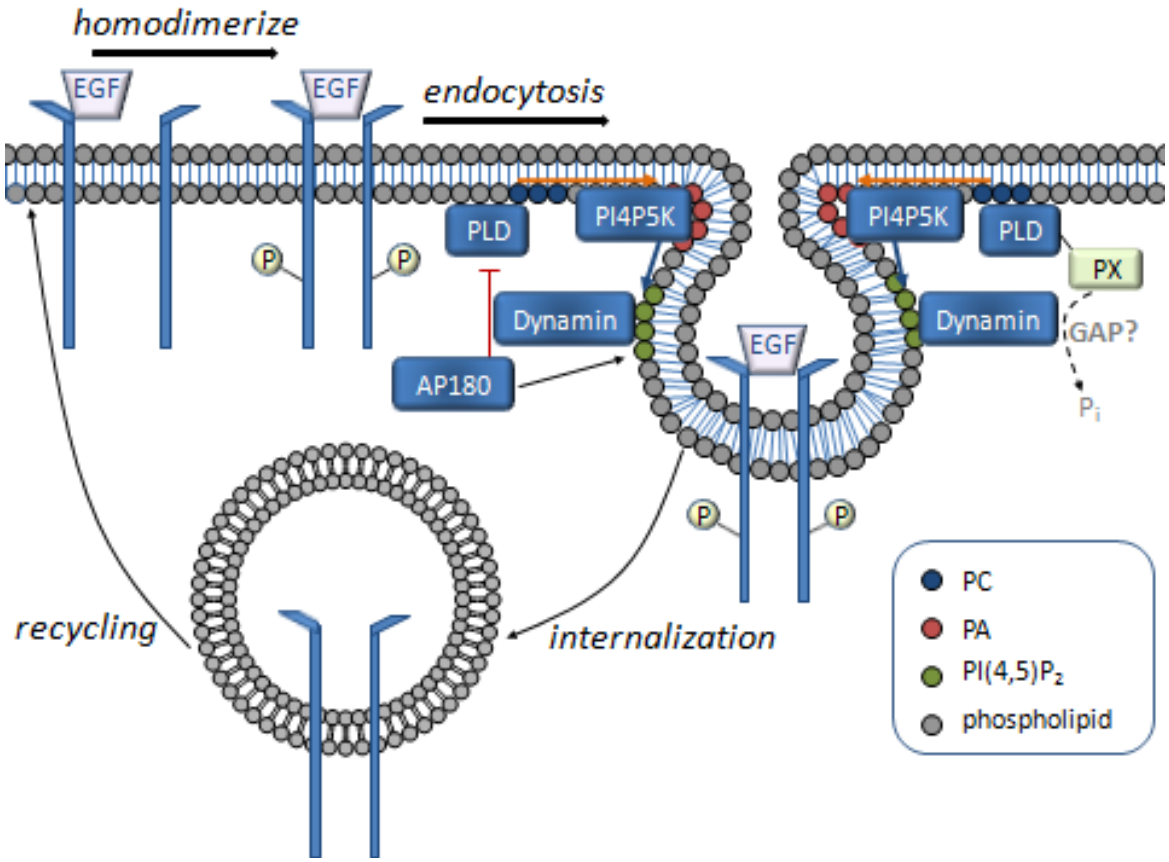


Figure 10. The role of PLD in endocytosis and receptor internalization (figure from [19]).

the second phase of the reaction mechanism (figure 5), resulting in formation of the transphosphatidylation product phosphatidylalcohol. Therefore these compounds do not inhibit PLD activity, a common misnomer in the literature. Instead, PA product formation is simply diverted to a more metabolically stable transphosphatidylation product. These characteristics make primary alcohols an attractive tool in cell signaling studies. As such it has historically been the most commonly utilized small molecule for studying PLD signaling. However, recent studies have suggested that primary alcohols may not effectively divert all phosphatidic acid formation, and that phosphatdidylalcohols such as phosphatidylbutanol may not be as physiologically inert as once predicted [269]. Also, some of the signaling capacities attributed to PLD through studies using alcohols do not hold true when RNAi and small molecule inhibitors are employed [237]. This suggests primary alcohols may have an off target effect on certain signaling pathways, as has been demonstrated in plants. The need to validate the role of PLD in signaling pathways and evidence that PLD may serve as a novel therapeutic target has been the impetus for the recent development of small molecule inhibitors for mammalian PLD (Chapter II).

Indirect PLD inhibitors

Historically, two classes of PLD inhibitors have been described- those that act indirectly or directly on the enzyme. Some groups have identified compounds that indirectly inhibit PLD activity. These compounds, highlighted in Figure 11

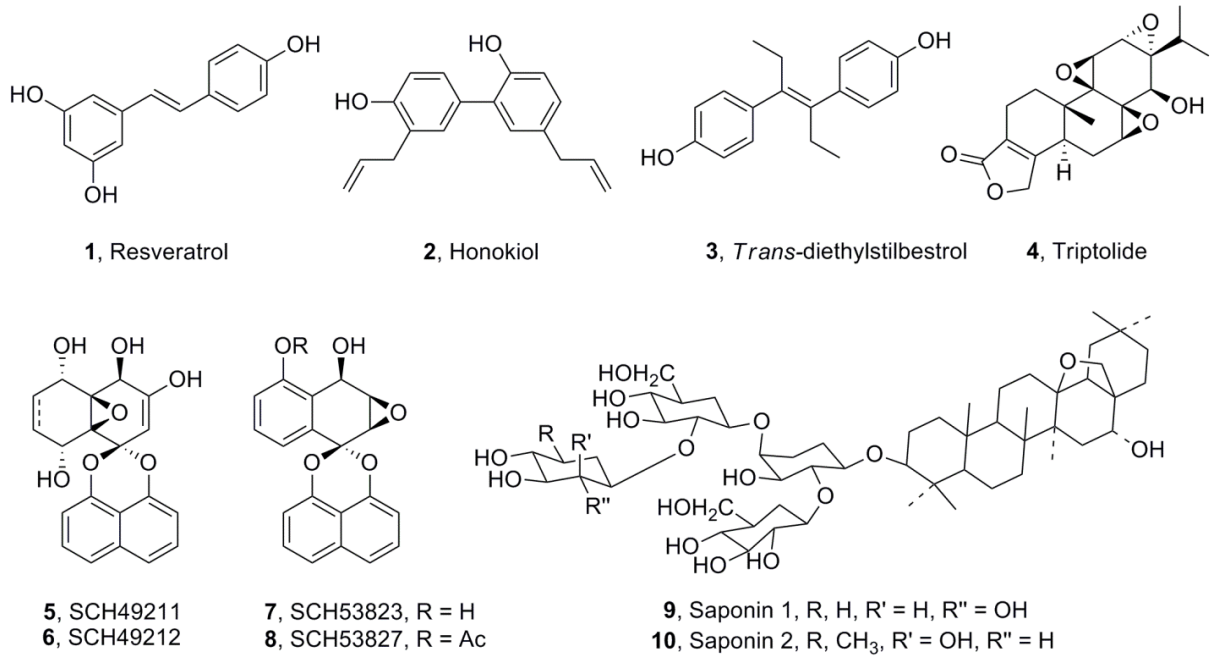


Figure 11. Reported indirect PLD inhibitors 1-10.

include natural compounds such as resveratrol(1), honokiol (2), and triptolide. Members of this class of compounds inhibit PLD activity in cells, but have not been demonstrated to inhibit PLD activity in the biochemical liposome reconstitution assay. Resveratrol, isolated from the skin of red grapes, yields an anti-inflammatory effect by indirectly inhibiting sphingosine kinase and PLD translocation and activity [280]. Honokiol, the natural anti-microbial compound derived from seeds of magnolia, inhibits Ras activation and thereby blocks Ras-dependent PLD acivity [281]. The natural anti-inflammatory compound, triptolide suppresses PLD1 and PLD2 protein expression by blocking NFκB activation [282].

Direct PLD inhibitors

Compounds that directly inhibit PLD activity, as demonstrated using purified recombinant protein in the biochemical liposome reconstitution assay, have also been identified (Figure 12) but many are not selective to mammalian PLD and hence are poor tools for studying cellular and *in vivo* functions of the enzyme. Such compounds include presqualene diphosphate (17) which was demonstrated to inhibit both mammalian PLD1b and scPLD (a non-HKD enzyme) in the biochemical reconstitution assay, but the mechanism was not determined [283]. Certain compounds in the selective estrogen receptor modulator (SERM) class (e.g. raloxifene (19) and 4-OH tamoxifen (20), further detailed in chapter IV) directly inhibit mammalian PLD isoforms [284]. Catalytic site inhibitors were identified from crystal structure characterization of bacterial PLD enzymes, and

were later validated for mammalian PLD (Henage, Selvy, and Brown, unpublished data). These compounds include the phosphate mimetics tungstate (12) and vanadate (13), which are thought to compete for phospholipid headgroup binding at the orthosteric site, but there is some evidence that these compounds may bind at a second allosteric site as these compounds bind with a two-site binding model, disrupt interfacial binding, and displace a select class of phospholipids that co-purify with recombinant PLD (Chapter IV).

More recent reports of direct PLD inhibitors have centered around a class of compounds based on the chemical structure of the anti-psychotic halopemide. From this class, novel isoform-selective inhibitors have been identified (referred to here as VU-series compounds) and structure-activity relationship (SAR) rigoursly characterized (Chapter II). Subsequent studies have characterized the molecular mechanism of action for this class of compounds and demonstrate their specificity towards mammalian PLD (Chapter III). The VU-series compounds are significantly more potent than previously reported PLD inhibitors, have desireable pharmacokinetics, are brain penetrant, and do not present toxicity issues when tested in humans, which was performed in the early 1980's as a function of screening this class of compounds as an experimental antipsychotic. These factors suggest the VU series compounds might be a good starting point for developing inhibitors to screen for systemic implications of PLD inhibition in concordance with disease studies such as those for cancer, Alzheimer's, and thrombosis.

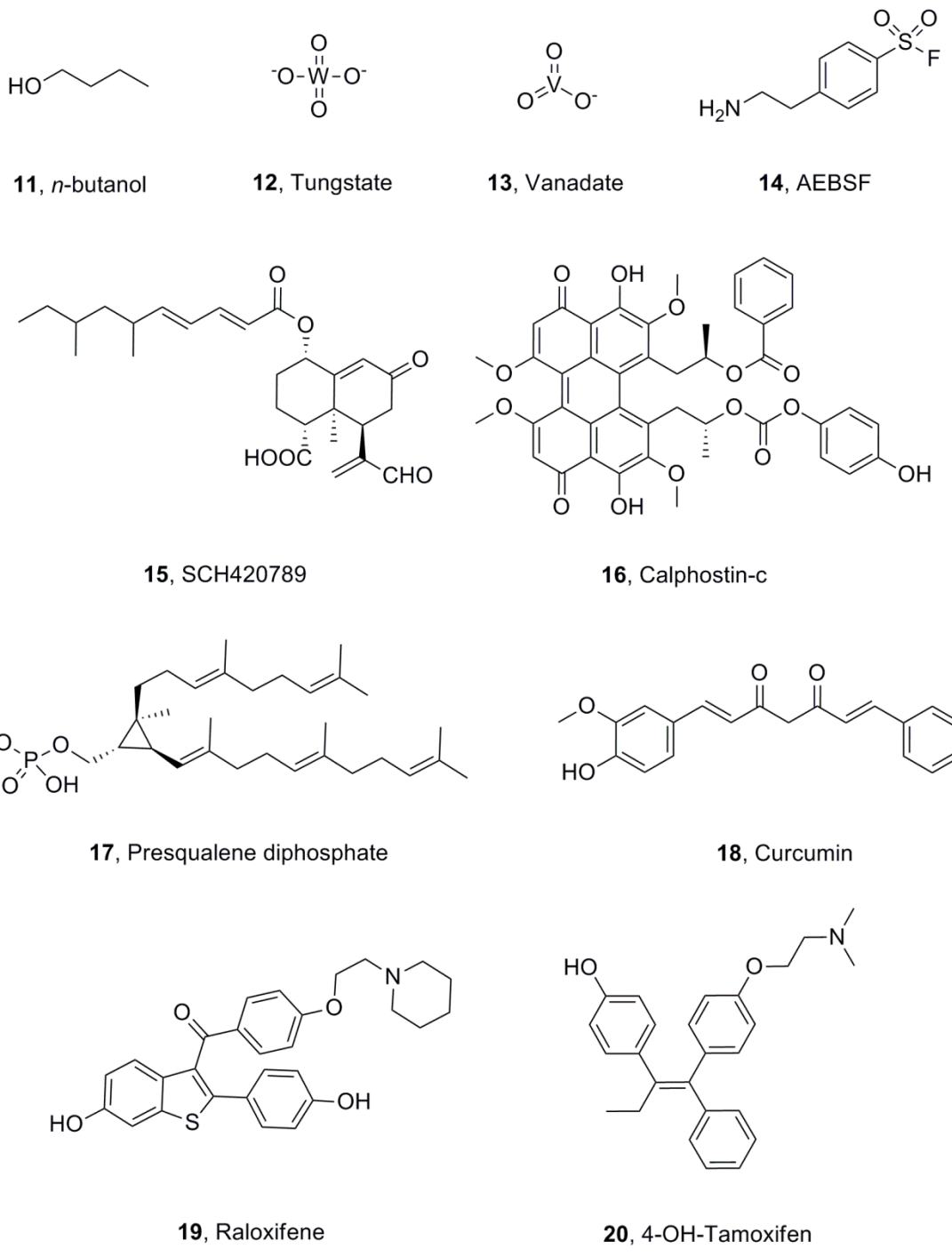


Figure 12. Reported direct PLD inhibitors 11-20.

PLD as a potential therapeutic target

Several groups have used a combination of RNAi and the newly available PLD1$^{-/-}$ or PLD2$^{-/-}$ knockout mice (for which no phenotype is observed in healthy mice) to validate a role for PLD in several disease states. Aberrant PLD activity has been implicated in several types of cancer, such as colon, breast, and glioma. Its role in oncogenesis is likely due to its ability to increase growth and proliferation, and to facilitate cancer cell invasion and metastases. Studies by Buchanan et al. demonstrated that overexpression of dominant negative PLD can impede the ability of rat fibroblasts transfected with H-Ras^{V12} to form tumors in soft agar or nude mice [285]. Exogenous supplementation of PLD product PA rescues the tumor-forming phenotype.

Dysregulated PLD activity has recently been validated as key player in Alzheimer's disease [7]. Oligomeric amyloid β increases PLD2 activity in cultures of neuronal cells. In the whole organism, increased PLD activity is observed in mice with a rodent model disease for Alzheimer's with a severe memory deficit phenotype. However, PLD2$^{-/-}$ mice with the same rodent model disease and the same amyloid β plaque load as a wildtype animals with the disease, do not present a memory deficit phenotype [286]. This suggests PLD2 might be a worthy therapeutic target for limiting the decreased memory phenotype in Alzheimer's patients.

PLD was also recently implicated in integrin-mediated thrombosis. Elvers, et al. demonstrated that PLD1$^{-/-}$ mice are protected from thrombotic formation

and ischemic brain injury without increasing bleeding time [237]. Chemically or mechanically induced arterial occlusion was significantly decreased in PLD1$^{-/-}$ mice. This provocative data suggests targeting PLD1 may protect against thrombotic disease without the compensatory increased risk of bleeding common for current therapies (e.g. aspirin and warfarin).

Chapter II

IDENTIFICATION AND DEVELOPMENT OF NOVEL ISOFORM-SELECTIVE PLD INHIBITORS

Recent studies using RNAi and PLD1$^{-/-}$ or PLD2$^{-/-}$ animals suggest both mammalian PLD isoforms are of potential value as therapeutic targets for a number of diseases. In conjunction with these whole animal studies, further interrogation of the role for PLD in critical stress-mediated signaling pathways is necessary. However, until recently the only small molecule that was both readily available and widely used as a validated tool for studying PLD signaling was primary alcohol. As mentioned in chapter I, although primary alcohols do divert product formation to a more slowly metabolized transphosphatidylation product that can also be monitored as a readout for PLD activity, recent studies have shown discrepancies between signaling roles of PLD elucidated using alcohol and other methods of blocking PLD-mediated PA formation (e.g. RNAi). Thus, it was necessary to identify and develop small molecule inhibitors of PLD that could readily be used in place of primary alcohol. The aim of these studies was to develop a class of compound that would not only be significantly more potent than previously-described inhibitors, but also lend isoform-selectivity for studying the roles of each isoform in isolation.

Halopemide

In 2007 a group from Novartis reported on a class of benzimidazolone-containing compounds that were identified in a highthroughput screen as novel PLD inhibitors [287]. This class, which includes halopemide an antipsychotic agent, was reported to inhibit PLD2 without mention of PLD1 or the type of screening assay used to determine this. In the late 1970's halopemide, a known antiemetic, was identified as a dopamine antagonist that also limits GABA uptake, but had micromolar potency and derivatives were ineffective *in vitro*, suggesting a metabolite was the active compound [288]. Human trials in the early 1980's identified halopemide as a non toxic effective anti-psychotic agent with psychic energizer properties that significantly benefitted withdrawn patients with schizophrenia and autism [289]. In the Novartis report, halopemide was identified as a low micromolar inhibitor of PLD2 (IC_{50}=1.5μM) [287], while in our own experience potency towards PLD1 was slightly greater (PLD1 IC_{50}=220nM versus PLD2 IC_{50}=310nM in biochemical assay). Follow up studies described nanomolar potencies for both isoforms for a halopemide derivative, coined FIPI (5-Fluoro-2-indolyl des-chlorohalopemide; PLD1 IC_{50}=25nM, PLD2 IC_{50}=25nM)[79]. Due to excellent potency and potential druggability of this class of compounds, halopemide was selected as the lead compound for our diversity-oriented medicinal chemistry project.

Development of novel isoform-selective compounds

In collaboration with Craig Lindsley's lab, an initial library of 263 compounds was generated to explore the chemical space and identify components that elicit isoform selectivity. Halopemide was divided into three structural elements (scaffold, linker, and eastern portion/ amide cap) that were synthetically varied in order to generate a diverse library of small molecules from which we could screen to identify isoform-selective PLD inhibitors.

This library of compounds was initially screened at a single micromolar concentration in the biochemical liposome assay to measure efficacy towards partially purified recombinant PLD1 and PLD2. A significant portion of the compounds were triaged in this first screen, and only compounds that demonstrated significant inhibition at the single concentration were then further characterized for isoform selectivity by generating concentration response curves for both PLD1 and PLD2. 30 compounds were secondarily screened in this manner, from which classes of compounds began to emerge (examples from each class shown in figure 13). Some small molecules inhibited both isoforms equivalently, which are referred to as dual isoform inhibitors. Other compounds elicited selectivity towards a single isoform and are referred to as PLD1- or PLD2-selective [290]. Striking chemical differences are apparent when comparing compounds from these three classes. Dual isoform and PLD1-selective compounds maintain the benzimidazolone scaffold present in halopemide (figure 14). Substitution of this scaffold to a triazaspirone engenders

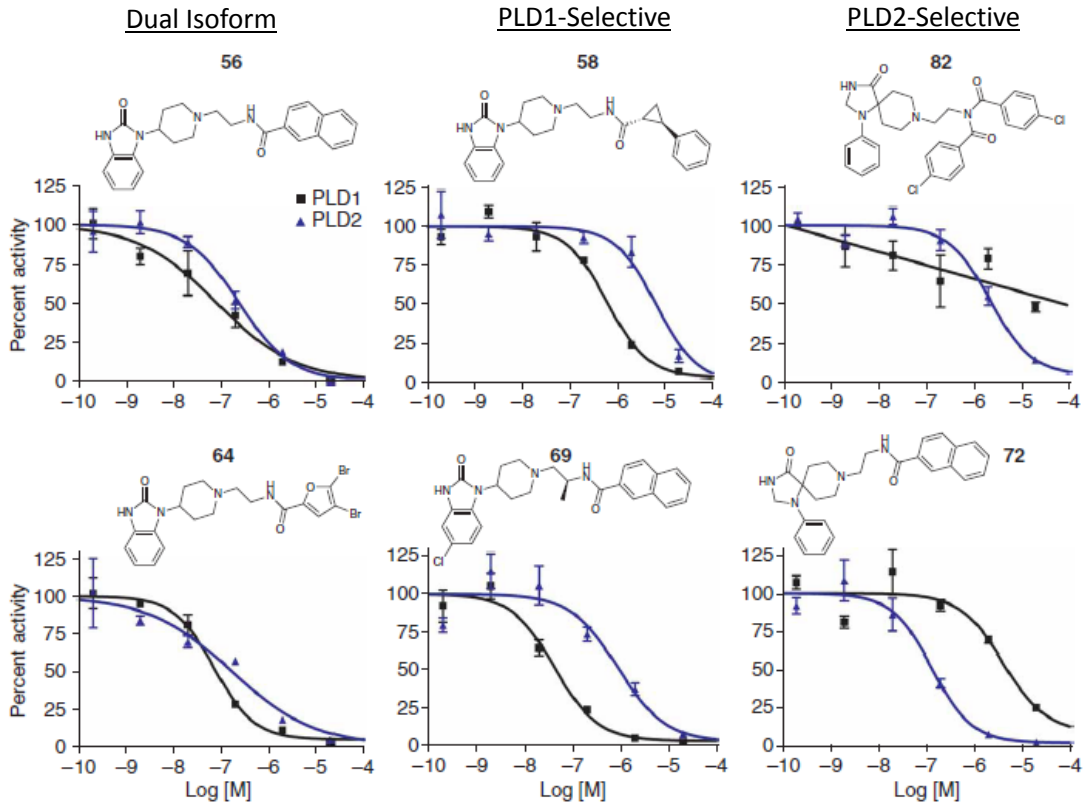
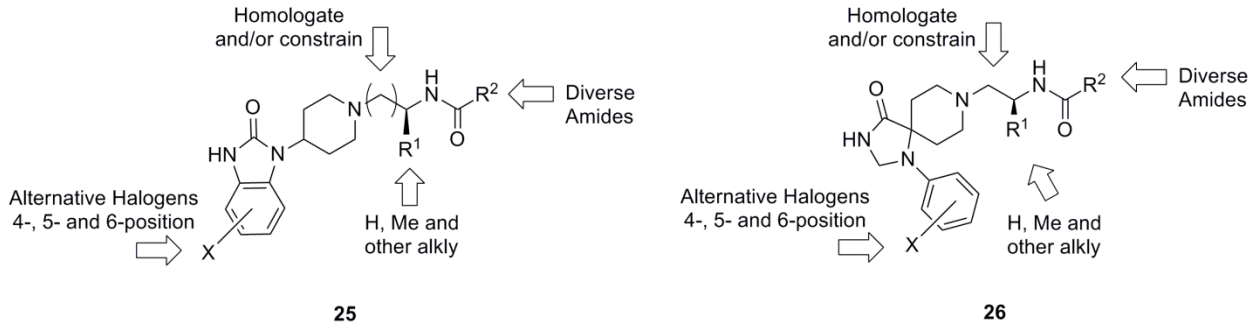


Figure 13. *In vitro* inhibition of recombinant PLD with select compounds. Graphs demonstrate CRCs for three classes of inhibitors on recombinant human PLD1 and PLD2 normalized to myr-Arf-1-stimulated activity +/-s.e.m. (representative data from a single 30-min experiment done in triplicate) (figure from [290]).

PLD2 selectivity. However, regardless of scaffold identity (either benzimidazolone or triazospirone)PLD1-selectivity can be dialed in with the addition of a *S*-chiral methyl to the linker region. An increase in overall potency of the benzimidazolone-containing compounds was observed by addition of a halogen, such as a chloro group, to the 5-position of the benzimidazolone.

In parallel to the biochemical screens, this library of compounds was also screened in a cell based assay previously described in detail elsewhere [78]. Sarah Scott, a postdoctoral fellow in our lab, performed the cell based screen, which uses two cell lines that owe the majority of their PLD activity to a single isoform (as determined by siRNA knockdown) [290]. ESI-MS was used to measure PtdBuOH formation as a readout for PLD activity in these cell lines following addition of small molecule and cell stimulation. Following concentration response curve generation for the same 30 compounds as characterized biochemically, similar classes of dual and isoform-selective inhibitors emerged (figure 15). Thus initial SAR held true for this new class of PLD inhibitors, referred to as the VU-series PLD inhibitors. However, every compound demonstrates increased potency in the cell based assay compared to the biochemical assay. This as of yet is unexplained, but there is evidence that these lipophilic compounds partition into PC-containing membranes possibly decreasing compound concentration accessible to recombinant protein in the biochemical assay (Selvy, Milne, Brown, unpublished data). Another possibility is, despite the fact that there is as of yet no evidence for off target effects in cells



25 **26**

Figure 14. Focused lead optimization strategy to improve PLD1 potency and selectivity within scaffold 25, and strategy to improve PLD2 potency and selectivity within scaffold 26 (figure from [19]).

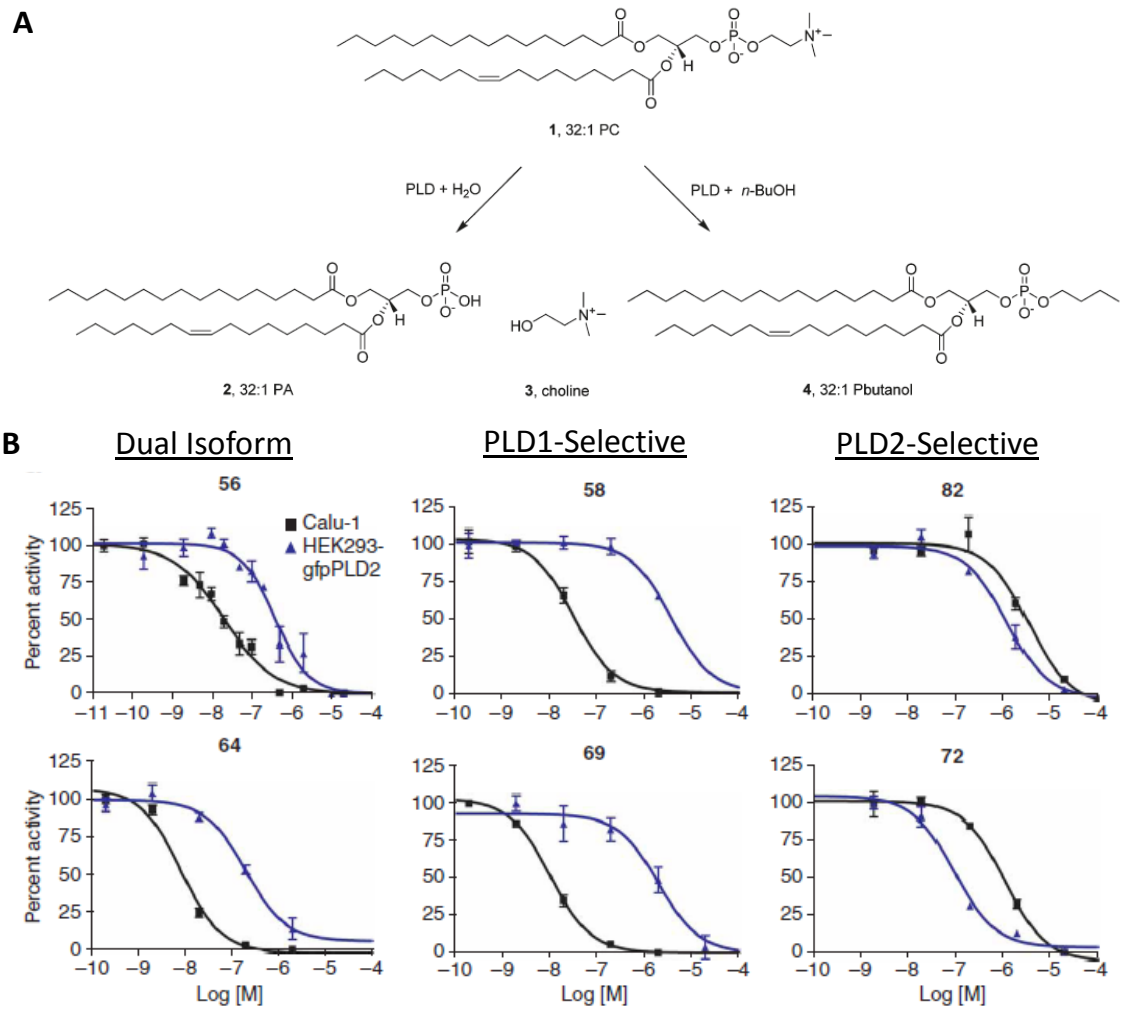


Figure 15. Cell-based activity assay utilizes deuterated *n*-butanol as a readout for PLD activity measured with ESI-MS (A). Small molecules potently inhibit cellular PLD activity. CRCs were obtained for multiple small-molecule PLD inhibitors for both PMA-stimulated Calu-1 cells (featuring predominantly PLD1 activity) and basal HEK293-gfpPLD2 stable cell line (featuring predominantly PLD2 activity) (figure modified from [291], [290]).

(off target screens have included other unrelated phospholipases, kinases, and receptors), the binding affinity of the compound may improve when then PLD protein is in a protein complex (as is possible in cells) rather that recombinantly expressed and purified (as in the biochemical assay). Regardless, rigorous characterization of the VU-series compounds to demonstrate both biochemical and cell-based efficacy suggests their potential power as a tool in studying PLD enzymology and signaling.

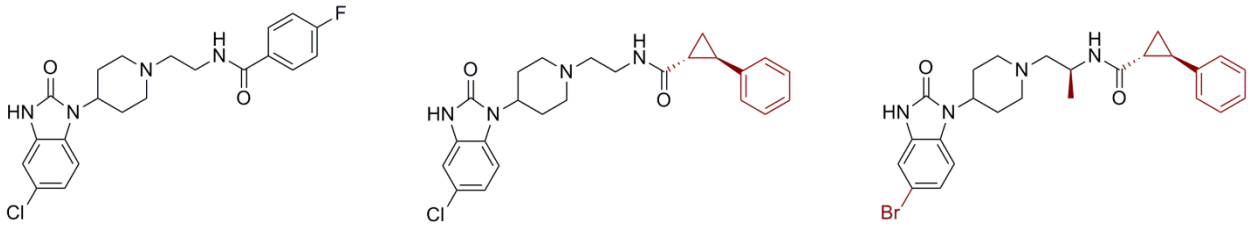
Structure-activity relationship characterization of novel PLD inhibitors

Following the initial description of the VU-series inhibitors, a larger library of compounds was generated in attempts to improve the potency and fold selectivity of these VU-series inhibitors. Extensive SAR characterization was undertaken for a library of more than 800 compounds generated in Dr. Craig Lindsley's lab. These compounds were screened for potency and selectivity in both biochemical and cell based assays. These studies lead to compounds with sub nanomolar potencies and in some cases more than 1700-fold selectivity.

SAR from this larger library tested the identity and position of the halogen substitution on the benzimidazolone, chemical space available around the ethyl diamine linker, and experimented with modifications or substitutions to the amide cap in the eastern portion of the molecule. Overall potency was almost always enhanced with halogen substitution of the scaffold, but the most significant increase in the potency of PLD1-selective compounds was afforded by bromo rather than chloro substitution of the 5-position on the benzimidazolone. There is

no flexibility in the length or size of constituents on the linker region. The *S*-methyl group continued to elicit PLD1 selectivity regardless of scaffold or amide cap identity. Finally, screening for optimal amide cap elements identified that the racemic transphenyl cyclopropane enhanced PLD1 selectivity over the diphenyl cap present in halopemide. Taken together, these modifications enhance PLD1 selectivity and potency (figure 16). These modifications yield VU0359595, our best PLD1-selective compound to date that has sub nanomolar potency and 1700-fold selectivity for PLD1 (in cells PLD1 IC_{50}=3.7nM versus PLD2 IC_{50}=6.4μM).

In contrast to PLD1-selective inhibitors, which were identified from direct modifications of the halopemide lead compound, potent PLD2-selective compounds were more elusive. From the initial screen for isoform-selective compounds the triazaspirone appeared to be a suitable alternative to the benzimidazolone scaffold that elicits PLD2-selectivity. However, the fold selectivity was not impressive, so in the subsequent SAR characterization, varied amide caps were screened. Substitutuion of a 2-quinoline amide congener yields increased PLD2 potency and selectivity (IC_{50}=90nM and 21-fold PLD2 selective). Further screening identified halogen substitution of a fluoro-group on the triazaspirone scaffold, which yields increased PLD2 potency and selectivity. This compound (VU0364739) at 75-fold PLD2-selective is the most selective and potent PLD2-selective compound reported to date (figure 17).

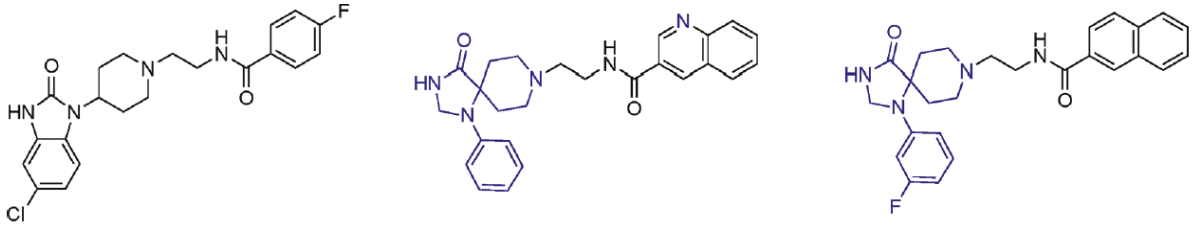


21, Halopemide
14-fold PLD1 selective
PLD1 IC_{50}= 21 nM
PLD2 IC_{50}= 300 nM

27, VU0285649
250-fold PLD1 selective
PLD1 IC_{50}= 2 nM
PLD2 IC_{50}= 520 nM

28, VU0359595
1700-fold PLD1 selective
PLD1 IC_{50}= 3.7 nM
PLD2 IC_{50}= 6,400 nM

Figure 16. The progression from halopemide, a 14-fold PLD1-selective inhibitor, to VU0359595, a 1700-fold PLD1-selective inhibitor. Functional groups shown in red conferred significant PLD1 selectivity (figure from [19]).



21, Halopemide
14-fold PLD1 selective
PLD1 IC_{50} = 21 nM
PLD2 IC_{50} = 300 nM

35, VU0285655
21-fold PLD2 selective
PLD1 IC_{50} = 1,900 nM
PLD2 IC_{50} = 90 nM

36, VU0364739
75-fold PLD2 selective
PLD1 IC_{50} = 1,500 nM
PLD2 IC_{50} = 20 nM

Figure 17. The progression from halopemide (21) to VU0364739 (36), a potent 75-fold PLD2-selective inhibitor. Functional groups shown in blue conferred significant PLD2 selectivity (figure from [19]).

Initial demonstration VU-series inhibitors act directly

Extensive SAR characterization has been performed using the biochemical liposome assay as a means of demonstrating that the compounds act directly to inhibit enzyme activity. Use of this assay in the initial screening is essential to confirm the compounds are not acting to inhibit activity through an upstream activator, as is possible when testing efficacy in the cell based assay alone. Such is the case for other reported PLD inhibitors that act indirectly, such as honokiol and resveratrol. However, the basal activity of full length PLD1 is very low, and to get the activity within the linear range of the biochemical assay the protein activator myristoylated Arf1 GTPase was included in every condition (including PLD2 screens for consistency). Therefore, it was important to demonstrate that these compounds inhibit PLD activity through direct interaction with the protein and not through the activator. For these studies an amino terminally-truncated form of PLD1, called PLD1.d311 (illustrated in figure 18) was used. PLD1.d311 can be purified to homogeneity and has significantly higher basal activity. Concentration response curves were generated using this truncation construct for two representative benzimidazolone-containing compounds in order to compare compound potency for basal versus Arf-activated activity. There is no shift in potency of the VU-series PLD inhibitors, demonstrating these compounds are acting directly to inhibit the enzyme.

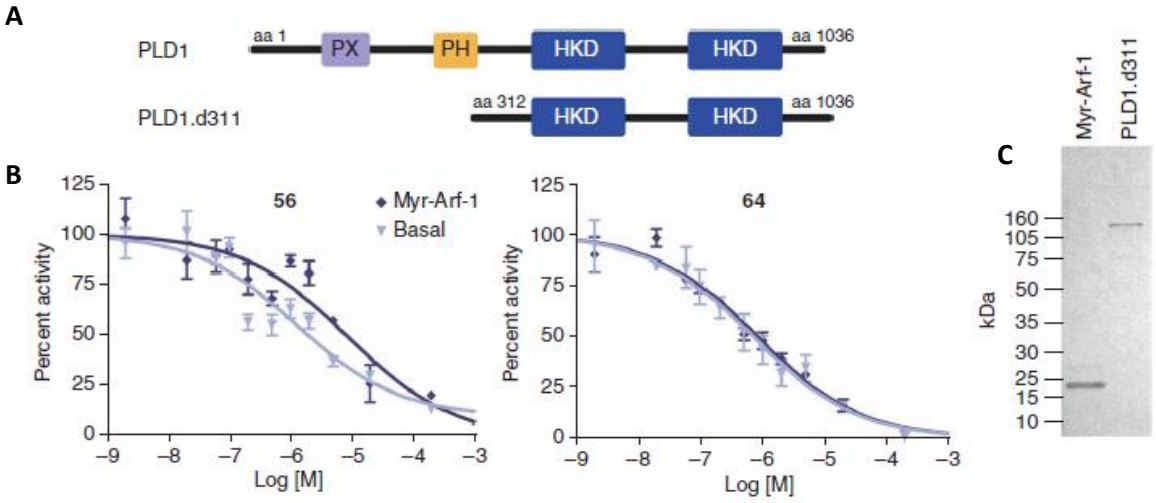


Figure 18. Direct small molecule inhibition of PLD. Schematic representation of truncated PLD1.d311 cosntruct (A). *In vitro* concentration response curves for two dual isoform compounds demonstrating direct inhibition of purified PLD1.d311 basal or myr-Arf-1-stimulated activity (B). The protein used for these studies was of high purity, as demonstrated with colloidal-stained SDS-PAGE gel (C) (figure from [290]).

A second possibility for how the inhibitors could be blocking PLD activity is through indirectly perturbing the liposome or lipid interface, such as partitioning into the lipid vesicle surface to impede access to substrate. Although we have demonstrated that the compounds do in fact partition into the liposome dependent on mole% PC (Milne, Selvy, and Brown, unpublished data), we used both scPLD (non HKD) and PMF PLD (HKD) and showed that these enzymes are not inhibited by the VU-series compounds (inhibition does occur in some cases at very high concentrations of compound, >10uM). Lack of inhibition towards these bacterial enzymes demonstrates that these compounds do not non-specifically inhibit non mammalian PLD enzymes, and do not disrupt substrate access or interface architecture.

The specificity of these compounds taken together with their ability to directly inhibit a highly purified amino-terminally truncated mammalian PLD demonstrates that the compounds allosterically inhibit the enzyme at a site that does not require the presence of the amino terminus. It is important to note that the potency of the compounds is shifted rightward for PLD1.d311, which may suggest a second compound binding site in the amino terminus, or that the amino terminus enhances potency through conformational change or provide support or stability to the small molecule binding site. The small molecule binding site and mechanism of action for these inhibitors is further addressed in chapter III.

Cellular ramifications for use of VU-series PLD inhibitors

Since the first report of the halopemide and VU-series PLD inhibitors, several groups have gone on to demonstrate their utility in studying the signaling roles for PLD. Other members in our group have demonstrated this class of compounds is of potential therapeutic value in blocking invasive migration, decreasing cancer cell viability, and inducing apoptosis. In the initial report of the VU-series compounds, our lab demonstrated that invasive migration was significantly blocked for three highly metastatic cancer cell lines (MDA-MB-231, mouse metastatic breast cancer 4T1, and PMT mammary tumors). Using the first generation of VU-series PLD inhibitors, both dual and isoform-selective compounds significantly decreased invasive migration at high concentrations (2-20μM). Recent studies using more potent and isoform-selective compounds demonstrate a time and dose-dependent decrease in proliferation and cell viability, particularly for the PLD2-selective inhibitors. The decrease in viability is enhanced under conditions of cell stress (e.g. serum-starvation), and the results are more significant for transformed versus non transformed cells. Increased apoptosis was also observed for cancer cells, as measured by caspase 3 and 7 activation. Taken together, these results suggest transformed cells rely heavily on PLD signaling for viability and proliferation, as other signaling pathways may not be intact. Thus preliminary studies suggest the VU-series PLD inhibitors may be effective therapeutically as anti cancer compounds.

Chapter III

MOLECULAR MECHANISM OF ISOFORM-SELECTIVE PHOSPHOLIPASE D INHIBITORS

Phosphatidic acid (PA) is a critical lipid second messenger that not only facilitates lipid bilayer curvature for membrane fusion, but also serves as a node for the recruitment and activation of signaling proteins at the plasma membrane and is a precursor to diacylglycerol and lysophosphatidic acid. These varied roles put PA at the intersection of cell signaling and metabolic pathways. PA is generated in response to receptor stimulation by phospholipase D (PLD) hydrolysis of phosphatidylcholine (PC) at the terminal phosphodiester bond. PLD activity is tightly regulated by a myriad of mechanisms that differ between the two canonical mammalian isoforms PLD1 and PLD2. PLD1 is directly activated by PKC, Arf and Rho GTPases and is basally localized to perinuclear, endosomal, and Golgi vesicles via protein-protein interactions at its Phox homology (PX) domain and palmitoylation in the PH domain [188]. PLD2 maintains higher basal activity and is constitutively localized at the plasma membrane. Upon activation PLD translocates to late golgi vesicles and the plasma membrane where it binds at the lipid interface to access phospholipid substrate. Multiple protein domains define PLD-lipid binding interactions (K_s) and control translocation and subsequent internalization. PX (aa 79-209) and pleckstrin homology (PH, aa

220-328) lipid binding domains at the amino-terminus promiscuously bind polyphosphatidylinositols and negatively-charged phospholipids. These domains are also predicted to elicit regulatory functions that may dictate differences in isoform subcellular localization, activity, and protein-protein interactions. There are also reports of a conserved $PI(4,5)P_2$ binding motif that lies between the conserved catalytic $H(x)K(\varphi)_4D$ motifs and facilitates translocation to $PI(4,5)P_2$-containing membranes and catalytic activity.

Until recently it has not been possible to acutely and pharmacologically inhibit PLD catalytic activity in order to study its enzymology and specific function in cell signaling pathways. Historically, RNAi and primary alcohols have been the only tools available. RNAi knocks down PLD protein production over a short period of time allowing for compensation within signaling pathways, while treatment with primary alcohol merely diverts product formation to phosphatidylalcohol. Primary alcohols exploit the transphosphatidylation reaction characteristic of PLD-family members, in which the primary alcohol is the preferred nucleophile to water during substrate hydrolysis. Recently, in response to the void in the field, we reported identification and characterization of a class of small molecules that potently and isoform-selectively inhibit PLD activity, called VU-series compounds (chapter II) [290]. Structure-activity relationship characterization of this class lead to the development of compounds that are >1700-fold PLD1-selective[292], and >75-fold PLD2-selective [291] to use as tools in delineating the distinct signaling roles of each isoform. Subsequent studies, in our own lab and in others, using these small molecule inhibitors have

yielded different results from those published using primary alcohols [79], [237]. This suggests that either the roles of PLD as discerned using primary alcohols have been widely overstated, or the mechanism of action of these small molecule PLD inhibitors extends beyond inhibiting PA formation. To address this discrepancy, we used backscattering interferometry (BSI), a highly sensitive and novel method for measuring protein-small molecule and protein-lipid binding affinities, to characterize the mechanism of action of these small molecule PLD inhibitors. For these studies we focus our characterization on the mammalian PLD1 isoform, and propose that a similar molecular mechanism also applies to VU-series-compound mediated PLD2 inhibition.

Here we demonstrate that these compounds directly target PLD1 and allosterically block lipid binding and catalytic activity. These *in vitro* findings are confirmed with cellular studies demonstrating that these compounds do not perturb basal PLD1 localization, but block stimulated enzyme translocation. This is a unique and underappreciated mechanism for inhibiting a lipid signaling enzyme, and is akin to the mechanism recently reported for an allosteric pharmacological inhibitor of Akt, Inhibitor VIII [293], [294].

Backscattering interferometry measures protein-ligand interactions

PLD is an interfacial enzyme that must bind at the lipid surface (S, figure 2) prior to binding and hydrolyzing substrate. As such, the nature of the lipid interface has a great impact on the binding affinity for the lipid surface ($1/K_s$), accessibility and affinity for substrate (K_m), and catalytic activity of the enzyme

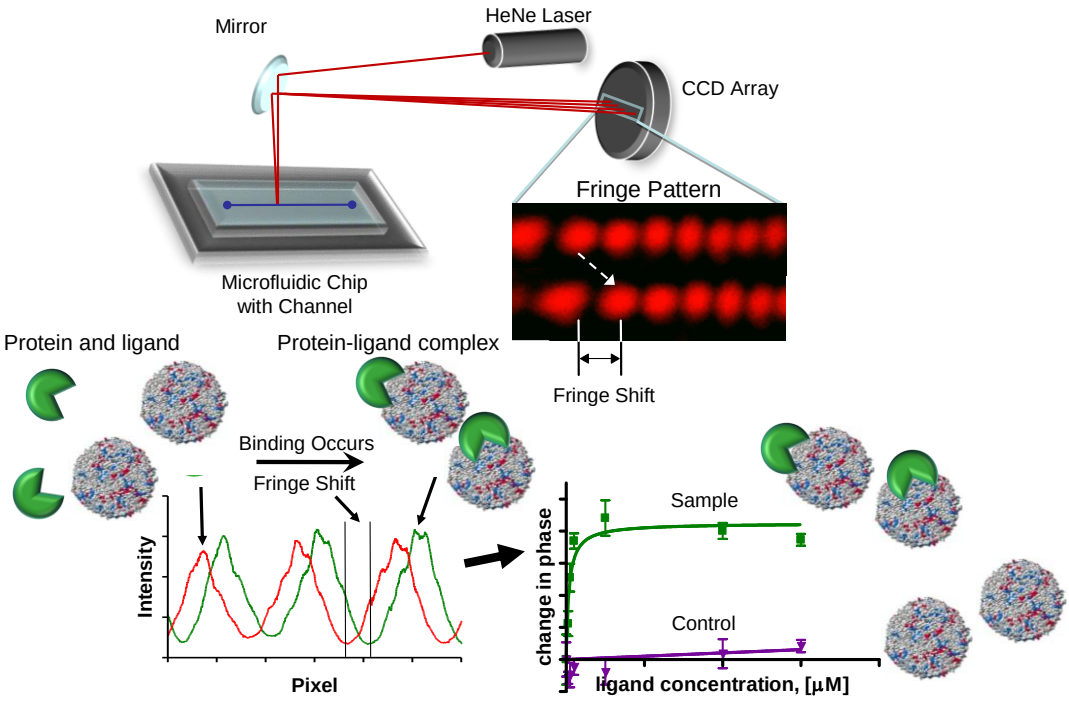


Figure 19. Backscattering interferometry (BSI) technique optimized for measuring protein-small molecule and protein-lipid binding affinities (figure modified from[295]).

(k_{cat}). Therefore, it was important to determine whether these small molecules were eliciting inhibition through direct interaction with soluble/non-lipid bound enzyme, or indirectly influencing the lipid interface, as is the case for neomycin [296] and tamoxifen [297] (unpublished data Selvy and Brown), or potentially selectively binding to interfacially-bound enzyme (E_s), as is the case for some PLA_2 inhibitors [298]. BSI has been validated as a method for measuring picomolar receptor-ligand binding affinities using nanomolar concentrations of protein [295], [299], [300], and was used here to measure protein-small molecule binding affinities for PLD1. In contrast to isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR), binding affinities can be measured using BSI in a microliter-scale format for untagged protein free in solution. In this technique, a laser is reflected and refracted through a sample contained within the channel of a microfluidic chip and an interference pattern is created. The interference pattern consists of a specific set of well defined fringe spots, and a shift in the fringe spots correlates to a change in refractive index due to a change in salvation of a protein upon ligand binding (i.e. change in protein conformation). A shift in fringe spots is quantified as a change in phase of a wave function using Fourier transform. The phase is measured for any set of samples and then graphed as the absolute change in phase relative to a control sample upon increasing ligand concentration.

Development of novel human PLD1 construct facilitates *in vitro* studies

To ensure phase changes, as recorded by BSI, are due to bimolecular interactions between PLD1 and small molecule, highly purified recombinant protein was necessary. Recombinant expression of full length PLD1 is poor and because of the myriad of protein binding partners, full length protein remains a heterogeneous population following multiple chromatographic purification steps. Therefore several robustly expressed and homogenously-purified truncation constructs of PLD1 were used to demonstrate direct bimolecular interactions. In our earlier report [290] we demonstrated that the VU-series compounds could inhibit an amino-terminally-truncated rat PLD1 construct lacking the first 311 amino acids. This construct comprises a truncation of the PX and PH domains, leaving 16aa of an alpha-helix at the *C*-terminus of the PH domain that is necessary for lipid binding (Henage, Selvy, and Brown, unpublished data).

For more rigorous mechanistic characterization of these VU-series compounds, however, we chose to study human PLD1. Characterization of human PLD1 splice variants using cDNA libraries detected a poorly studied PLD1c form. This PLD1 splice variant was initially reported by Steed et al. [173] as a shortened protein whose transcript contains a single nucleotide deletion that results in a frame shift in protein translation. In nature, the PLD1c frame shift results in an early stop codon and a protein that is truncated following the first HKD motif (figure 20). Insertion of a single nucleotide corrects the frameshift and results in a catalytically active 2 HKD PLD enzyme with an 18 amino acid stretch of residues in the catalytic loop region. The shorter catalytic loop region,

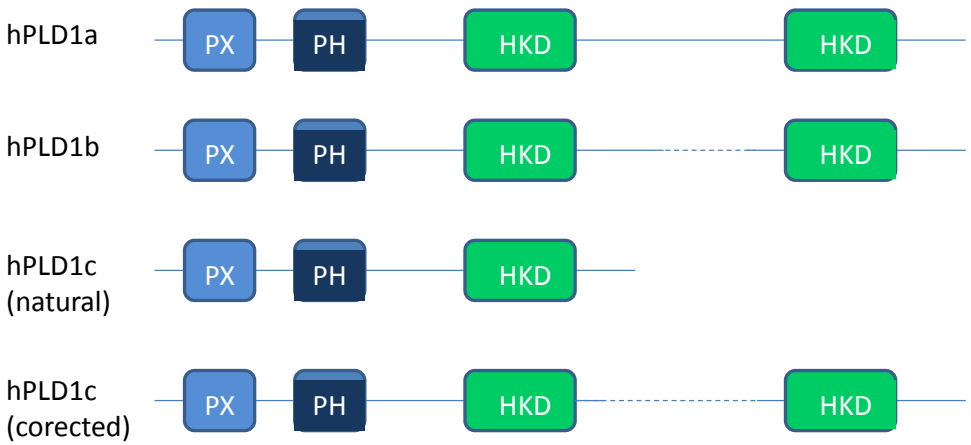


Figure 20. Illustration of the domains of the human PLD1 splice variants.

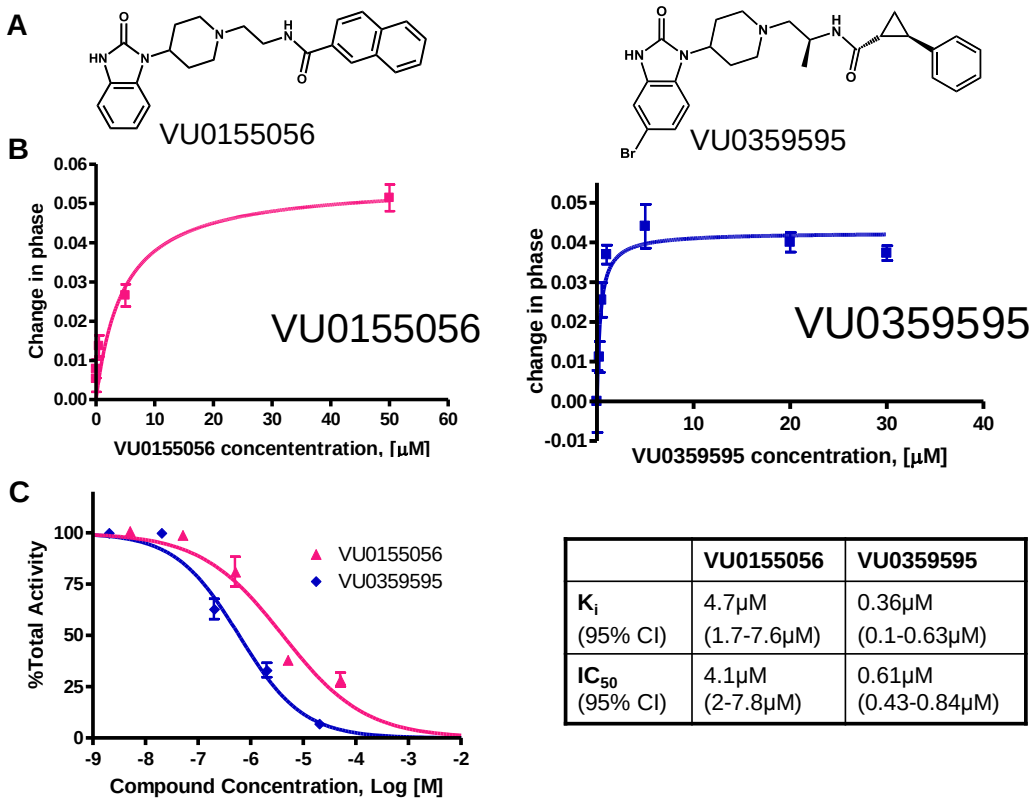
as compared to the loop regions found in PLD1a and PLD1b splice variants, facilitates robust recombinant expression, enhanced protein stability, and straightforward chromatographic purification. Corrected PLD1c maintains all the same biochemical and cellular characteristics of the natural PLD1a and PLD1b, including protein-activator response and robust receptor-stimulation.

VU-series PLD inhibitors directly interact with enzyme

Using BSI and silver-stain pure preparations of the amino-terminally truncated PLD1c splice variant, referred to here simply as PLD1.d311, we demonstrate this construct directly binds the compounds in a one-site binding model in the absence of lipid (figure 21). The K_i for benzimidalozone-containing small molecules (VU0155056 and VU0359595) was similar to IC_{50}s measured for respective compounds in the biochemical liposome reconstitution assay. Because the affinities and potencies for the benzimidalozone-containing compounds are similar for their respective compounds, we conclude that these compounds are acting directly on the enzyme and not indirectly inhibiting activity by disrupting access to $PI(4,5)P_2$ or substrate at the lipid interface.

Small molecule PLD inhibitors block lipid binding

PLD's phospholipid substrate is located at a lipid interface and is not freely soluble, therefore classic Michaelis-Menten assumptions do not apply. In order to study PLD kinetics and the mechanism of action for these small molecule



	VU0155056	VU0359595
K_i (95% CI)	4.7µM (1.7-7.6µM)	0.36µM (0.1-0.63µM)
IC_{50} (95% CI)	4.1µM (2-7.8µM)	0.61µM (0.43-0.84µM)

Figure 21. PLD1c.d311 directly binds VU-series PLD inhibitors to inhibit catalytic activity. *A,* Structures of VU-series small molecule PLD inhibitors *B*, K_i small molecule binding affinities were measured for two representative benzamidazolone-scaffold VU-series PLD inhibitors (VU0155056 and VU0359595) using BSI. These compounds directly bind to purified PLD1.d311 (aa 312-966) with similar binding affinities (K_i) to IC_{50} values measured from concentration response curves using a reconstituted liposome activity assay.

inhibitors, the interfacial binding affinity must first be measured (figure 2, K_s), and subsequent studies must saturate lipid binding in order to apply Michaelis-Menten assumptions. Historically, intricate and imperfect methods have been applied to measure K_s (FRET and lipid binding "strips" of immobilized lipid). Despite its resource and time-consuming nature, the sucrose-loaded vesicle (SLV) binding assay[72] has been the standard method for measuring cosedimentation of protein with increasing concentrations of bulk lipid. Using a defined vesicle composition (87%mol PE, 8%mol PC, 5%mol $PI(4,5)P_2$), PLD1.d311 cosedimentation was measured for increasing bulk lipid concentrations (figure 22a). The K_s value derived from the SLV assay was then compared to the K_s value obtained using BSI as a means of measuring protein-lipid binding (figure 22b). Using a similar vesicle compostion, phase change was measured for PLD1.d311 with increasing concentrations of extruded large unilamellar vesicles (LUV). Similar K_s values were obtained for both methods, validating the use of BSI as a novel and highly efficient method for measuring protein-lipid binding.

Regardless of assay method, the PLD1-selective small molecule inhibitor, VU0359595, noncompetitively blocked bulk lipid binding at absolute inhibitory concentrations (10μM). Reports in the literature propose $PI(4,5)P_2$-dependent binding at a polybasic region in the catalytic domain is solely responsible for PLD translocation to the plasma membrane. Therefore, it was important to determine whether the *in vitro* lipid binding measured here was $PI(4,5)P_2$ -dependent. Protein lipid binding was measured for vesicle compositions lacking $PI(4,5)P_2$.

Despite the fact that K_s was significantly shifted to the right in its absence, bulk lipid binding (and catalytic activity, data not shown) was detectable and saturable without $PI(4,5)P_2$ (figure 22c). In fact, bulk lipid binding also occurs in the absence of PC or PE, the preferred and secondary substrate, respectively, as is found when measuring K_s for PG-only vesicles. PG is not a substrate as confirmed by LC/MS (Milne, Selvy, and Brown, unpublished data). For each vesicle composition VU0359595 noncompetitively blocked bulk lipid binding (figure 22c). From these studies we conclude *in vitro* PLD binds and hydrolyzes lipid in the absence of $PI(4,5)P_2$, and the small molecule PLD inhibitor non competitively blocks this interaction regardless of lipid vesicle composition.

In order to demonstrate that these small molecules do not nonspecifically disrupt interfacial binding for other lipid binding proteins, we also measured VU-series interactions with phospholipase C delta 1 (PLCδ1, provided by Ken Harden's lab), a well characterized phospholipase which binds $PI(4,5)P_2$ with high affinity via its PH domain in order to hydrolyze $PI(4,5)P_2$. In the absence of lipid, VU0359595 does not interact with or bind PLCδ1, and protein-lipid binding is not perturbed due to the compound (figure 22d). The absence of any interaction with an unrelated phospholipase demonstrates that the VU-series compounds do not nonspecifically perturb protein-lipid binding. The non competitive disruption of PLD-lipid binding, regardless of lipid identity, suggests these compounds are allosterically impacting a hydrophobic phospholipid binding site on the catalytic domain likely through inducing a significant conformational change.

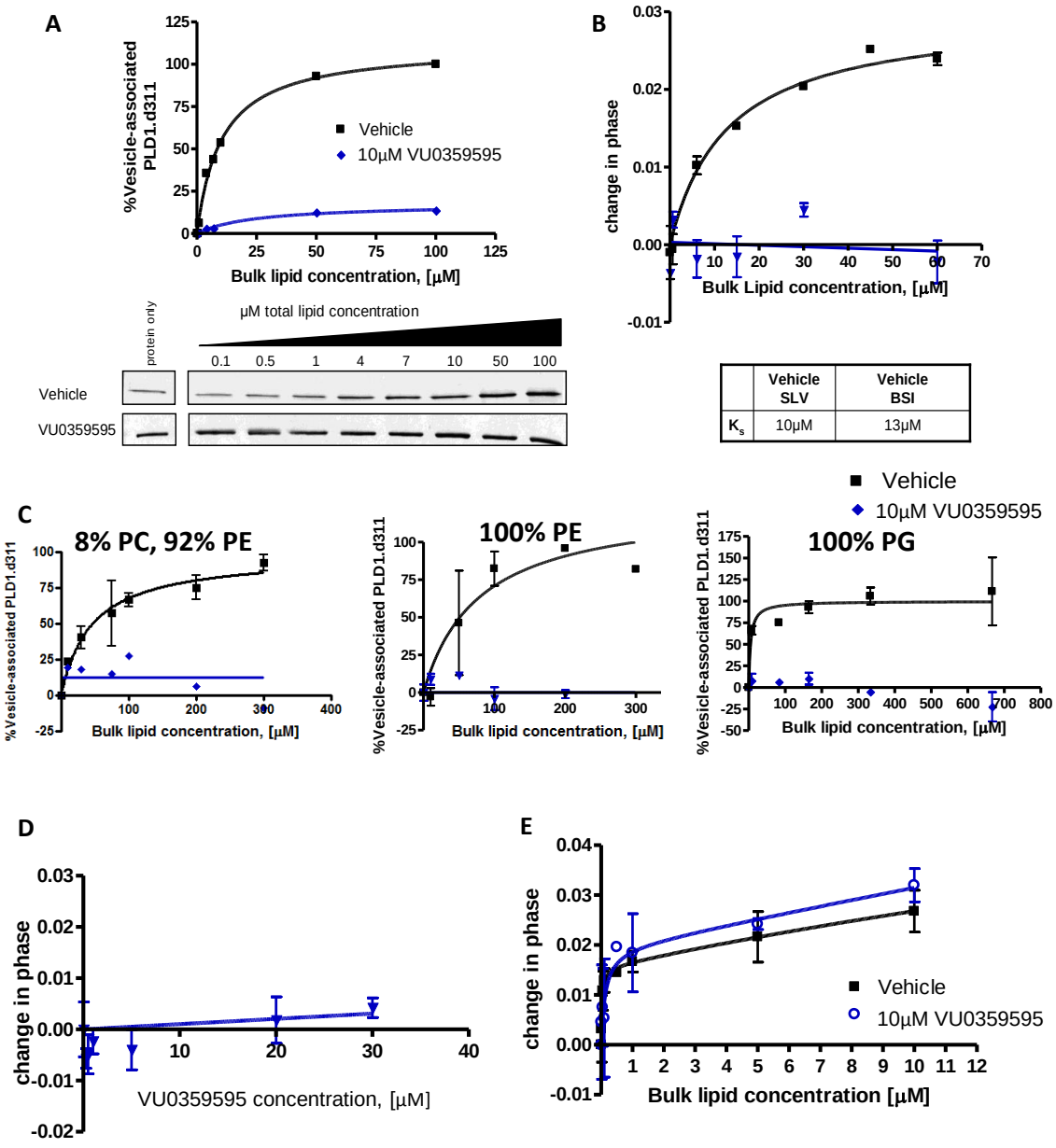
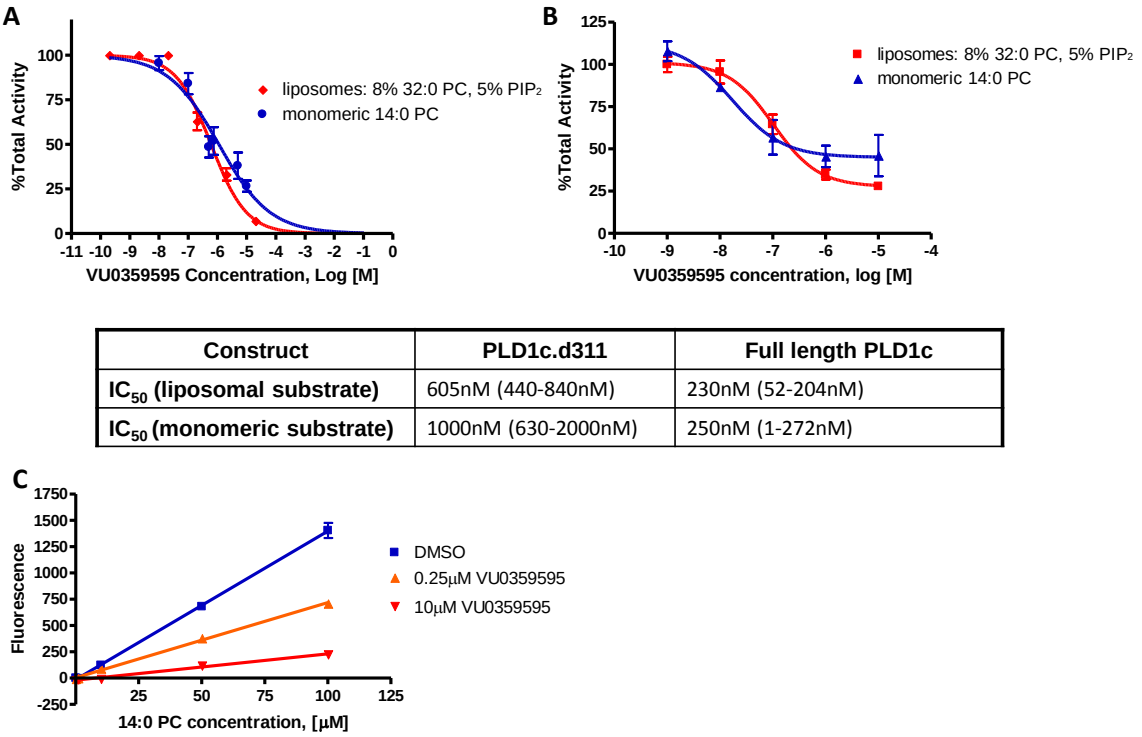


Figure 22. VU-series PLD inhibitors block bulk lipid binding regardless of lipid interface composition. Bulk lipid binding was measured for purified PLD1.d311 using the classic sucrose-loaded vesicle technique (*A*), and backscattering inerferometry (*B*). These techniques give similar K_s, validating the BSI technique, and demonstrating that the small molecule inhibitor blocks bulk lipid binding. *C*, PLD1.d311 bulk lipid binding was measured for varied lipid vesicle compositions omitting $PI(4,5)P_2$ and substrate (PC and PE). Regardless of lipid vesicle composition, inhibitory concentrations of VU0359595 continue to noncompetitively disrupt bulk lipid binding. Inhibition of bulk lipid binding is not mediated through indirect disruption of the interface as the VU-series compounds do not nonspecifically bind PLCδ1, another lipid binding protein (*D*), and do not perturb PLCδ1 bulk lipid binding (*E*).

VU-series compounds inhibit catalytic activity in the absence of a lipid interface

Subsequent to demonstrating that the VU-series compounds allosterically disrupt lipid binding, it was important to interrogate whether the small molecules perturbed catalysis for substrate not present at an interface. PLD activity was monitored in the absence of the lipid binding component (K_s) by measuring hydrolysis of monomeric 14:0 PC (used below the critical micelle concentration) via direct and indirect techniques (i.e. LC/MS or amplex red reagent, respectively). Concentration response curves of VU0359595 generated for different substrate presentations (e.g. lipid-bound (32:0 PC) or monomeric 14:0 PC) were comparable for PLD1.d311 and full length PLD1 (figure 23a and b, respectively). IC_{50}'s are similar for *in vitro* concentration response curves generated using these different assay formats, demonstrating VU0359595 potency is unchanged regardless of substrate presentation. This suggests that the VU-series compounds mediate inhibition not only by blocking lipid binding but also through inhibiting catalytic activity. Soluble monomeric PC allows measurement of catalysis in the absence of the lipid binding component, which ultimately allows for Michaelis-Menten kinetic analysis. Upon increasing substrate concentration, the activity of PLD1.d311 was measured in the presence of vehicle or constant concentrations of VU0359595 (near the IC_{50}= 0.25μM, and well above= 10μM; figure 23c). In both VU0359595-treated cases, catalysis was decreased from that of vehicle-treated, and increases in substrate could not compete for its inhibitory effect. This demonstrates that the compounds non



Construct	PLD1c.d311	Full length PLD1c
IC_{50} (liposomal substrate)	605nM (440-840nM)	230nM (52-204nM)
IC_{50} (monomeric substrate)	1000nM (630-2000nM)	250nM (1-272nM)

Figure 23. VU-series compounds inhibit PLD1.d311 activity regardless of substrate presentation. VU035959 inhibits PLD1.d311 (*A*) and full length PLD1c (*B*) with the same potency regardless of substrate presentation, as determined from concentration response curves in liposome and monomeric substrate assays. *C*, VU-series compounds do not compete for substrate binding, as determined using an Amplex Red assay with varied monomeric substrate (14:0 PC) concentrations for VU0359595 near K_i (0.25uM) and >>K_i (10uM). This demonstrates the VU-series compounds inhibit catalytic activity at a site allosteric to the substrate binding site.

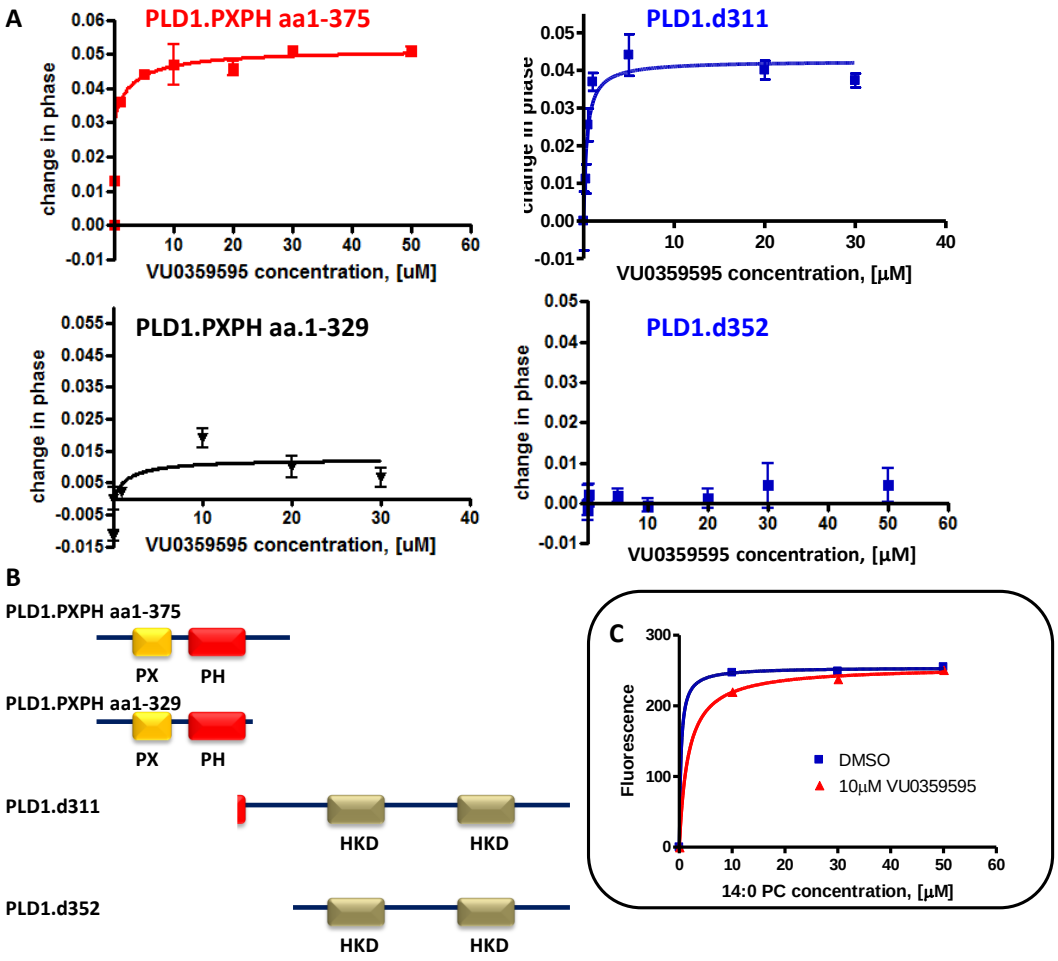
competitively inhibit catalytic activity by directly interacting with the enzyme at a site allosteric to the substrate binding pocket.

Protein truncation constructs illuminate the small molecule binding site

Following demonstration that the compounds directly interact with PLD1.d311 to disrupt both protein-lipid binding and catalytic activity, we went on to determine the allosteric site of small molecule interaction. Using several protein truncation constructs composed of lipid binding or catalytic domains, we measured small molecule binding affinities (illustrated in figure 24b). These studies allowed us to narrow the small molecule binding site to a previously uncharacterized region of PLD1 that lies between the PH and catalytic domains. This stretch encompasses amino acids 329-352 and is predicted to be a loop region, which because of its proximity to the PH and catalytic domains is hereafter called the linker loop.

A catalytic domain construct lacking the PXPH domains and the entire linker loop (PLD1.d352) is inactive towards liposomal substrate but can hydrolyze monomeric substrate. As measured in the amplex red reagent assay using monomeric substrate, PLD1.d352 catalytic activity is not inhibited by VU0359595 (figure 24c). Also, this construct does not directly interact with the small molecule as determined by BSI (figure 24a). The inhibitor resistant nature of PLD1.d352 in conjunction with data from PLD1.d311 suggested that the region encompassing amino acids 311-352 were critical for small molecule efficacy.

In our first report on the VU-series compounds we described a 3 to 24-fold shift in potencies of the VU-series inhibitors between full length PLD1 versus PLD1.d311[290]. This suggested the PLD1.d311 truncation construct lacked a portion of the small molecule binding site or lacked a second small molecule binding site. To better understand these discrepancies in IC_{50}s we used purified amino-terminal constructs encompassing the PX and PH domains (aa1-375, aa1-329) and used BSI to measure K_d for the benzimidalozone compound VU0359595 (figure 24). Truncation construct PLD1.PXPH aa1-375 directly interacts with the small molecule, but does so in a two-site binding model ($p=0.0003$ for two-site over one-site model) with a high affinity and low affinity binding site. The shortened construct PLD1.PXPH aa1-329, which lacks the linker loop region (residues 329-352), also directly interacts with the small molecule, but does so in a one-site binding model with a single low affinity binding site. Consistent with truncation data from the catalytic domain, differences in VU0359595 binding for the PXPH constructs suggest the high affinity binding site lies within the linker loop region. Based on the somewhat hydrophobic nature of the VU-series compounds it is not surprising that a low affinity binding site remains for the PXPH construct even in the absence of the linker loop. This second site of small molecule interaction may be at one of several remaining lipid-binding hydrophobic patches on the protein surface, or may loosely contribute to the tertiary small molecule binding site in the full length enzyme. However, as the affinity observed for the low affinity binding is well outside of the range that elicits potent isoform-selective inhibition of PLD1, we



Construct	K_d	B_{max}
aa 1-375 2-site	K_{d1}=0.014µM (0.001-0.0282µM) K_{d2}=2.7µM (0-10.79µM)	B_{max1}=0.019 (0.008-0.030) B_{max2}=0.032 (0.019-0.045)
aa 1-329 1-site	K_d=1.2µM (0-4.9µM)	B_{max}=0.012 (0.006-0.018)
aa 311-966 1-site	K_i=0.36µM (0.1-0.63µM)	B_{max}=0.042
aa 352-966 1-site	No binding	No binding

Figure 24. **Truncation constructs suggest VU-series PLD inhibitors directly bind PLD in a loop region C-terminal to the PH domain.** ***A,*** Using BSI, small molecule binding affinities were measured for several PLD1c truncation constructs. VU0359595 directly binds PLD1.PXPH aa 1-375 at two distinct sites, only one of which is high affinity. PLD1.PXPH aa 1-329 only encompases the lower affinity binding site. PLD1.311 binds VU0359595 with high affinity, while PLD1.d352 does not bind the compound. ***B***, Cartoon alignment of conserved domains within these truncation constructs illustrates that PLD1.PXPH aa 1-375 and PLD1.d311 overlap at a region C-terminal to the PH domain, while PLD1.PXPH aa 1-329 and PLD1.d352 do not include this region. This suggests this site encompasses

the VU0359595 high affinity binding site. ***C***, To further demonstrate that this region encompasses the binding site we examined the activity of PLD1.d352 +/- VU0359595. This construct retains catalytic activity towards monomeric substrate, but VU0359595 does not inhibit activity.

propose that this second site does not contribute to the mechanism of small molecule inhibition and is not relevant for further analysis in these studies.

An important observation from the binding curves for these two PXPH constructs is the significant difference in B_{max} (phase change). As mentioned above, the phase measurement in BSI is essentially the refractive index of the protein-ligand solution. Changes in refractive index are a measurement of change in protein solvation (i.e. protein conformation) upon protein-ligand binding. Therefore, the larger the change in phase, the larger the predicted change in protein conformation upon ligand binding. From PXPH binding curves it is evident that PLD1.PXPH 1-375 undergoes a significantly larger change in protein conformation upon small molecule binding than PLD1.PXPH 1-329. From these protein-small molecule binding studies, we conclude that these small molecule inhibitors directly interact with high affinity at the linker loop, a region of the protein encompassing amino acids 329-352 that has a predicted loop-like secondary structure, and subsequently undergo a significant change in conformation.

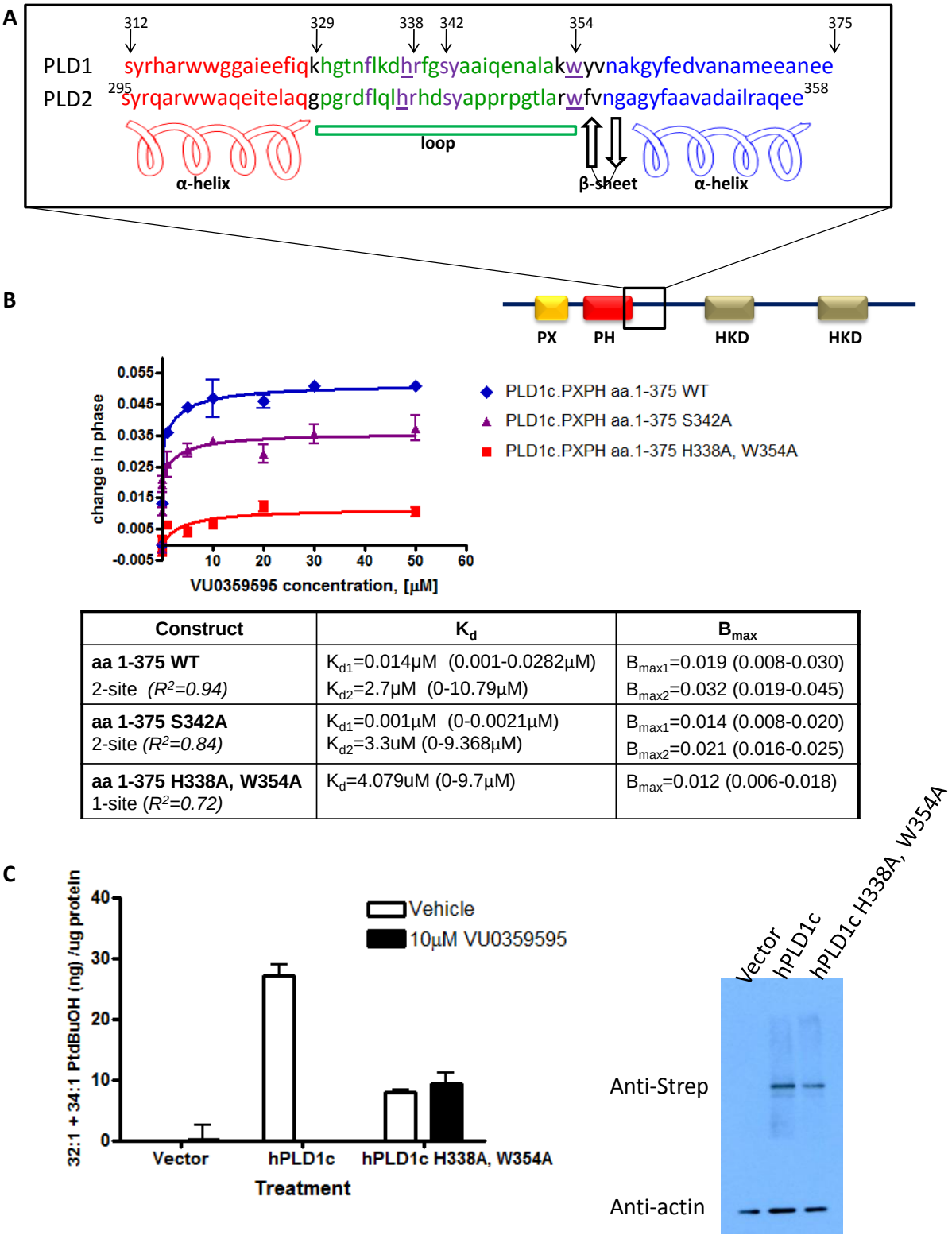
Confirmation of small molecule binding site with point mutant constructs

Upon further analysis of the linker loop (the region of predicted high affinity VU-series compound interaction) we observe a predicted loop region flanked by

more rigid structural elements (e.g. alpha-helices and a short beta sheet, figure 25a). Linker loop sequence comparison between PLD1 and PLD2 demonstrates some amino acid variability, while the predicted secondary structural elements are conserved. SAR studies (chapter II) suggest these compounds bind at a similar site on the two isoforms, but certain chemical modifications of the compounds are able to dial in selectivity by taking advantage of some unknown differences in the specific isoform binding site. Comparison of the linker loop sequence for PLD1 and PLD2 highlights a few conserved residues that may be integral for VU-series compound binding. To secondarily confirm the linker loop is the site of VU-series compound binding, these conserved residues (shown in purple in figure 25) were systematically point mutated to alanines in the full length PLD1c enzyme. The potency of VU0359595 was subsequently measured for each mutant *in vitro*. From these studies, His338Ala and Trp354Ala mutations significantly decreased the potency of VU0359595, but did not ablate inhibitory action. Therefore, a dual mutant in which both His338 and Trp354 were mutated to alanines was generated and assessed for inhibitor resistance using several methods. The small molecule binding affinity of PLD1.PXPH 1-375 H338A, W354A was compared to PLD1.PXPH 1-375 S342A (a mutation which yields no shift in VU0359595 potency) and wildtype PLD1.PXPH 1-375. Consitent with S342A mutation not impacting VU0359595 potency in the full length enzyme, PLD1.PXPH 1-375 S342A demonstrated a two-site binding model ($p<0.0001$) with large change in phase similar to wildtype PLD1.PXPH 1-375. However, mutation of both H338A and W354A significantly alters the binding curve profile

(figure 25b). This double mutant construct yields a single low affinity binding site with a small change in phase, comparable to the PLD1.PXPH 1-329 construct that entirely lacks the linker loop. This binding data is consistent with the suggestion that His338 and Trp354 contribute to the small molecule binding site in the linker loop.

In order to demonstrate that the double mutation of both His338 and Trp354 does not significantly alter the folding of the enzyme and non specifically alter VU0359595 affinity, we measured PLD activity for this construct transfected into an HEK293 cell. Following serum-starved conditions, cellular PLD activity was measured for vector, wildtype PLD1c, and PLD1c H338A, W354A. Although double mutant activity is ~30% of the wildtype activity (consistent with preliminary *in vitro* studies of the double mutant) this activity is measureable above vector control, suggesting that the protein is correctly folded and a viable construct to test for inhibitor resistance. An inhibitory concentration of VU0359595 does not affect double mutant activity (figure 25c). This cell based activity data is consistent with the PXPH binding experiments demonstrating that His338 and Trp354 are integral for high affinity small molecule binding, as mutation of these residues generates inhibitor resistant PLD activity. *In vitro* studies will further demonstrate that these conserved residues in the linker loop are integral components of the high affinity binding site for the VU-series compounds.



Construct	K_d	B_{max}
aa 1-375 WT 2-site *(R^2=0.94)*	K_{d1}=0.014μM (0.001-0.0282μM) K_{d2}=2.7μM (0-10.79μM)	B_{max1}=0.019 (0.008-0.030) B_{max2}=0.032 (0.019-0.045)
aa 1-375 S342A 2-site *(R^2=0.84)*	K_{d1}=0.001μM (0-0.0021μM) K_{d2}=3.3uM (0-9.368μM)	B_{max1}=0.014 (0.008-0.020) B_{max2}=0.021 (0.016-0.025)
aa 1-375 H338A, W354A 1-site *(R^2=0.72)*	K_d=4.079uM (0-9.7μM)	B_{max}=0.012 (0.006-0.018)

Figure 25. Point mutations confirm VU-series PLD inhibitors directly bind PLD in a loop region C-terminal to the PH domain. *A*, Closer analysis of the protein sequence in the linker loop for both human PLD1 and PLD2 isoforms demonstrates a conserved pattern

of predicted secondary structure (PredictProtein) and conserved residues (shown in purple, numbered residues correspond to PLD1 sequence) in a region predicted to be a loop (shown in green). *B*, An H338A, W354A double mutant was generated to ablate the high affinity binding site. This double mutant was generated in PLD1.PXPH aa 1-375, and VU0359595 binding was measured using BSI to demonstrate that the high affinity binding site was ablated, similar to the PLD1.PXPH aa 1-329 construct that lacks the linker loop region entirely. An inert mutation (S342A) that does not shift the potency of VU0359595 also does not perturb high affinity binding. *C*, The H338A, W354A double mutant was generated in the full length PLD1c. While the overall activity of the double mutant is decreased as compared to wildtype, resistance to VU0359595 is observed for double mutant activity as measured in the cell. This data taken as a whole confirms the linker loop contributes to the high affinity small molecule binding site. Western blot analysis was used to demonstrate similar transcription efficiency and protein expression of the strep-tagged PLD1c constructs, anti-actin demonstrates equal loading between lanes.

Small molecule PLD inhibitors block cell signal-mediated translocation

Subcellular protein localization studies were performed to test conclusions from the *in vitro* studies in a cellular context. EGFP-tagged PLD1 was transfected into Cos7 cells, 24 hours later cells were serum-starved, and the following day visualized with live-cell fluorescent-microscopy. Basal localization was observed following 5 minute pre-treatment with vehicle or 10μM VU0359595, while stimulus-induced translocation was demonstrated following 1 hour treatment with phorbol ester. In the presence of vehicle, EGFP-tagged PLD1 is basally localized to perinuclear membranes and early endosomes (not nucleus as determined by DAPI-stain, figure 26). This is consistent with basal localization of PLD1 previously reported in Cos7 cells.[188] Upon stimulation with 1μM PMA (figure 26) or 10-20%serum (not shown), the protein is observed to translocate to plasma membrane or late endosomes, respectively, for stimulation types.[188]

Basal localization of EGFP-tagged PLD1 is unchanged in the presence of VU0359595. This observation is consistent with other reports in the literature that claim protein-protein interactions and palmitoylation-modifications mediated by the PXPH domain control basal localization of PLD1 in Cos7 cells rather than protein-lipid interface interactions[188]. However in the presence of VU0359595, upon cellular stimulation EGFP- tagged PLD1 translocation to late endosomes or the plasma membrane is blocked. This observation is consistent with *in vitro* data presented herein, where interaction between PLD and lipid interface is blocked in the presence of the inhibitor. Truncation studies suggest the high affinity small molecule binding site lies in the linker loop (a loop region C-terminal to the PH domain and N-terminal to the start of the catalytic domain). Cellular data suggests that small molecule binding does not perturb protein-protein and palmitoylation-mediated interactions known to maintain basal PLD1 localization, but likely results in a conformational change that inhibits general bulk lipid binding (both PI(4,5)P_2- and non PI(4,5)P_2-mediated interactions). Therefore VU-series compounds inhibit PLD in a bimodal format through binding with high affinity at the linker loop. These compounds disrupt both cell stimulation-induced translocation to the plasma membrane and catalytic activity.

Model of VU-series compound mechanism of inhibition

These studies define the binding site and mechanism of action for the potent and isoform-selective VU-series PLD inhibitors. Using BSI, a novel and highly sensitive method for measuring bimolecular interactions, we demonstrate

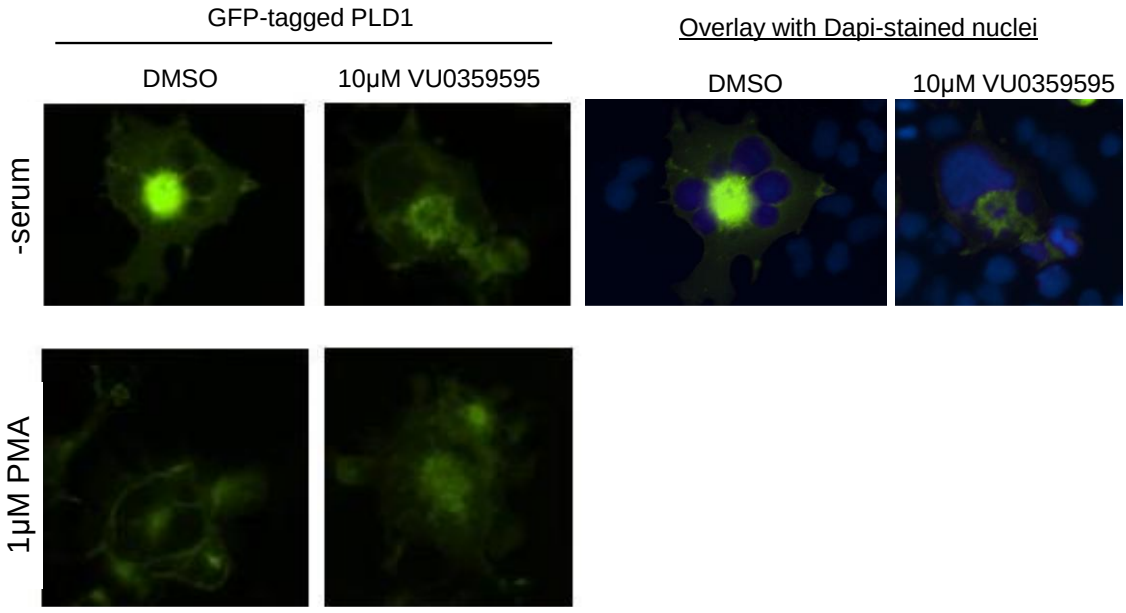


Figure 26. PLD1 basal localization is unperturbed, but stimiulus-induced translocation is blocked by treatment with 10μM VU0359595.

that the VU-series compounds directly interact with purified PLD1 enzyme at a defined loop region resulting in non competitive inhibition of protein-lipid interface interaction, regardless of the interface composition. Through binding at this single high affinity binding site these compounds also allosterically inhibit catalytic activity towards monomeric substrate. Cellular protein localization data supports this mechanism, demonstrating that while basal localization of PLD1 is undisturbed by inhibitory concentrations of VU0359595, stimulation-induced translocation of the protein is inhibited. These results are consistent with reports that protein-protein or palmitoylation mediated interactions maintain basal protein localization, while lipid interactions are important for stimulation-induced translocation to the plasma membrane and late endosomes.

The biochemical data herein demonstrates VU-series compounds directly interact with the enzyme at a high affinity binding site within a loop region at the junction between the PH domain and the start of the catalytic domain. This previously uncharacterized region of the enzyme, herein called the linker loop, shares little homology with a loop region at the N-terminus of the *Streptomyces* PMF PLD and *Streptomyces antibioticus* PLD catalytic domains. Through binding at this allosteric site, this class of compounds has tapped into a previously unappreciated regulatory region of the protein that is both specific for mammalian PLD (as bacterial enzymes are unaffected by these compounds) and can be exploited to elicit isoform selectivity. With the composite biochemical and cellular

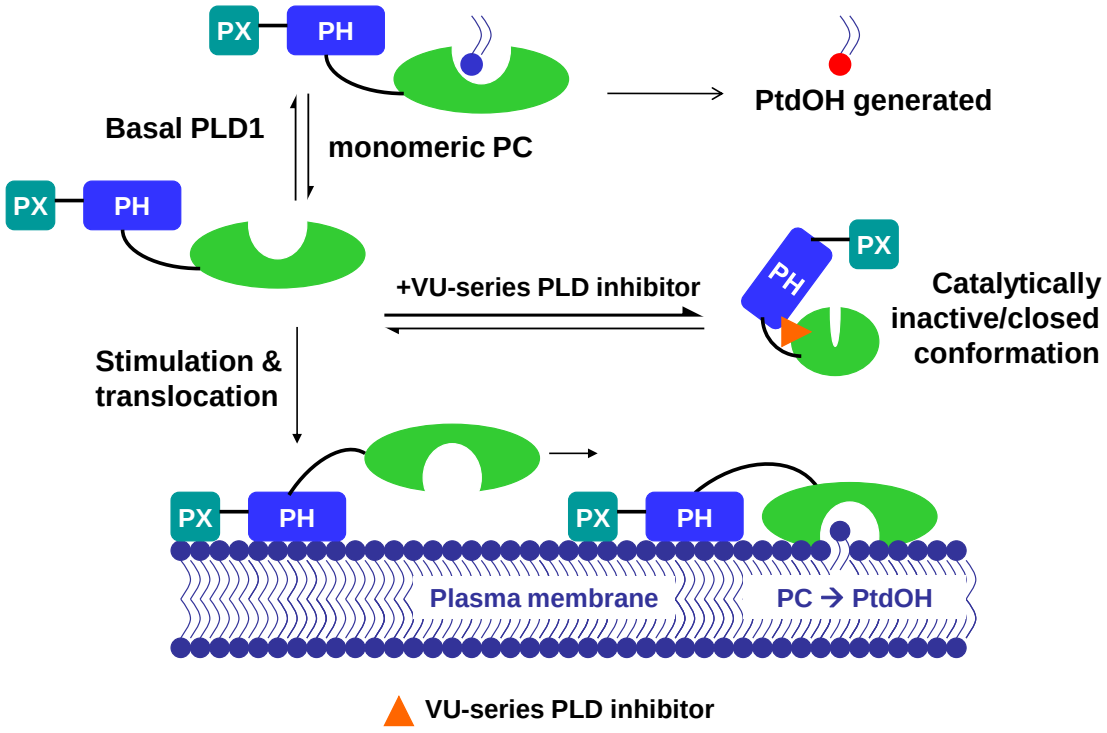


Figure 27. Model of the molecular mechanism of action for the VU-series PLD inhibitors. The VU-series compounds directly bind PLD at a loop region C-terminal to the PH domain to allosterically block both bulk lipid interface interaction and catalytic activity. This is similar to the mechanism of action recently described for the Akt I/II inhibitor, in which small molecule binds at the interface of the PH lipid binding and catalytic domain. This induces a closed protein conformation that prevents translocation to the lipid interface as well as catalytic activity.

data we propose a mechanism of action for the VU-series compounds (figure 27) in which the small molecule directly binds the enzyme at this linker loop resulting in a significant conformational change that inhibits both interfacial interactions (e.g. translocation to plasma membrane) and catalytic activity.

This proposed mechanism is similar to that recently described for a small molecule inhibitor of Akt, a serine/threonine kinase that is activated downstream of the PI3K pathways. Similar to the domain structure for mammalian PLD, Akt consists of an N-terminal PH domain, bilobal kinase domain, and C-terminal hydrophobic motif (HM). This enzyme is basally localized in the cytosol but upon PI3K activation Akt translocates to the plasma membrane where it binds PIP_3 via its PH domain. At the plasma membrane Akt is subsequently activated by two sequential phosphorylation events: PDK1 phosphorylates Akt at threonine 308 (in the activation loop), and mTORC2 subsequently phosphorylates Akt at serine 473. Orthosteric and allosteric inhibitors of Akt have been described that block kinase activity through divergent mechanisms. Orthosteric inhibitors include pan-kinase inhibitors that bind at the active site in an ATP-competitive manner. These compounds do not affect PH-mediated translocation to the plasma membrane, and therefore leave the compound in what is termed the PH-out conformation (figure 28a). Allosteric inhibitors of Akt, including the dual Akt 1/2 inhibitor, inhibitor VIII, block catalytic activity by binding at the interface of the PH and kinase domain, inducing a PH-in conformation (figure 28b). As visualized in a recent crystal structure of this protein-compound complex[294], the benzimidazolone scaffold of inhibitor VIII binds in a cavity within the PH domain

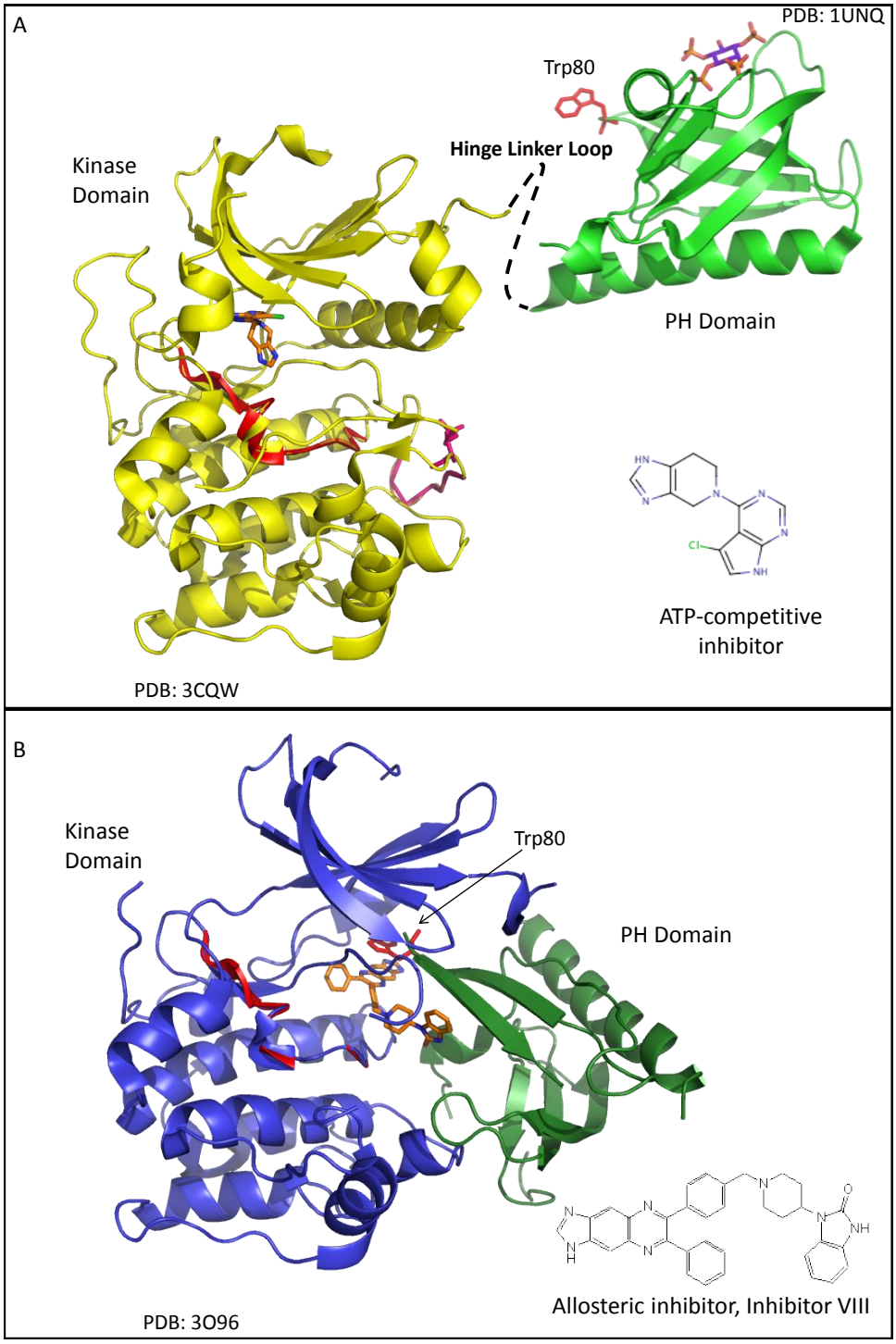


Figure 28. Crystal structures demonstrating the mechanism of orthosteric or allosteric Akt inhibition. *A*, ATP-competitive inhibitors (orange) bind in the orthosteric site alongside the catalytic loop (red). These inhibitors merely compete for ATP, and do not change the overall conformation of the enzyme. In this PH-out conformation, the PH domain (green) is still accessible for PIP_n binding (shown bound to IP4 in this crystal structure) and the activation loop harboring Thr308 in the kinase domain is accessible

for phosphorylation. *B*, Allosteric inhibitors of Akt, such as inhibitor VIII, bind at the interface of the PH and kinase domains. This locks the enzyme in a PH-in conformation in which the PH domain is inaccessible from binding PIP_n and the activation loop (not shown in this structure) is shielded by the PH domain. The significant difference in position of Trp80, integral for inhibitor VIII binding, is shown in both structures.

and the eastern portion of the molecule locks the enzyme in a PH-in comformation that is mediated by hydrogen bonding, for which Trp80 is a notable contributor (as deletion of Trp80 generates an inhibitor resistant enzyme).[293] *In vitro* studies with recombinant full length Akt demonstrate that inhibitor VIII non competitively blocks PIP_3 binding to the PH domain.[293] In cells inhibitor VIII does not change basal localization of Akt, but stimulus-induced translocation to the plasma membrane is blocked.

Allosteric Akt inhibitors, including derivatives of inhibitor VIII such as MK2206, are proving highly beneficial as therapeutics. Since these compounds specifically target Akt (unlike the pan-kinase ATP-competitive compounds) and allosterically prevent translocation to the plasma membrane by inducing a conformation in which the PH domain is inaccessible, these compounds are useful against many cancers in which Akt is found constitutively localized to the plasma membrane. In these cancers a somatic mutation of glutamate 17 to lysine allows for PIP_2 rather than PIP_3 mediated translocation, bypassing PI3K signaling.

The inhibitor VIII mechanism of inhibition is strikingly similar to the mechanism of action proposed here for VU-series PLD inhibitors. The VU-series compounds enable isoform-selectivity because they non competitively bind in an allosteric and previously-unappreciated regulatory region of mammalian PLD,

and as such render a novel means of inhibiting a lipid-modifying enzyme. We propose that, similar to the PH-in conformation observed following Akt-inhibitor VIII interaction, the VU-series compounds induce a significant conformational change in the enzyme that prevents both protein-lipid and protein-substrate interaction. Preliminary evidence obtained by a postdoc in the lab (Sarah Scott) also supports this proposed conformational change. A decrease in potency is observed for VU0359595 when a lyso-lipid substrate is used versus a diacyl substrate in the amplex red reagent assay. This suggests that the conformational change in the active site sterically blocks access to bulky diacyl substrates, but less effectively prevents lyso lipid binding.

We go on to propose that the molecular mechanism outlined here for PLD1 may also extend to the PLD2 isoform. Rigorous SAR characterization supports the existence of a single high affinity small molecule binding site for both isoforms. Preliminary *in vitro* data from several PLD2 constructs suggests the VU-series PLD inhibitors bind the enzyme in the linker loop. BSI studies show that the compounds directly interact with PLD2.PXPH domains in a two-site binding model (one high affinity, and one low affinity site) and a catalytic domain construct similar the PLD1.d311 (called PLD2.d308, which maintains the lnker loop) is also inhibited by the VU-series compounds. In contrast to the PLD1 isoform, PLD2 is constitutively localized to the plasma membrane and maintains high basal activity. Therefore it is possible that if a similar mechanism of inhibition applies to PLD2, different patterns of cellular localization might emerge (i.e. basal cellular localization to the plasma membrane may be perturbed).

Further studies are necessary to thoroughly interrogate the mechanism of inhibition for PLD2 and possible differences in functional outcomes of VU-series compound use for each isoform.

This report represents the first detailed mechanistic characterization of the VU-series PLD inhibitors. Using the unique and highly sensitive BSI method for measuring binding affinities, these studies provide insight into the regulation and enzymology of mammalian PLD1. These studies offer a unique model for targeting and inhibiting other lipid signaling enzymes.

Chapter IV

CONCLUSIONS AND FUTURE DIRECTIONS

The PLD superfamily is a diverse collection of enzymes from multiple species that serve a varied range of functional roles and hydrolyze a range of substrates. The common denominator to superfamily members is thought to be a conserved core structure of the catalytic domain that likely means these enzymes share a similar S_N2 reaction mechanism for hydrolyzing phosphodiester bonds. Mammalian PLD serves multiple functional roles in cell signaling pathways highlighted in chapter I. Aberrant mammalian PLD activity has been implicated in disease states including several cancers, neurodegenerative diseases, and thrombosis. Recent studies with RNAi and knockout animals suggest PLD might be a good therapeutic target because healthy animals are unaffected with a single isoform knockout, but disease-related stress-mediated pathways that signal through PLD are perturbed, thereby ablating aberrant PLD-mediated signaling, and preventing the disease-related phenotype.

Efforts described in chapter II detail the identification and characterization of the VU-series PLD inhibitors, the first class of druggable PLD inhibitors specific to mammalian PLD that can selectively inhibit each isoform. Rigorous SAR characterization describes the chemical space and components that elicit isoform selectivity. The studies described in chapter III go on to characterize the

mechanism of action for the VU-series compounds. Assays using BSI were optimized and used to efficiently measure binding to demonstrate these compounds directly inhibit PLD in a noncompetitively bimodal manner. In cells this translates to PLD1 basal localization being unchanged, but stimulated translocation being blocked. This cell-based data supports the biochemical studies detailed in chapter III and suggests some possible ramifications to using these compounds as tools to study PLD signaling. In addition to blocking product formation, as is expected for an enzyme inhibitor, these compounds likely change protein-protein interactions that occur at the plama membrane thereby disrupting the role of PLD1 as a scaffolding protein. It is important to be aware of the mechanistic effects of applying a small molecule to a whole-cell, and care is necessary when interpreting signaling results. Others in the lab are following up on the effects of the VU-series inhibitors on known protein-protein interactions.

Still to be characterized are protein structural changes that occur in response to small molecule binding. NMR studies of small molecule binding in the linker loop, both alone and in the context of the PH domain, have been initiated in collaboration with Chuck Sanders. Specific residues that are found to be critical for small molecule interactions may explain some of the isoform-selective SAR that has been observed and point to other possible compound modifications that may icrease potency, selectivity, or broaden the chemical diversity of this class of compounds that may prove necessary as whole animal pharmacokinetic studies commence. Ultimately a mammalian PLD protein crystal structure, both with and without inhibitor, will be necessary to truly

understand the conformational changes that occur upon small molecule binding. These structures may further elucidate the molecular mechanism described herein. However, given the position of the small molecule binding site and the noncompetitive bimodal mechanism of inhibition, it is proposed that the small molecule-induced conformational change results in a closed enzyme. This is predicted to be similar to the Akt-inhibitor-induced conformational change where the N-terminal PH domain folds over the catalytic kinase domain preventing access to both lipid binding sites and the orthersteric site.

In collaboration with Eric Dawson, homology modeling efforts are currently underway to model the mammalian PLD1 catalytic domain onto the *Streptomyces* bacterial PLD structures. The linker loop has preliminarily been modeled into position in reference to the 1st HKD motif of the catalytic domain (figure 29), however this loop shares little homology with any sequence in the bacterial enzyme, and therefore *de novo* modeling and more structural restraints are necessary in order to model the accurate orientation of this loop region in relation to the catalytic domain as well as the possible conformational changes that occur upon small molecule binding. Biochemical data suggests His338 and Trp354 are integral for small molecule binding in the linker loop, but these residues are quite a distance apart according to both linear sequence and preliminary homology modeling of the linker loop (roughly 19 , figure 29). Based on the significant conformational changes that occur in Akt upon inhibitor VIII binding (figure 28), it is proposed that in the tertiary PLD1 small molecule binding

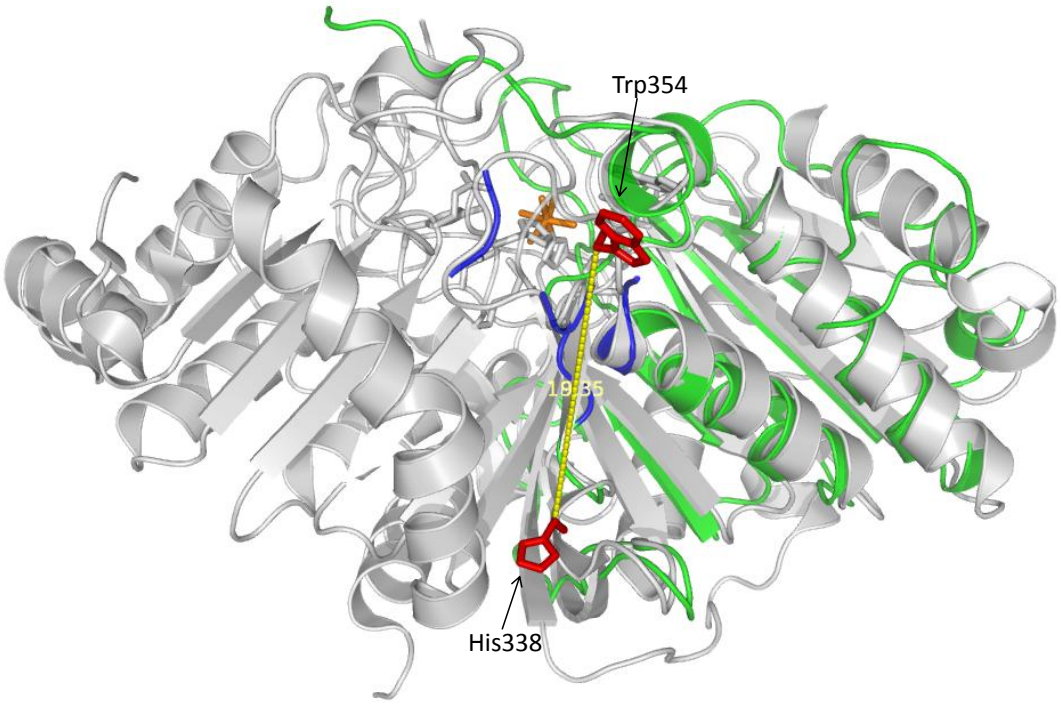


Figure 29. Homology model of human PLD1 (green, amino acid 312 through first HKD modeled) onto the C-alpha backbone of the bacterial *Streptomyces* PLD crystal structure that is complexed with tungstate (orange, observed in the orthosteric site). Highlighted in blue, the small molecule binding loop is loosely modeled onto an amino-terminal region of the protein. (Model provided by E. Dawson).

site His338 and Trp354 are brought within close proximity of the compound and stabilize the interaction through as yet uncharacterized means.

The biochemical studies herein have enhanced our understanding of mammalian enzymology. Future related studies may consider biochemically characterizing the mechanism of action for other classes of direct PLD inhibitors, regardless of their specificity for PLD in the cellular setting. Such studies might follow up on some preliminary observations made for the SERM class in which raloxifene and 4-OH tamoxifen were determined to be direct inhibitors of PLD1.d311, while tamoxifen indirectly inhibited (figure 30a). This is likely through a known mechanism of tamoxifen partitioning into $PI(4,5)P_2$ membranes and sequestering the lipid binding cofactor/enhancer. CRC studies with tamoxifen and 4-OH tamoxifen also demonstrate that the PLD1 and PLD2 isoforms respond differentially to these small molecules, while PLD1.d311 behaves more similarly to *Streptomyces* PMF PLD, an enzyme that also lacks the PXPH domain (figure 30b). These differential responses suggest the N-terminal lipid binding domains, which are not highly conserved between mammalian PLD isoforms, may play a role in SERM mechanism of inhibition. Early SLV binding studies with raoxifene further support differences in the small molecule effects on the catalytic versus the PXPH domains (figure 30c). Using the SERMs as tools, differences in the mammalian isoforms and the multifaceted functional contributions of both the PXPH and catalytic domains could be explored.

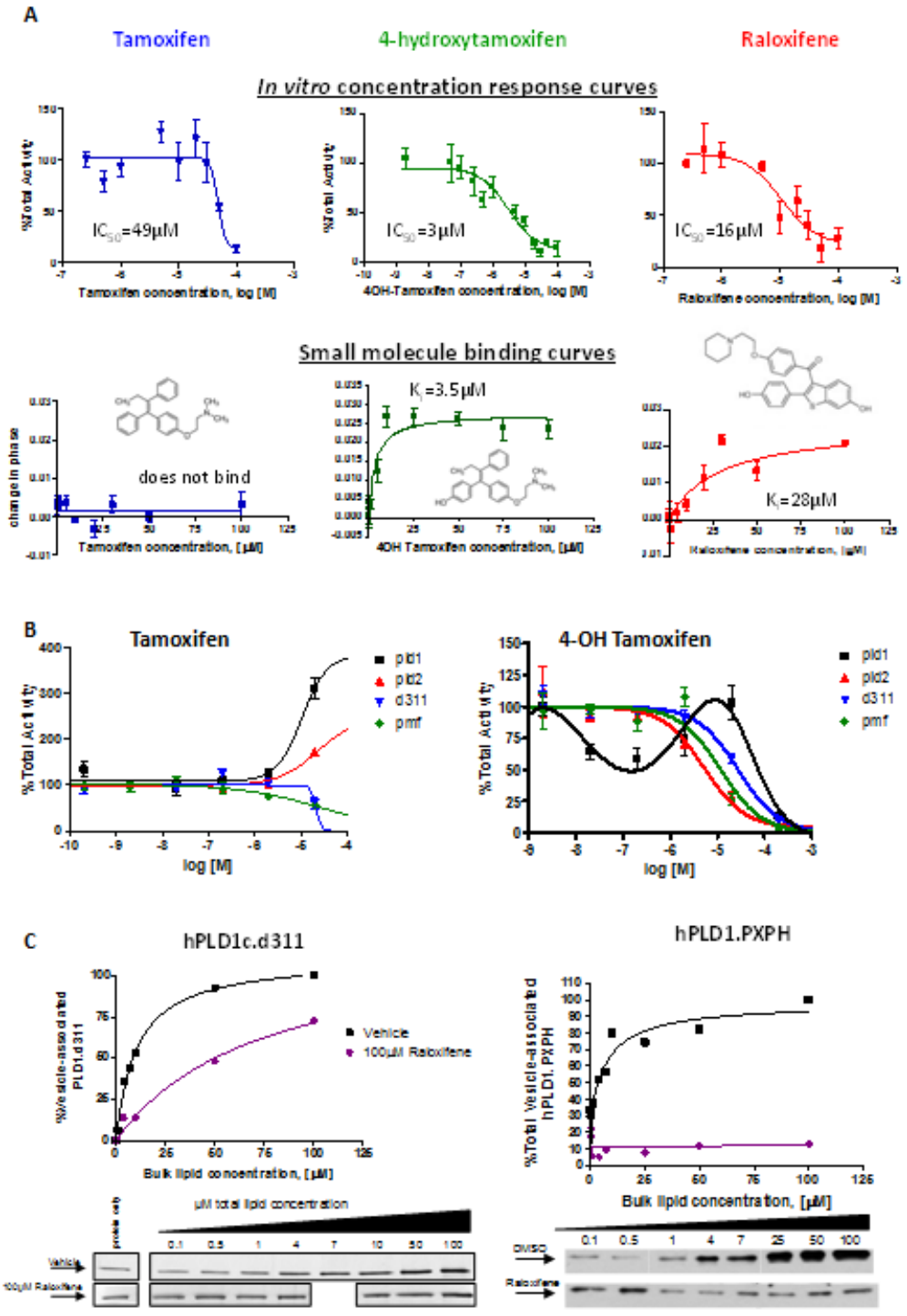


Figure 30. Preliminary mechanistic studies performed with the SERM class of PLD inhibitors. BSI was used to determine whether three representative small molecules from this class of compounds directly inhibited PLD1.d311. BSI-derived K_d were compared to IC_{50} values from biochemical concentration response curves (A). Biochemical concentration response curves were performed for tamoxifen and 4-OH

tamoxifen with purified recombinant enzyme (B). Preliminary SLV experiments were performed with raloxifene to compare the impact of the compound on protein-lipid binding for the PXPH and catalytic domains of the protein (C).

Other interesting small molecule mechanistic findings include differences in the composition of lipids that co-purify with PLD, or differences in gel filtration elution profiles dependent on small molecule present. These findings might contribute to further enzymological and mechanistic understanding of the protein-small molecule interaction (i.e. dodecyl maltoside detergents trigger monomeric and dimeric protein states, tungstate displaces phosphatidylinositolphosphate, VU-series compounds result in protein bleeding off the size exclusion column). This knowledge might also be used in conjunction with self-interaction chromatography (SIC) to identify protein buffer components that facilitate protein self-interactions ideal for protein cyrstalization. SIC measures the second virial coefficient (B_{22}) which describes all possible interactions between two protein molecules in a dilute solution. Proteins aggregate to form crystals in a narrow range of B_{22} values, which can be determined using SIC. Preliminary SIC results, obtained in collaboration with Larry DeLucas, identified reagents that shifted the B_{22} from an initial value of -100 (severely aggregated) to zero (just outside the crystallization slot). Overall yield and separation off gel filtration chromatography was significantly improved in the presence of these additives. Small molecule inhibitors might be ideal components to improve the B_{22} and facilitate protein crystallization.

Overall, the dissertation studies described herein have added to the tools with which we have to biochemically study mammalian PLD and has enhanced

our understanding of its enzymology. Completion of these studies leaves us with a novel class of potent and isoform-selective small molecules that specifically target mammalian PLD, for which we have determined the mechanism of action and region of the protein that encompasses the high affinity small molecule binding site. In characterizing these novel compounds we have optimized and validated the use of BSI for measuring both protein-small molecule and protein-lipid binding affinities. This technique in combination with the VU-series PLD inhibitors significantly expands our biochemical capabilities and provides excellent opportunities for further characterizing mammalian PLD enzymology.

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