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Structure and Regulation of Human Phospholipase D

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Abstract

Mammalian phospholipase D (PLD) generates phosphatidic acid, a dynamic lipid secondary messenger involved with a broad spectrum of cellular functions including but not limited to metabolism, migration, and exocytosis. As a promising pharmaceutical target, the biochemical properties of PLD have been well characterized. This has led to the recent crystal structures of human PLD1 and PLD2, the development of PLD specific pharmacological inhibitors, and the identification of cellular regulators of PLD. In this review, we discuss the PLD1 and PLD2 structures, PLD inhibition by small molecules, and the regulation of PLD activity by effector proteins and lipids.

Keywords

Phospholipase D; Cell Signaling; Phosphatidic Acid; Cancer; Protein Structure

Introduction

Mammalian phospholipase D (PLD) is a transphosphatidylase that in physiological conditions primarily hydrolyzes the membrane-lipid phosphatidylcholine (PC) to generate the dynamic lipid second messenger phosphatidic acid (PA).(Möhn, et al., 1992) PA signals to a variety of downstream effectors and is involved in a broad spectrum of cellular functions including but not limited to increased cell metabolism, migration, and exocytosis.(Barber, et al., 2018; Jenkins and Frohman, 2005) PLD has been identified as a promising therapeutic target with activity and expression linked to tumor growth and metastasis(Chen, et al., 2012; Gomez-Cambronero, et al., 2018; Roth and Frohman, 2018), neurodegeneration(Lindsley and Brown, 2012), and thrombotic disease.(Elvers, et al., 2010) In addition, human PLD activity has recently been found to be critical for hippocampal axis organization(Santa-Marinha, et al., 2020) and proper cardiac development.(Lahrouchi, et al., Unpublished results)

There are two canonical PLD isoforms in mammals. Human PLD1 and PLD2 are similar in overall structure with 57% amino acid conservation. Both contain conserved tandem PX and PH domains, two HKD catalytic domains that combine to form a single active site, and a conserved C-terminus.(Sung, et al., 1999; Sung, et al., 1999) A flexible loop of

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approximately 140 amino acids between the two catalytic HKD domains distinguishes PLD1 from PLD2.

Despite the high structural similarity, the cellular localization and regulation of the two isoforms differs significantly (Fig. 1). Localization of the enzymes varies based on cell type and the effect of overexpression, but in general PLD1 localizes to intracellular membranes and upon activation translocates to the plasma membrane. In contrast, PLD2 constitutively resides at the plasma membrane and undergoes translocation to internal cellular membranes upon signaling events.(Jenkins and Frohman, 2005)

Mammalian PLDs depend on the membrane-lipid phosphatidylinositol-(4,5)-bisphosphate (abbreviated PI(4,5)P_2) as a cofactor. PLD1 is activated by several protein effectors (Protein Kinase C and members of the Arf and RhoA small G-protein families) both *in vitro* and *in vivo*.(Hammond, et al., 1997) PLD2 is generally considered constitutively active with the same protein activators displaying little effect on PLD2 activity.(Colley, et al., 1997)

Knowledge of the biochemical mechanisms of PLD regulation and the design of a clinically useful PLD inhibitor was previously limited by the lack of structural information. Structures of bacterial PLDs and endonucleases have revealed the general architecture of the PLD catalytic core and reaction mechanism.(Leiros, et al., 2004; Stuckey and Dixon, 1999) 15 years after the publication of the bacterial PLD structure, the crystal structures of plant PLDα and human PLD1 and PLD2 isoforms were all determined within 6 months of each other.(Bowling, et al., 2020; Li, et al., 2020; Metrick, et al., 2020) In contrast to mammalian PLD, bacterial PLDs are constitutively active and are not inhibited by PLD small molecule inhibitors, thus the mammalian structures have provided new insights into mammalian PLD regulation and inhibition.

In this review we compare the human PLD1/2 crystal structures and discuss biochemical insights gleaned from the structures. We then describe the regulation of PLD1 and PLD2 by lipids and proteins.

Structural Information

Overall Structure

Canonical phospholipase D enzymes are defined by the conserved catalytic motif $HxKx_4Dx_6G(G/S)$ (X is any amino acid) commonly referred to as the HKD domain. (Hammond, et al., 1995; Koonin, 1996) The architecture of HKD catalytic domain was first established from structures of the endonuclease Nuc (Fig. 2a).(Stuckey and Dixon, 1999) Nuc contains a single HKD domain that homodimerizes into a symmetrical beta sandwich with a single active site. In contrast, bacterial PLD contains two HKD domains that are within the same polypeptide. The HKD domains of bacterial PLD self-dimerize to form an active site that resembles the homodimer of Nuc (Fig. 2b). The beta sandwich core of both Nuc and bacterial PLD are flanked by peripheral alpha helices, with bacterial PLD containing additional alpha helices and a beta strand that connects the two beta sheets.(Ingar Leiros, 2000)

The general architecture of the HKD beta sandwich core is conserved in human PLD1, PLD2, and plant PLDα, however additional elements create a more elaborate structure (Fig. 2c,d,e).(Bowling, et al., 2020; Metrick, et al., 2020) Most of the novel structural elements are found in the C-terminal region that is not conserved with Nuc or bacterial PLD. For example, there are two unique beta hairpins of unknown function that are found on the membrane and cytoplasmic faces of the enzyme. The membrane interface hairpin forms one side of a deep pocket of unknown function. The membrane interface also contains several additional flexible loops that form the entrance to the active site tunnel. The two isoforms' structures are highly similar (RMSD = 0.559Å) with the exception of a small alpha helix at the membrane interface of PLD2, which is discussed below. Both PLD1 crystal structures were determined by deleting the distinguishing flexible loop of PLD1, which would be another site of structural variation. In addition, the current set of PLD1/2 structures also lack the N-terminal PX and PH domains that are not found in Nuc, bacterial PLD, and plant PLDα.

The C-termini of all eukaryotic PLD structures form a short six residue alpha helix that is part of the active site tunnel.(Bowling, et al., 2020; Li, et al., 2020; Metrick, et al., 2020) Modification to the eukaryotic C-terminus abolishes PLD activity.(Lerchner, et al., 2006; Sung, et al., 1999; Xie, et al., 2000) The structural basis of this requirement was not known since the crystallized bacterial homolog lacks this feature. A conserved threonine residue in the C-terminus plays two important structural roles. First the carboxylate group of the C-termini interacts with an alpha helix proximal to the active site. Second, the side chain of the conserved threonine residue forms a hydrogen bond with the backbone amide nitrogen of D916 and R917 (PLD2 - D776/R777). Addition of a C-terminal epitope tag or mutation of the final residue would disrupt these interactions and provides a structural explanation for the reported loss of activity. Similar evidence is seen in plant PLDα, where mutation of the C-terminal threonine residue to aspartic acid abolished activity, but mutation to serine, which is able to hydrogen bond with the adjacent alpha helix, retained 20% activity.(Li, et al., 2020)

The Active Site—The PLD active site contains the conserved histidine and lysine residues of the HKD domain whereas the conserved aspartic acids are distant on the exterior of the enzyme. The two conserved histidine residues hydrolyze PC using a ping-pong catalytic mechanism.(Stuckey and Dixon, 1999) In this mechanism, one histidine residue acts as a nucleophile to attack the phosphorous atom of phosphatidylcholine to release choline and form a covalent intermediate between the catalytic histidine and PA. Next, the other conserved histidine activates a water molecule that leads to hydrolysis of the phosphoryl-histidine bond with PA to regenerate the catalytic histidine and release the product PA. The active site lysine residues assist in substrate coordination by interacting with the phosphate of the incoming substrate. In both human isoforms an aspartic acid and a glutamic acid each coordinate the catalytic histidines to potentially increase their nucleophilicity and orient the histidines for proper catalysis.(Bowling, et al., 2020) The general PLD catalytic mechanism was first proposed with the detection of a radioactive phosphoenzyme that indicated a covalent intermediate.(Gottlin, et al., 1998) A co-crystal structure of short chain PA

covalently bound to the histidine residue of bacterial PLD confirmed the catalytic mechanism.(Leiros, et al., 2004)

The initial nucleophilic histidine is bound to a phosphate molecule in one of the PLD1 structures. A water molecule was found above the bound phosphate ready for nucleophilic attack upon activation. The bound phosphate resembles an earlier structure of PC soaked *Streptomyces* bacterial PLD.(Leiros, et al., 2004) The biological importance of the phosphohistidine is still unknown and was not visible in the inhibitor bound co-crystal PLD1 structure.(Metrick, et al., 2020)

Substrate Specificity—Mammalian PLD is substrate selective for phosphatidylcholine. Superimposition of a bacterial enzyme co-crystallized with phosphatidic acid with the human PLD1 structure identified several residues that interact with the glycerol backbone and acyl chains of the incoming PC substrate. Attempts to dock the substrate phosphatidylcholine failed to place the substrate in the active site.(Bowling, et al., 2020) Comparisons with the bacterial PLD and plant PLDα, which are not PC specific, do not reveal any unique residues responsible for the PC selectivity.(Leiros, et al., 2004; Li, et al., 2020) The inability of two independent groups to satisfyingly dock the substrate PC to identify the choline-interacting residues suggests that a conformational change may be required for hydrolysis to occur.(Bowling, et al., 2020; Metrick, et al., 2020)

Basal Regulation—PLD2 is constitutively active while PLD1 requires additional protein activators. Comparison of the two structures may give insight to PLD1 conformational changes induced by the protein activators. PLD1 contains a flexible loop near the substrate tunnel entrance (PLD1 – residues 827–836, PLD2 – residues 687–696). In PLD2 the same stretch of residues forms an alpha helix that is oriented away from the active site entrance. This is the only significant secondary structure difference between the two isoforms. The alpha helix formation leads to a dilated active site entrance in PLD2 in comparison to PLD1.

Both PLD1 structures are believed to capture the structure of the enzyme in the quiescent, basal state as they lack bound activators. PLD2 is constitutively active and the larger active site entrance may be the structural basis of this difference in regulation. PLD1 interaction with a protein activator may induce a similar alpha helix formation and lead to increased activity. Despite the difference in structure the amino acid sequence of the dynamic loop is completely conserved. Notably, the PLD2 alpha helix displaces a partially conserved flexible loop. One potential hypothesis is that the differing residues in the displaced loop may contribute to the isoform specific regulation of PLD1 vs. PLD2.

The plant PLDα active site entrance is similarly occluded and requires a conformational change for activation.(Li, et al., 2020) The apo-structure of PLDα includes an autoinhibitory alpha helix that blocks the active site tunnel. A co-crystal of short chain PA covalently bound to PLDα revealed that the inhibitory alpha helix unfolds into a flexible loop to allow substrate access. A calcium binding site in the catalytic domain of PLDα is believed to regulate this conformational change. The calcium binding domain and alpha helix are not conserved in PLD1/2, but may demonstrate a similar activation mechanism to the one proposed in this review. Further study is needed to examine possible autoinhibitory effects

from the dynamic loop and potential induced conformational changes from PLD1 protein activators.

Pharmacological Inhibitors

PLD1 and PLD2 have emerged as promising pharmaceutical targets with increased PLD1/2 activity and expression linked to tumor growth, neurodegeneration and various blood disorders.(Bruntz, et al., 2014; Elvers, et al., 2010; Roth and Frohman, 2018) Knockout PLD animal models demonstrate reduced tumor growth and metastasis with few negative health effects.(Roth and Frohman, 2018)

Halopemide, a psychotropic drug, was identified as a PLD1/2 inhibitor in a broad chemical screen.(Monovich, et al., 2007) Optimization of halopemide led to the design of 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), which improved the IC_{50} for PLD2 to 20 nM, a 75-fold improvement in potency. FIPI is now used widely in cell and animal studies of PLD1/2 inhibition.(Frohman, 2015) FIPI phenocopied the reduction in tumor metastasis seen in PLD1 knockout mouse cancer models.(Chen, et al., 2012)

Isoform specific inhibitors have been designed optimizing the same halopemide framework. (Lewis, et al., 2009) Dehalogenation of the FIPI benzimidazolinone scaffold by addition of a chiral methyl on the amide linker, and a racemic trans-phenyl cyclopropane amide led to 1700 fold higher PLD1 specificity. Replacement of the halopemide benzimidazolinone scaffold with oxo-triazaspiro and further optimization conveyed an 80 fold PLD2 specificity. (Lewis, et al., 2009; O'Reilly, et al., 2013; O'Reilly, et al., 2015) The isoform selective inhibitors enable the probing of isoform specific cellular functions and effects on cancerous cells such as PLD2 specific mediation of anti-apoptotic signals.(Bruntz, et al., 2014; Burkhardt, et al., 2015) However, their short half-life in animals has hindered potential pharmaceutical applications.(Scott, et al., 2009) These inhibitors are unable to inhibit the bacterial PLD homolog which has limited structure guided inhibitor design.

The inhibitory mechanism of halopemide derived inhibitors was hypothesized to involve binding to both the PH and catalytic domains.(Ganesan, et al., 2015) Similar binding of a halopemide-derived PLD2 specific inhibitor to full length PLD2 and the catalytic domain alone then suggested the catalytic domain as the sole interaction site.(Metrick, et al., 2020) A co-crystal structure of PLD2 and halopemide revealed that the inhibitor acts competitively, binding deep in the catalytic pocket of PLD2.(Metrick, et al., 2020) Bacterial PLD may be unable to bind human PLD inhibitors due to a comparably shallower active site pocket. Halopemide makes hydrogen bonds with PLD2 residues Q642, N773, and R464. The bound inhibitor also interacts with W364, W365, L514, W519, R777, and other residues through hydrophobic and cation-π interactions.

In addition to the halopemide-based inhibitors, PLD2 co-crystal structures with an additional dual PLD1/2 and PLD2 specific inhibitor with different scaffolding were determined. (Metrick, et al., 2020) The discovery of a novel active site cavity guided the design of a new improved oxo-triazaspiro PLD inhibitor with 8-fold higher potency compared to an inhibitor of similar structure. A PLD1 co-crystal with the designed inhibitor allowed comparison of the isoform specific interactions.

Benzimidazolinone scaffold inhibitors contain an extra bond compared to oxo-triazaspiro inhibitors. The added methyl group on benzimidazolinone scaffolds, which creates PLD1 specificity, extends deeper into the active site pocket due to the additional scaffold bond. The PLD1 isoform was found to have a larger active site pocket that can accommodate the added methyl group, whereas PLD2 residue L514 would clash with the inhibitor. A π-stack of W519 and the inhibitor's naphthalene ring appears to be the structural basis for PLD2 specific inhibitors. In PLD1, F614 is believed to alter the placement of the conserved tryptophan and disrupt the interaction. Residues that contribute to the smaller pocket of PLD2 were mutated to the respective PLD1 residues to expand the space of the active site pocket. This modified PLD2 chimera responded to PLD1 selective inhibitors, thus supporting the proposed structural basis of inhibitor selectivity.

The co-crystal structures have already enabled structure guided inhibitor design and will continue to allow optimization of the halopemide derived inhibitors. In addition, the co-crystal structures now allow *de novo* design of inhibitors with novel scaffolds, expanding the possibilities for PLD inhibition.

Cellular Activators

Lipids

Phosphoinositides—Phosphoinositide lipids facilitate mammalian PLD localization and activity depending on the phosphorylation pattern of the lipid's inositol ring. Mammalian PLDs depend on $PI(4,5)P_2$ for activity and there are two sites that have been identified that bind $PI(4,5)P_2$.(Liscovitch, et al., 1994) One is the N-terminal PH domain, which is required for proper cell localization, but is not required for catalytic activity.(Henage, et al., 2006; Hodgkin, et al., 2000; Sciorra, et al., 1999; Sung, et al., 1999; Sung, et al., 1999) Biochemical assays of the PLD catalytic domain alone showed that it required $PI(4,5)P_2$ for activity, which suggested that there should be an interaction site on the PLD catalytic domain.(Bowling, et al., 2020; Sciorra, et al., 1999; Sung, et al., 1999; Sung, et al., 1999) A mutational analysis originally proposed a polybasic motif in the catalytic domain as the $PI(4,5)P_2$ interaction site.(Sciorra, et al., 1999) However, the PLD1 and PLD2 structures revealed this polybasic motif was distant from the membrane interface calling into question its role as the site of $PI(4,5)P_2$ interaction.(Bowling, et al., 2020; Metrick, et al., 2020)

Both human PLD structures contain a polybasic pocket at the membrane interface (Fig. 3). The basic residues of the pocket are distant in the primary sequence but cluster in the folded protein structure. In the $PI(4,5)P_2$ independent plant PLDα the pocket's basic residues are replaced by acidic and neutral residues incapable of interacting with the negatively charged $PI(4,5)P_2$ head group.(Li, et al., 2020) Mutating the conserved basic residues in PLD1 abolished activity and significantly reduced $PI(4,5)P_2$ membrane binding.(Bowling, et al., 2020) The polybasic pocket may facilitate human PLD regulation through properly orienting the enzyme against the membrane to engage the PC lipid substrate. Co-crystal structures with $PI(4,5)P_2$ or the head group IP_3 could demonstrate the molecular interaction and demonstrate any induced conformational change that might enhance PLD activity, however these are yet to be determined.

The other PI(4,5)P_2 binding site lies in the N-terminal PH domain. The presence of a PH domain in PLD1/2 and PI(4,5)P_2 binding residues (PLD1 – K252/R253/W254, PLD2 – K235/R236/W237) were determined with sequence alignment with other known PH domains.(Holbrook, et al., 1999; Sciorra, et al., 2002; Steed, et al., 1998) Mutation of the conserved basic residues in PLD1 reduced basal and stimulated activity in COS-1 cells. (Hodgkin, et al., 2000)

The PLD PH domain structure has yet to be determined. The PH domain fold is characterized by an antiparallel beta sheet followed by one or two alpha helices.(Yamamoto, et al., 2020) Canonical PH domain phosphoinositide binding is carried out by a KX_n(K/R)XR sequence motif between the first and second beta strands in the beta sheet. The PLD PH domain lack this exact motif but the loop between the two predicted beta strands contains several basic residues that may facilitate PI(4,5)P_2 binding.

PH domain cysteine residues are post-translationally modified by palmitoylation to add a lipid anchor to further facilitate membrane binding.(Du, et al., 2003; Sugars, et al., 1999) Mutation of the PLD1 cysteine residues to serine, removing the lipid anchors, changes the cell localization to the cytosol while retaining the ability for translocation to the plasma membrane upon stimulation.

PI(3,4,5)P_3 stimulates PLD activity to a lesser extent compared to PI(4,5)P_2, while other PIPs have significantly less stimulatory properties.(Hammond, et al., 1997; Liscovitch, et al., 1994) The N-terminal PLD1 PX domain was found to bind to several different phosphoinositides with rat PLD1 PX domain binding PI(3,4,5)P_3 with the highest affinity of the phosphoinositides tested.(Lee, et al., 2005; Stahelin, et al., 2004) Similar to the PH domain, removal of the PX domain affects cell localization and *in vivo* activity while not disturbing *in vitro* activity.(Du, et al., 2003) PLD1 residues R118, F120, and R179 are believed to facilitate PI(3,4,5)P_3 binding and membrane interaction.(Du, et al., 2003; Lee, et al., 2005) In PLD2 R118 and F120 are replaced by a lysine and tyrosine residue respectively.

The PX domain fold is three antiparallel β-strands followed by three alpha helices. Residing between the first and second alpha helices is a stretch of residues known as the PPK loop. The PPK loop is defined as ψPxxPxK (ψ – large aliphatic amino acids: V, I, L, and M) and acts as the as the binding site for the PX canonical binding partner, PI3P.(Chandra, et al., 2019) Additional residues (RYR) before and after the PPK loop act as the PI3P binding residues. The PX domain of PLD1/2 lacks the lysine residue in the PPK loop, which is replaced by a serine and alanine respectively. In PLD1 the additional PI3P binding residues are replaced by KFR and in PLD2 KYK. The change in residues may alter phosphoinositide specificity.

A study attempting to characterize all human PX domains was unable to purify the PLD1/2 PX domain.(Chandra, et al., 2019) The study organized the human PX domains into groups by structure examination and secondary structure-guided sequence alignment. A group of PX domains was determined that lack the canonical PI3P binding site but instead contain a distinct secondary PI(4,5,)P_2/PI(3,4,5)P_3 binding site. The PLD1/2 PX domain was grouped into this category by sequence alignment lacking any structural information. This secondary

binding site is defined by histidine, tyrosine, and basic residues. Notably this region in PLD1/2 is longer than in all of the other PX domains. PLD1/2 PX domain residues found to be critical to phosphoinositide binding all sit outside this newly defined phosphoinositide binding site. Structural determination and mutational analysis of residues identified in the hypothesized secondary binding site may reveal further biochemical details surrounding the human PLD1/2 PX domain.

The SWISS-MODEL webserver was used to generate homology models of the PLD1/2 PX/PH domain using the yeast Bem3 tandem PX/PH domain (pdb code: 6fsf) as a template (Fig. 4).(Waterhouse, et al.) The two palmitoylated cysteines (PLD1 - C240/241 PLD2 – C223/224) are on a flexible loop not present on the Bem3 PX/PH domain structure. The PSIPRED server predicts short beta strands in this sequence so secondary structure may form in the PLD1/2 enzyme.(Jones, 1999) The bound membrane may also induce secondary structure formation. Both domains hypothesized phosphoinositide binding residues were found to be spatially adjacent despite their distance in the primary sequence. The residues cluster in a membrane facing pocket that may be a single site for phosphoinositide binding. This binding mechanism could resemble the polybasic pocket in the catalytic domain hypothesized to be the site for $PI(4,5)P_2$ binding.(Bowling, et al., 2020) Structural determination of both PX and PH domains are obviously still required to identify the respective PIP binding sites and any interaction between the two membrane binding domains.

Monounsaturated Fatty Acids—While phosphoinositides are direct activators of both PLD isoforms, additional lipids are capable of indirectly stimulating PLD2. The monounsaturated fatty acid oleate was found to specifically stimulate PLD2 activity in a variety of cell types and *in vitro*.(Chalifour and Kanfer, 1982; Kim, et al., 1999; Kobayashi and Kanfer, 1987; Okamura and Yamashita, 1994) Other unsaturated acids including linoleate and arachidonate stimulated PLD2 activity whereas saturated acids such as myristate, palmitate and arachidate are unable to elevate PLD2 activity. PLD1 has been reported to be unresponsive or even inhibited by oleate addition.(Hammond, et al., 1995)

Oleate synergistically stimulates PLD2 with $PI(4,5)P_2$ at low concentrations, but at higher concentrations has an inhibitory effect potentially through substrate dilution. It is unclear how oleate stimulates PLD2 activity and how the activation is selective for PLD2. Direct interaction between PLD2 and unsaturated fatty acids has not been recorded. It is tempting to suspect the addition of unsaturated fatty acids affects the membrane fluidity to potentially alter PLD2 membrane binding or substrate availability. However, this mechanism would most likely affect PLD1 activity as well.

Lysophosphatidylcholine—The inflammatory lipid lysophosphatidylcholine (lyso PC) specifically stimulates PLD2 activity in coronary endothelial and PC12 macrophage cells.(Cox and Cohen, 1996; Gómez-Muñoz, et al., 1999; Yun, et al., 2006) Stimulation is not expected to be direct as lyso PC was unable to activate PLD2 *in vitro*.(Kim, et al., 1999) Lyso PC activation may act through Protein Kinase C as PKC inhibitors or PKC downregulation attenuated PLD stimulation.(Cox and Cohen, 1996; Gómez-Muñoz, et al., 1999) Platelet activating factor receptor (PAF) may also play a role as PAF antagonists such

as pertussis toxin, which prevents PAF from interacting with G protein effectors, reduced lyso PC-mediated PLD stimulation. Further study is required to elucidate why lyso PC is selective for PLD2 stimulation as several of the potential mediating factors are attributed to either PLD1 selective activation or capable of activating both isoforms.

Canonical Protein Activators

Arf: Addition of GTPγS and cytosol was found to stimulate PLD activity in HL60 and mature granulocyte membranes.(Cockcroft, et al., 1994; H.Alex Brown, 1993) GTPγS alone was unable to activate partially purified PLD1 and required cytosol for activation. This indicated a GTP dependent cytosolic factor was responsible for PLD activation. The stimulating factor was purified and sequenced, which identified the small GTPase proteins Arf1 and Arf3.(Brown, et al., 1993; Cockcroft, et al., 1994)

Arf small GTPase proteins belong to the ras GTPase family of proteins and play key roles in vesicular trafficking and cytoskeleton rearrangement.(Kaczmarek, et al., 2017) Mammalian arf proteins are divided into three subclasses by gene organization, class I (Arf1,2,3), class II (Arf 4,5) and class III (Arf6) with arf proteins from each subclass capable of stimulating PLD activity to varying efficacy.(Massenburg, et al., 1994) Arf4 has been reported to specifically activate PLD2 as well.(Kim, et al., 2003) Arf is post-translationally modified with an N-terminal myristate lipid anchor, which in addition to GTP, is required for complete PLD activation.(H.Alex Brown, 1993; Hammond, et al., 1995)

Arf stimulation of PLD activity is specific to the PLD1 isoform.(Colley, et al., 1997) Arf1 coimmunoprecipitates with PLD1 upon incubation with GTPγS, which suggests a direct interaction.(Kim, et al., 1998) Incubation with activated Arf1 increases the catalytic rate constant of purified PLD1.(Henage, et al., 2006) The molecular activation mechanism and interaction site still remains unknown. Activation of various PLD1 constructs has narrowed down the potential site of interaction to the catalytic domain.(Sung, et al., 1999) Arf1 synergistically activates PLD1 with RhoA, which suggests different sites of interaction. (Hammond, et al., 1997; Henage, et al., 2006) Surprisingly when the PLD2 PX and PH domains are truncated the enzyme becomes sensitive to Arf activation, whereas full length PLD2 is relatively resistant to Arf activation.(Sung, et al., 1999) Arf1 interaction and sensitivity can also be induced by the *in vitro* PLD2 activator GM_2 activator protein.(Sarkar, et al., 2001)

The region within Arf1 that facilitates PLD1 activation has been narrowed down to residues 35–94.(Liang, et al., 1997) This region includes the interswitch and switch 1 and 2 regions that undergo significant conformational change upon GTP binding.(Pasqualato, et al., 2001) The combination of myristylation and a membrane sensing N-terminal helix in Arf facilitate membrane interaction, which allows GTP activation.

Further details of the interacting residues are lacking and in need of study to give insight to the isoform specificity and mechanism of activation.(Sung, et al., 1999)

Protein Kinase C: Conventional protein kinase C (PKC) enzymes are activated by diacylglycerol and elevated calcium.(Singh, et al., 2017) Activated PKC then phosphorylates

the serine/threonine residues on a variety of effectors. Addition of the diacylglycerol mimic phorbol 12-myristate 13-acetate (PMA) to cells stimulated PLD activity suggesting a PKC dependent PLD activation pathway.(Billah, et al., 1989; van Blitterswijk, et al., 1991) The PMA dependent PLD activating factor was isolated and confirmed to be protein kinase C. (Singer, et al., 1996) Protein kinase C alpha and beta are the most potent activating isoforms. (Conricode, et al., 1994; Lopez, et al., 1995)

PLD1 is generally thought to be the main PLD effector of PKC, with PKC having little effect on PLD2 activity *in vitro*.(Sung, et al., 1999) However, PLD2 has been reported to be stimulated in Sf9 insect cells with PKCα co-expression.(Siddiqi, et al., 2000) Unique to PLD2, co-expression of the PKCδ isoform also increased activity.

Surprisingly, PLD activation by PKC is ATP independent.(Conricode, et al., 1992; Hodgkin, et al., 1999; Singer, et al., 1996) While PMA stimulation does leads to PLD1 phosphorylation at Thr147, the activation mechanism is suspected to occur through a direct protein-protein interaction of the PKC regulatory domain with the N-terminus of PLD1. (Singer, et al., 1996; Zhang, et al., 1999) Proteolysis of PKC that split the enzyme into the regulatory and kinase domains revealed that the regulatory domain is capable of activating PLD activity by itself.(Singer, et al., 1996) Kinase specific inhibitors such as staurosporine have little effect on PLD activation, whereas PKC regulatory domain inhibitors such as calphostin block PLD activity.(Zhang, et al., 1999) It should be noted calphostin is reported to also directly interact and inhibit PLD.(Zhang, et al., 1999)

The PX domain of PLD1 is believed to be the site of PKC interaction. A study to find the interaction site on PLD1 found that the removal of the 115 N-terminal residues from PLD1 selectively abolished PMA stimulation.(Park and Exton, 1998; Sung, et al., 1999) Supporting this finding, insertion of five residues at PLD1 residue 87 selectively ablated PKC activation *in vitro* and *in vivo*.(Zhang, et al., 1999) Further attempts of point mutations to identify residues of interaction failed to replicate the loss of PKC activity, which suggested that the five residue insertion allosterically alters the protein structure as opposed to directly interacting with PKC. The five-residue insertion did not affect T147 phosphorylation further confirming the phosphorylation-independent activation mechanism.

Further studies are needed to discern the effect of the T147 phosphorylation. The PKC interaction region and PKC mediated activation mechanism may be determined with further structural studies of the N-terminal domains, which have so far eluded structural characterization. A co-structure of PLD1 and PKC may reveal conformational changes induced by the kinase that increase PLD activity. The interaction with PKC may also facilitate increased membrane interaction and proper orientation as activated PKC translocates to the membrane.(Igumenova, 2015)

Rho-Family GTPases: Addition of cytosol to HL60 and rat liver plasma membranes stimulates PLD activity with GTP dependency.(Bowman EP, 1993; Kenneth C. Malcolm, 1994) Purification of the stimulating factor identified RhoA as a GTP dependent PLD activator. The Rho family of GTPases includes RhoA, Cdc42, and Rac and are part of the larger Ras enzyme superfamily that includes the previously mentioned PLD activator Arf.

RhoA GTPases activity is linked to several cytoskeleton rearrangement, cell proliferation, and apoptosis.(Boureux, et al., 2007)

RhoA activation is specific to the PLD1 isoform and increases PLD's affinity for PC. (Henage, et al., 2006; Sung, et al., 1999) The Rho family members cdc42 and rac1 also activate PLD1.(Bae, et al., 1998; Henage, et al., 2006) Upon RhoA activation with GTP, RhoA translocates to the plasma membrane to activate PLD1.(Ohguchi, et al., 1996) Immunoprecipitation and yeast two-hybrid systems have demonstrated that after translocation, RhoA then directly interacts with PLD1 for activation.(Bae, et al., 1998; Su, et al., 2006; Yamazaki, et al., 1999)

Many structural details of the RhoA activation have been documented. Rho family members are post-translationally modified with a geranylgeranyl lipid anchor at the C-terminus.(Bae, et al., 1998) The lipid anchor is not required for PLD interaction but is critical for complete activation.(Bae, et al., 1998; Walker, et al., 2000) The switch I region of RhoA, residues 34–42, changes in conformation upon GTP binding. This switch region is crucial for PLD activation.(Bae, et al., 1998)

A Rho specific insert helix has varying effects on PLD activation depending on the Rho family member. Replacement of the helix in Cdc42 with respective Ras residues does not disturb PLD binding but does block activation with further mutational assay delineating S124 as the key activation residue.(Walker and Brown, 2002; Walker, et al., 2000) Interestingly the Cdc42 Ras-insert chimera also blocked activation by PKC and Arf. While this insert is critical for Cdc42 activation, replacement of the Rho insert helix residues in RhoA and Rac1 had little effect on PLD1 activation suggesting either different mechanisms of binding or activation.(Su, et al., 2006; Walker and Brown, 2002) The insert helix may also have allosteric effects on the structure of Cdc42 interfering with the PLD1 interaction interface.

RhoA was found to interact with residues 712–1074 in the C-terminus of PLD1.(Yamazaki, et al., 1999) Expression of these PLD1 residues as a peptide immunoprecipitates RhoA and specifically prevents RhoA mediated PLD1 stimulation.(Yamazaki, et al., 1999) RhoA in the same study was found not to bind PLD2 or the yeast PLD-homolog Spo14. Several point mutations were identified that disrupted RhoA binding and activation in the C-terminal region.(Du, et al., 2000) The point mutations were later mapped onto the PLD1 crystal structure. They clustered on one face of PLD1, potentially defining the RhoA interaction surface.(Bowling, et al., 2020) The activation mechanism of RhoA still remains unknown which might be revealed with further structural studies capturing the protein-protein complex.

Non-canonical PLD Activators

The canonical and most studied PLD activators are Arf, PKC, and RhoA. The research on the following activators is limited in comparison. Some of the reported activation and interaction studies are disputed, which will be discussed.

Small GTPases

Rheb: The small Ras-family GTPase protein Rheb moderates cell growth through direct interaction and subsequent activation of the metabolic sensor mTOR.(Long, et al., 2005) A secondary pathway of indirect mTor activation by Rheb was discovered through PLD1 activation as PA increases interaction between mTor and its co-factor raptor.(Sun, et al., 2008; Toschi, et al., 2009)

Rheb activation is believed to be PLD1 specific.(Mukhopadhyay, et al., 2015; Xu, et al., 2011) Direct interaction between Rheb and PLD1 is disputed and needs further study. Initial findings indicated direct interaction through a pulldown of PLD1 with GST-Rheb.(Sun, et al., 2008) Purified Rheb activated precipitated HA-PLD1 *in vitro* in a GTP dependent manner. Rheb C181S, a mutant lacking the C-terminal farnesylated lipid anchor had reduced PLD1 activation *in vivo*, similar to the effect seen with RhoA activation. D60K Rheb, a mutant lacking the ability to activate mTor correspondingly abolished PLD1 activation ability. Contrasting these initial findings, a review by Bruntz and colleagues reported an inability to replicate the activation and interaction between Rheb and PLD1 utilizing purified recombinant proteins.(Bruntz, et al., 2014) A potential intermediate protein may be necessary to mediate the interaction and PLD1 activation.

RalA: The oncogenic viral gene v-Src is known to induce ras-mediated PLD activation. (Jiang, et al., 1995) Two ras effectors, Raf-1 and PI3K, were found to not be required for v-Src induced PLD activation leading to the study of another ras effector, the small GTPase RalA.(Jiang, et al., 1995) GST-RalA co-localizes with PLD at the plasma membrane and precipitates PLD activity in a GTP dependent manner.(Jiang, et al., 1995; Luo, et al., 1997; Vitale, et al., 2005) The precipitated PLD was PI(4,5)P_2 dependent and Arf1/3 stimulated, and was confirmed to be PLD1 using PLD1 specific antibodies.(Kim, 1998; Luo, et al., 1997) RalA D49N increased PLD1 precipitation, while deletion of the initial eleven RalA residues strongly reduced interaction.(Jiang, et al., 1995; Luo, et al., 1997) Purified RalA is unable to precipitate purified PLD2, which suggests there is no interaction.(Luo, et al., 1997)

Overexpression of RalA increased PLD activity in v-Src transformed cells, while having no effect on untransformed parental cells.(Jiang, et al., 1995) RalA has a slight stimulatory effect on precipitated PLD1, but stronger synergistic stimulation in combination with Arf1. (Kim, 1998) Arf1 was able to bind to a PLD1-RalA complex, but not to RalA alone. In combination with the synergistic effect, this suggests that there are separate binding PLD1 binding sites. The RalA mechanism of activation appears to be Arf dependent, potentially forming a protein complex that increases the affinity of Arf1 for PLD1.

Kinases

Protein Kinase N: Protein kinase N (PKN) is a serine/threonine kinase that shares a similar kinase domain to protein kinase C.(Oishi, et al., 2001) Structural overlap with protein kinase C, another PLD1 activator, in combination with similar membrane localization led researchers to hypothesize an interaction between PLD1 and PKN.(Oishi, et al., 2001) PLD1 was able to immunoprecipitate PKN, thus confirming the hypothesized interaction. PKN

was able to activate both immunoprecipitated PLD1 and PLD1 in PKN overexpressing COS-7 cells.

Attempts to find the interacting region of PKN found both N-terminal and C-terminal fragments of PKN bound to PLD1. The interacting residues of PLD1 was narrowed down to residues 1–598, which includes the PX, PH, and first conserved catalytic HKD domain. PKN is expected to have a different binding and activation mechanism from PKC as PLD1 interacts not with the shared kinase domain, but instead with the PKC specific regulatory domain.(Mukai, 2003; Singer, et al., 1996) Additionally PKN interacts and is activated by RhoA.(Maesaki, et al., 1999) The PKN-RhoA interaction site does not overlap with the PLD1-RhoA interaction model making a potential complex of the three proteins a possibility.

Ribosomal S6 Kinase 2: Ribosomal S6 Kinase 2 (RSK2) directly interacts and phosphorylates PLD1 residue T147 in the PX domain.(Zeniou-Meyer, et al., 2008) Potassium stimulation of RSK2 leads to subsequent PLD1 activation and leads to the release of growth hormone from neuroendocrine cells.(Zeniou-Meyer, et al., 2008) Erk is believed to be the upstream kinase that activates RSK2, thus activating PLD1 activity.(Zeniou-Meyer, et al., 2009; Zeniou-Meyer, et al., 2008) PLD1 T147A, incapable of phosphorylation, was unable to restore depleted PLD1 activity when stimulated by potassium, while a phosphomimic mutant was able to restore PLD activity and downstream growth hormone secretion. The activation mechanism through PX phosphorylation is unclear, but it may activate PLD1 through the assistance of further protein-protein or membrane interaction. T147 is the same residue phosphorylated after PMA stimulation that is believed to be phosphorylated by PKC.

P38: P38 MAP kinase directly interacts and phosphorylates PLD1 and PLD2.(Natarajan, et al., 2001) While p38 activation by diperoxovanadate increases PLD1/2 phosphorylation and activation *in vivo*, only the increase in phosphorylation was replicated *in vitro*. Stress induced PLD activation is partially inhibited by p38 specific inhibitors.(Banno, et al., 2001) Nerve growth factor induced PLD2 activation and induced neurite growth was found to be downstream of p38 kinase activity.(Watanabe, et al., 2011) The many downstream effectors of the MAPK pathway and inability to replicate PLD activation *in vitro* leads us to suspect another protein is involved in PLD activation.

Aurora Kinase A: Aurora Kinase A (AURA) is a cell division regulatory protein involved in spindle assembly and microtubule nucleation around chromatin. Increased PLD activity and phosphorylation of PLD2 was reported in AURA overexpressing COS-7 cells. (Mahankali, et al., 2015) PLD2 overexpression in COS-7 cells led to increased AURA catalytic activity, which is blocked by the addition of the PLD inhibitor FIPI. A positive feedback loop was proposed by which AURA activation of PLD2 generates PA, a known AURA activator.

Direct interaction between the two proteins was determined through co-immunoprecipitation of PLD2 by anti-AURA antibodies and vice-versa. It is unknown which PLD2 residue is phosphorylated by AURA and how this leads to activation. The AURA phosphorylation

consensus motif is RX(S/T)(L/V/I) with X being any amino acid.(Ohashi, et al., 2006) PLD2 contains one instance of this motif at residue 396. It is partially solvent exposed and resides near the membrane interface.(Metrick, et al., 2020) The serine residue is replaced by a phenylalanine in PLD1, which suggests isoform selectivity. Further study of the AURA-PLD2 interaction is needed potentially to replicate the phosphorylation *in vitro* to determine if any accessory proteins are required.

Cyclin Dependent Kinase 5: Cyclin dependent kinase 5 (Cdk5) is a regulatory serine/threonine kinase involved in brain development and sensory pathways.(Lee, et al., 2008) In diabetic animals, PLD activity is inhibited. Insulinoma cells, a pancreatic tumor cell line that continuously secretes insulin, incubated with the cdk5 inhibitor roscovatine had decreased EGF-dependent PLD activity and insulin secretion. Basal levels of PLD activity and insulin secretion were unaffected. The observed phenotype was also observed in Cdk5 double negative cells.

Purified Cdk5 phosphorylates immunoprecipitated PLD2. The phosphorylation was found to be PLD2 specific as treatment of immunoprecipitated EGF with activated Cdk5 only increased in PLD2 phosphorylation, but not PLD1 phosphorylation.

PLD2, but not PLD1, has a Cdk5 consensus phosphorylation sequence, (S/T)PX(K/R), at residues 134–137. The S134A mutant interacted with Cdk5 but failed to become phosphorylated by EGF stimulated Cdk5 (with the Cdk5 activity confirmed using phospho-Cdk substrate antibody). The S134A mutant also lacked the ability to increase insulin secretion when stimulated with EGF suggesting that the phosphorylation is key to the Cdk5 mediated activation.

Miscellaneous Proteins

C-Terminal Binding Protein 1: C-terminal-binding protein/brefeldin A ADP-ribosylation substrate (CtBP1/BARS) is a metabolic sensor, transcriptional co-repressor, and regulator of membrane fission.(Valente, et al., 2013) The shared role of PLD and CtBP1/BARS in membrane remodeling, specifically macropinocytosis, the formation of endocytic vesicles at ruffling membrane domains, inspired study of the relationship between the two proteins. (Haga, et al., 2009) Knockdown of both PLD1 and PLD2 reduced EGF-induced macropinocytosis with PLD1 knockdown having the stronger effect. CtBP1 was found to co-localize with PLD1/2 in fluorescent microscopy and a direct interaction was shown with immunoprecipitation. Whereas PLD2 co-precipitated with CtBP1 under basal conditions, PLD1 precipitation was serum dependent.

Purified CtBP1/BARS stimulated purified PLD1 activity, whereas there was no effect on purified PLD2.(Haga, et al., 2009) PLD1 activation was synergistic with Arf1, Arf6, and RhoA in a GTP dependent manner, again suggesting different sites of interaction. CtBP1/BARS directly interacts with Arf, thus leaving the potential for a PLD1-Arf-CtBP1/BARS complex.(Paliwal, et al., 2006) PKCα failed to synergistically activate with CtBP1/BARS, potentially due to a shared activation mechanism or interaction site. Phosphorylation of CtBP1 residue S147 by p21 activated kinase is important for membrane translocation. A

S147D phosphomimic produced a twofold higher PLD1 stimulation than the WT protein, which suggested that membrane localization is key to PLD interaction and activation.

Haga and colleagues hypothesize that CtBP1/BARS is the unidentified 50 kDa (CtBP1/BARS – 47 kDa) PLD1 activating cytosolic factor previously found in granulocytes and HL60 cells.(Bourgoin, et al., 1995; Lambeth, et al., 1995) Both the 50 kDa stimulating factor and CtBP1/BARS synergistically activate PLD1 with Arf1 and treatment of cytosolic fractions with anti-CtBP1/BARS antibody leads to the reduction of PLD stimulatory factors around the 50 kDa fraction.

Phosphocofilin: Cofilin is an actin binding protein that regulates and disassembles actin microfilaments.(Yang, et al., 1998) Upon phosphorylation by LIM kinase, the interaction between cofilin and actin is halted, which then mediates PLD stimulation by muscarinic acetylcholine receptors (mAChR).(Han, et al., 2007) A phosphomimic (S3D) version of cofilin also increased mAChR based PLD stimulation. Purified phosphocofilin bound strongly to immobilized GST-PLD1, while the mutation S3A failed to bind. Phosphocofilin and S3A cofilin failed to bind to PLD2, which suggested phosphocofilin selectively interacts and activates PLD1. In addition to increased activity, coexpression of PLD1 and cofilin induced cellular redistribution of PLD1 to the plasma membrane where the two proteins interact as detected by immunoprecipitation between PLD1 and cofilin.

Phosphocofilin activation of PLD1 also occurs *in vitro*. PLD activity is increased 3-fold when cofilin is primed with ATP and LIM kinase. Co-immunoprecipitation of various PLD1 fragments with cofilin narrowed the interaction site to PLD1 residues 585–712 which includes the flexible loop and the second catalytic HKD domain. Interestingly WT cofilin co-immunoprecipitated more strongly than the phosphomimic S3D with the PLD1 fragment 585–712. No other fragments of PLD1 or PLD2 bound cofilin. The PLD1 fragment found to precipitate with cofilin had inhibitory effects on mAChR stimulation but not for other receptors, strengthening this fragment as the phosphocofilin- interacting site.

PEA-15: The 15 kDa death effector domain protein PEA-15 was discovered to be a PLD1 interacting protein in a yeast two-hybrid screen.(Zhang, et al., 2000) PEA-15 bound residues 762–801 of PLD1 in the second catalytic HKD domain.(Doti, et al., 2010) The PEA-15 binding site is distinct from the RhoA binding site as PEA-15 was unable to inhibit RhoA stimulation.(Zhang, et al., 2000) PEA-15 does not directly modulate PLD1 activity but rather increases PLD1 activity through increased PLD1 protein expression levels. A hypothesized model is proposed with PEA-15 acting as a stabilizing factor or folding chaperone increasing the amount of PLD1 available for catalysis. Further study is needed to determine how the binding of PEA-15 potentially stabilizes the PLD enzyme and its biological significance.

Growth Factor Receptor-bound Protein 2: Growth factor receptor-bound protein 2 (Grb2) is involved with Ras signaling and is another promising anticancer therapy target.(Lung and Tsai, 2003) Grb2 binds to the phosphorylated tyrosine residues 169 and 179 of PLD2 via an SH2 domain.(Di Fulvio, et al., 2006) Mutation of either tyrosine to phenylalanine reduces Grb2 binding with the double mutation abolishing binding.(Di Fulvio, et al., 2006) Grb2 is

critical to PLD2 *in vivo* activity.(Di Fulvio, et al., 2007) Grb2 constitutively associates with PLD2 and upon stimulation redistributes PLD2 localization to perinuclear membranes.(Di Fulvio, et al., 2007)

Grb2 is proposed to act as a scaffolding protein that links PLD2 to various proteins. Upon EGF stimulation, the phosphatase PTP1B forms a complex with PLD2 through Grb2 binding and increases PLD2 phosphorylation and activity.(Horn, et al., 2005) EGF stimulation recruits PLD2 and Grb2 to membrane ruffles.(Mahankali, et al., 2011) At the membrane ruffles the actin regulatory Wiskott–Aldrich Syndrome protein (WASp) forms a complex with PLD2-Grb2 through the SH3 Grb2 domains.(Kantonen, et al., 2011) It is hypothesized that the formation of the complex, anchors WASp to the membrane where PLD2 generated PA activates WASp indirectly through the generation of additional $PI(4,5)P_2$ by PI5K.(Kantonen, et al., 2011) Sos is also recruited to the PLD2-Grb2 complex and leads to PLD activation. This potentially links PLD2 activity to the downstream activation of Rac2 and the MAPK pathway.(Di Fulvio, et al., 2006) Upon dephosphorylation of Y169 and Y179, CD45 binds and inhibits PLD2 *in vitro* demonstrating the phosphorylation of PLD2 as a dynamic regulation feature.(Henkels, et al., 2009) Grb2 interaction with PLD1 has not been studied and may be capable of interacting as both tyrosine residues are conserved, however basal cell localization may prevent this interaction.

PLC-γ: Phospholipase C-gamma (PLC-γ) co-immunoprecipitates with PLD2 in EGF stimulated COS-7 cells.(Jang, et al., 2003) The interaction was repeated *in vitro* with purified PLC-γ and purified PLD2, which suggested a direct protein-protein interaction. Precipitation with both GST-PLC-γ and GST-PLD2 fragments revealed that the PX domain of PLD2 binds to the SH3 domain of PLC-γ. PLD2 SH3 binding is specific to PLC-γ, as PLD2 did not bind to the SH3 domains of AbI or CrkI. PLD2 P145L/P148L abolished PLC-γ binding and reduced EGF stimulated activity.

The effect of PLC-γ on PLD2 activity has not been examined *in vitro*, so it is unclear if PLC-γ directly affects PLD2 activity. Additionally, PLC-γ mediates PKC stimulation, which can also activate PLD1. The PLD interaction region is conserved in both isoforms and it would be of interest to study PLC-γ binding and potential activation of PLD1.

Activation Summary

Upon review of the current armamentarium of the PLD activation literature, the general depiction of PLD1 activity being highly regulated and PLD2 as being constitutively active requires a more nuanced view. While the canonical activators Arf1, PKC, and RhoA all appear to be PLD1 specific, there are several lipids and various proteins that have been shown to specifically activate PLD2.

The molecular mechanism of PLD activation remains unknown. The numerous PLD activators detailed in this review interact through the N-terminal PX and PH domains, catalytic HKD domains, and even the PLD1 specific flexible loop. The multiple sites of interaction and reported synergistic activation of Arf1 with other PLD1 activators leads to the hypothesis of multiple mechanisms of activation. Some activators may share a similar

mechanism of activation and PLD modifications may serve as clues to link activators. For example, T147 phosphorylation by PKC and RS6K2 may suggest similar mechanisms.

A common feature of many PLD activators is a dependency on membrane interaction. Removal of the membrane anchors in Arf1 and RhoA lead to a reduction in activation efficacy. Protein Kinase C binds directly to diacylglycerol drawing the protein to the membrane surface. Membrane interaction may assist in increasing the local concentration of the interacting protein partners, thus increasing PLD binding and activation. The membrane may also correctly orient the PLD active site towards the lipid substrate. Localization to membrane domains of higher substrate concentration may also be a potential activation mechanism.

Until further structural and biophysical studies are carried out, the mechanism of PLD activation remains a mystery. A combination of allosteric activation and influences on membrane affinity is the most likely hypothesis to explain the diverse proteins and lipids capable of increasing PLD activity.

PLD Inhibitors

Cytoskeletal Proteins

α-Actinin: An immunoblot assay probing PLD interacting proteins from rat heart lysate identified α-actinin as a PLD2 specific-interacting protein.(Park, et al., 2000) α-actinin binds actin and is key in cytoskeletal regulation and structure. Purified actinin co-immunoprecipitated with purified PLD2 when incubated with PLD antibodies. Actinin and PLD2 were also found to be enriched in the sarcolemmal membranes of heart tissue.

Purified α-actinin inhibited PLD2 in a concentration dependent manner in both $PI(4,5)P_2$ and oleate activated assays. The PLD2 interaction site was narrowed to the N-terminal PX domain as N-terminal deletions (1–185, 1–308) of PLD2 were unable to bind to, or be inhibited by α-actinin. Addition of Arf1 abolished α-actinin inhibition. Arf1 additionally interrupted α-actinin binding, but the interaction sites are not expected to be shared as N-terminal deleted PLD2 is activated by Arf1.(Sung, et al., 1999) Further study is needed to dissect the mechanism of α-actinin inhibition, which might potentially work by blocking of the PLD2 N-terminus from binding to membranes.

β-Actin: Precipitation of PLD2 from rat brain co-precipitated a 43 kDa protein identified as the microfilament cytoskeleton protein β-actin.(Lee, et al., 2001) Precipitation of GST-PLD2 fragments with β-actin narrowed the interacting region of PLD2 with β-actin to residues 613–723. The residues form three solvent exposed alpha helices on a single face of the PLD2 structure.(Metrick, et al., 2020) β-actin was found to interact with PLD1 with equal affinity. Immunostaining and expression of GFP-PLD constructs showed co-localization of β-actin with both PLD isoforms at the plasma membrane.

β-actin inhibits both PLD isoforms in a concentration dependent manner. β-actin reduces binding of the other PLD inhibitor actinin, but differing sites of interaction suggest different inhibition mechanisms. It is unclear how β-actin reduces actinin binding. Incubation with

Arf1 was able to activate PLD2 but did not affect β-actin binding as was seen with actinin binding.(Park, et al., 2000)

Tubulin: The cytoskeleton protein tubulin inhibits PLD2 in a concentration dependent manner.(Chae, et al., 2005) Tubulin co-precipitated with PLD2 overexpressed in COS-7 cells. Utilization of GST-PLD2 fragments narrowed the interaction region to residues 476–612. Purified tubulin inhibited PLD2 in both $PI(4,5)P_2$ and oleate stimulated assays ruling out $PI(4,5)P_2$ sequestration.

Tubulin binding increased with nocodazole incubation, which depolymerizes microtubules thereby increasing free tubulin concentration. Incubation with the microtubule-stabilizing drug taxol decreased PLD2 binding. Paradoxically PLD inhibition inversely correlated with tubulin binding.

Muscarinic receptor activation induces tubulin inhibition of PLD2. (Chae, et al., 2005) Initial PLD activity increases after carbochol mediated muscarinic receptor activation with sharp returns to basal activity levels after 2 minutes. Muscarinic receptor activation has been reported to lead to phosphocofilin mediated PLD1 activation, which may be the source of the initial spike in activity.(Han, et al., 2007) The timeline of reduced activity correlated with binding and co-localization of tubulin with PLD2 at the plasma membrane. Further study is needed to determine the inhibition mechanism and potential interactions with PLD1. A cytoskeleton regulation mechanism may utilize the interplay of PLD1 activation by phosphocofilin and PLD2 inhibition by tubulin.

Gelsolin: PLD purified from rabbit brain co-purified with a 90 kDa stimulatory factor later identified as the cytoskeleton regulatory protein gelsolin.(Steed, et al., 1996) Purified gelsolin was found to stimulate rabbit brain PLD *in vitro*, while the presence of ATP induced inhibitory effects. Direct interaction between PLD and gelsolin was hypothesized as anti-gelsolin antibodies were able to precipitate PLD activity.

A later study identified gelsolin as an inhibitor of PLD1 and PLD2, but surprisingly this mechanism of inhibition was indirect.(Banno, et al., 1999) Purified gelsolin inhibited Sf9 expressed PLD1 and PLD2 activity. The gelsolin inhibition was repeated *in vivo* in 3T3 cells with gelsolin overexpression reducing PLD activity. Precipitation of PLD demonstrated no interaction with gelsolin, contrary to the previous study. The mechanism of inhibition was hypothesized to be the indirect inhibition of the PLD activator protein kinase C through the inhibition of phospholipase C. Gelsolin inhibits PLC *in vitro* and *in vivo* by preventing the generation of diacylglycerol, a PKC activator. Incubation with PMA, a diacylglycerol mimic capable of activating PKC circumvented PLC dependency, increased PLD activity and was unaffected by gelsolin addition. Of note gelsolin incubation also decreased RhoA expression, another potential indirect mechanism to inhibit PLD1 activity.

If gelsolin inhibition of PLD activity is indirect through the consequent inhibition of protein kinase C, it would be expected that the PKC independent PLD2 isoform would be independent of gelsolin inhibition. Instead, gelsolin also inhibited PLD2 activity. Further study is needed to identify the potential several mechanisms of PLD inhibition by gelsolin.

Brain Enriched Proteins

Amphyiphysin: Chromatography of rat brain cytosol identified a PLD inhibitory peak of two proteins later determined to be amphiphysin I and II in a heterodimer.(Lee, et al., 2000) Amphiphysin is enriched in brain tissue and is involved in endocytosis in nerve terminals and outside synapses. Purified GST-amphiphysin I/II both inhibited and co-precipitated with partially purified PLD1 and PLD2 *in vitro*. PLD1/2 *in vivo* inhibition and interaction were repeated in both BHK and PMA stimulated COS-7 cells.

Removing the N-terminal amphiphysin residues (1–373) abolished PLD binding and inhibition. The N-terminal residues contain the amphiphysin BAR domain. The BAR domain is key for membrane interaction and its removal may also have had effects on PLD interaction and binding as opposed to directly facilitating the interaction. The mechanism of inhibition and the site on PLD1/2 for amphiphysin binding is yet to be determined.

AP3/AP180: A PLD inhibitory protein isolated from rat brain cytosol was identified as AP3/AP180.(Lee, et al., 1997) AP3/AP180 is enriched in the brain and facilitates clathrin-mediated intracellular vesicle trafficking. AP3/AP180 binding and inhibition is PLD1 specific.(Cho, et al., 2011)

The PLD1-interacting residues of AP3/AP180 were delineated to residues 310–314 with phosphorylation of S313 being critical to PLD1 binding and inhibition.(Cho, et al., 2011; Lee, et al., 1997) AP3/AP180 binds to the C-terminus of PLD1 (residues 820–1074). Synthetic peptides of AP3/AP180 (residues 310–314) inhibit PMA-simulated PLD1 activity. PMA stimulation of PLD1 is PKC mediated and takes place through the interaction of PKC with the N-terminus of PLD1. This difference in binding sites leads to a hypothetical allosteric mechanism of inhibition or one that potentially affects membrane interaction. The mechanism of inhibition is unknown with further studies needed potentially examining the biochemical kinetics of AP3/AP180 inhibition or effect on PLD1 membrane binding.

Collapsin Response Mediator Protein-2: A blot overlay assay with rat brain cytosol identified a 62 kDa PLD2 interacting protein. The protein was purified and sequenced identifying it as Collapsin Response Mediator Protein-2 (CRMP-2).(Lee, et al., 2002) CRMP-2 is a phosphoprotein activated by semaphorin 3A that is involved in hippocampal neuron growth.(Inagaki, et al., 2001) Overlay of CRMP-2 with GST-PLD2 fragments found that CRMP-2 interacted with residues 65–192 of the PX domain, and residues 724–825 in the C-terminal catalytic domain. PLD2 was found to interact with CRMP-2 residues 243–300. The interaction between PLD2 and CRMP-2 was replicated *in vivo* with immunoprecipitation in transfected COS-7 cells.

CRMP-2 inhibited PLD2 *in vitro* in a concentration dependent manner with a CRMP fragment of residues 243–573 retaining similar PLD2 inhibitory properties to WT CRMP, while residues 301–573 lacked all PLD2 inhibition and did not bind PLD2. PLD2 inhibition was replicated *in vivo* in PC12 cells with co-localization in the distal tips of neurites. Lee and colleagues hypothesize the mechanism of inhibition is disruption of the catalytic domain formation due to the binding of both the N-terminal PH domain and C-terminal catalytic domain. Expression of the catalytic domain alone and structural studies have determined the

catalytic domain folds independently of the PH domain, raising the question of how this mechanism would occur.(Metrick, et al., 2020; Sung, et al., 1999)

Alpha-Synuclein: A 20 kDa cytosolic factor from mouse and bovine brain inhibited purified PLD2.(Jenco, et al., 1998) Purification and sequencing identified the inhibitory factor as alpha-synuclein.(Jenco, et al., 1998) Alpha-synuclein is primarily found in the brain and while its cellular function is relatively unknown, alpha-synuclein is used as a Parkinson's disease biomarker.(Malek, et al., 2014) PLD inhibition by synuclein has been controversial with conflicting reports.

Overexpression of synuclein in HEK cells inhibited PLD1 and PLD2 activity.(Ahn, et al., 2002) Synuclein Y125F, a mutant that prevents tyrosine phosphorylation by Src kinase, increased PLD inhibition.(Ahn, et al., 2002) A phosphomimic Y125D abolished PLD2 inhibition.(Payton, et al., 2004) In contrast, however, a later study found no PLD inhibition, endogenous or overexpressed, by alpha-synuclein in HEK cells.(Rappley, et al., 2009)

Purified PLD2 was inhibited *in vitro* by purified alpha-synuclein in a concentration dependent manner that increased the K_m for bulk lipid.(Payton, et al., 2004) Purified synuclein inhibited PLD1 with either Arf, Rho, Rac, or PKC present.(Jenco, et al., 1998) A later study argued against the *in vitro* inhibitory effect and found a lack of inhibition of either purified PLD1 or PLD2 by purified synuclein.(Rappley, et al., 2009)

Direct interaction between synuclein and PLD1/2 was shown utilizing immunoprecipitation *in vivo* in HEK cells and *in* vitro with purified synuclein and PLD1 from rat brain cytosol. (Ahn, et al., 2002) Precipitation with GST-fragments of synuclein identified the PLD interaction region to residues 1–95(Ahn, et al., 2002). Deletion of residues 56–102 or 130–140 of synuclein were found to reduce synuclein induced PLD2 inhibition *in vitro*.(Payton, et al., 2004) Precipitation of GST-fragments of PLD1 identified the N-terminal PX/PH domains (1–331) as the synuclein interacting region. In contrast, another study failed to co-immunoprecipitate synuclein and PLD2.(Rappley, et al., 2009)

It is apparent that further study is required to examine the potential inhibition of PLD by synuclein. Synuclein and PLD1 were found to associate with protein kinase C and synuclein has been reported to inhibit PKC activity.(Ahn, et al., 2002) Indirect inhibition of PLD1 through inhibition of PKC may be a potential indirect inhibition mechanism.

Munc-18-1: Rat brain PLD coprecipitated with a 67 kDa protein later identified as Munc-18-1.(Lee, et al., 2004) Munc-18-1 is a member of the Sec1 family of proteins and is involved in the regulation of neuron exocytosis.(Tomas, et al., 2008) Munc-18-1 was found to bind to both isoforms of PLD. Munc-18-1 binding with GST-PLD2 fragments narrowed the interaction region to the N-terminal PX domain (167–195). GST-munc-18-1 fragments all bound to PLD2, thus suggesting multiple PLD2 binding sites. A potential mechanism of inhibition may be the disruption of the PX domain with $PI(3,4,5)P_3$ or a protein-protein interaction critical for PLD activity and localization.

Munc-18-1 inhibited PLD activity *in vitro* and *in vivo* when transfected in COS-7 cells. Addition of epidermal growth factor induces a translocation of Munc-18-1 away from the

membrane where PLD resides and alleviates inhibition. Addition of Arf *in vitro* abolished the munc-18-1 inhibitory effect, potentially indicating a shared interaction site.(Lee, et al., 2004) Lee and colleagues hypothesize Arf may mediate the EGF induced disruption of Munc-18-1/PLD interaction as Arf4 was earlier identified as a mediator of EGF induced PLD activation. The binding of PLD is hypothesized to be akin to Munc-18-1 binding to syntaxin, wherein multiple ,munc-18-1 domains form a binding pocket potentially overlapping with PLD activator binding sites.

Miscellaneous PLD Inhibitors

Aldolase: A 40 kDa PLD2 interacting protein from cytosolic rat brain fractions was identified as the metabolic protein aldolase C.(Kim, et al., 2002) The interaction was repeated with co-immunoprecipitation of FLAG-aldolase and PLD2. Pull downs of GST-fusion fragments of PLD2 identified the PH domain as the aldolase interaction site. Aldolase was found to specifically interact with PLD2 PH domain with no binding to PLC, DGK, or Akt1 PH domains.

Aldolase A was found to inhibit PLD2 activity and inhibition increased when incubated with aldolase metabolites. As aldolase was found to interact with the PH domain, which primarily binds to $PI(4,5)P_2$, the predicted mechanism would be to disrupt $PI(4,5)P_2$ binding thereby preventing proper membrane interaction. Surprisingly it was reported that the inhibitory effect was independent of the presence of $PI(4,5)P_2$. Further study will be needed to determine the inhibition mechanism and downstream effects of aldolase-based inhibition.

Gβ1γ: Addition of aluminum fluoride to rat myometrial samples was found to inhibit PLD activity.(Le Stunff, et al., 2000) Aluminum fluoride is a heterotrimeric G protein activator but is incapable of activating small G proteins such as Arf/RhoA. PLD1 inhibition by Gβ1γ was then confirmed *in vitro* using purified proteins.(Preininger, et al., 2006) The Gβ1γ2 complex more potently inhibits PLD activity. Gβ1γ1 and Gβ1γ2 contain different lipid anchors, which may be the contributing factor for inhibitory potential. Gβ1γ1 is farnesylated, while Gβ1γ2 is geranylgeranylated. Geranylgeranylation of RhoA increases PLD activation potential and the specific lipid anchor may increase PLD interaction.

Gβ1γ inhibits both basal PLD activity and notably Arf, PKC, and RhoA stimulated activity. Gβ1γ is expected to interact in the N-terminal PX/PH binding domain, as a truncated construct lacking the N-terminal 311 residues was not inhibited or able to bind Gβ1γ. An N-terminal 311 PLD1-MBD fusion protein bound Gβ1γ in a concentration dependent manner. PLD inhibition was replicated *in vivo* in MDA-MD-231 cells basally and upon PMA or LPA stimulation. As Gβ1γ prevents activation by several classic PLD activators, the proposed mechanism for Gβ1γ mediated PLD inhibition is expected to be independent of protein activation, and potentially through disruption of membrane binding or active site interaction.

Inhibitor Summary

The cellular inhibitors of both PLD1 and PLD2 are lacking in the level of study in comparison to PLD activators. The inhibitory effect of many cytoskeleton proteins including α-actinin, β-actin, and tubulin is of interest, as PLD activity is linked to the migration of

cells utilizing cytoskeleton protrusions. The inhibitory effect may be a part of a negative feedback loop allowing controlled migration. Many of the inhibitors reviewed in this study appear to bind in the N-terminal membrane binding PX/PH domains, where the mechanism of inhibition may affect the membrane binding and cellular localization of PLD. Studies measuring membrane affinity or membrane localization may elucidate potential inhibitory mechanisms.

This review focused on cellular inhibitory factors that directly interact with PLD but several other proteins indirectly inhibit PLD activity through interaction with PLD activators. One example is the $PI(4,5)P_2$ hydrolyzing protein synaptojanin.(Chung, et al., 1997) Hydrolysis of the critical lipid cofactor prevents membrane interaction and thus PLD activity. The recently solved crystal structures open the possibilities of co-crystal structures that detail the binding mechanisms and potential conformational changes that affect activity.

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Abbreviations :

PLD	Phospholipase D
PC	Phosphatidylcholine
PA	Phosphatidic Acid
$PI(4,5)P_2$	Phosphatidylinositol 4,5-Bisphosphate
FIPI	5-Fluoro-2-indolyl des-chlorohalopemide
Lyso PC	Lysophosphatidylcholine
PKC	Protein Kinase C
PMA	Phorbol 12-myristate, 13-acetate
PKN	Protein Kinase N
RSK2	Ribosomal S6 Kinase 2
AURA	Aurora Kinase A
Cdk5	Cyclin Dependent Kinase 5
CtBP1	C-terminal-binding protein
BARS	Brefledin A ADP-ribosylation substrate
Grb2	Growth Factor Receptor-bound Protein
WASp	Wiskott–Aldrich Syndrome protein

PLC-γ	Phospholipase C Gamma
EGF	Epidermal Growth Factor
CRMP2	Collapsin response mediator protein 2

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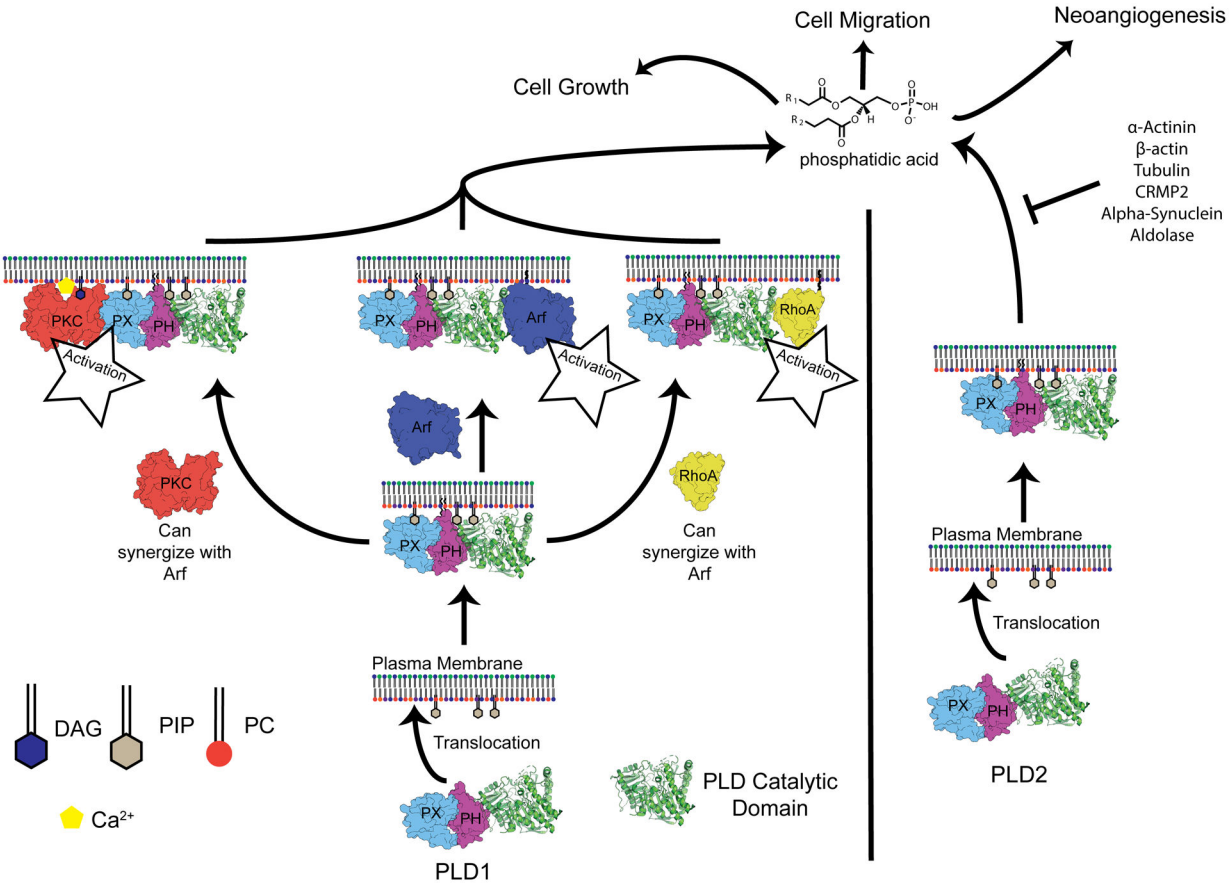

Fig. 1. Activation schematic of PLD1 and PLD2.

PLD1 translocates to the plasma membrane in response to extracellular signals. Interaction with a combination of PI(4,5)P_2, PI(3,4,5)P_3, and protein activators lead to PLD1 activation and the generation of phosphatidic acid. PLD2 translocates to the plasma membrane and the presence of PI(4,5)P_2 and PI(3,4,5)P_3 activate PLD2. Inhibitory proteins listed can halt the generation of phosphatidic acid.

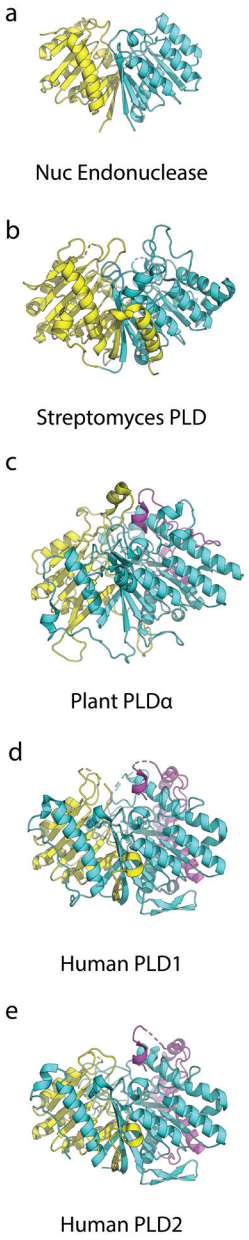

Fig. 2. Structural comparison of PLD family phosphodiesterases catalytic domains.

All enzymes form a catalytic core of a symmetrical beta sandwich flanked by α-helices. a, Two identical subunits of Nuc endonuclease dimerize to form a single active site. (PDB entry 1BYS). b, Streptomyces PLD that self-dimerizes into a single catalytic domain. (PDB entry 1v0y). c. Plant PLDα forms the conserved beta sandwich core with additional asymmetric secondary structures including the eukaryotic specific C-terminal domain. An autoinhibitory alpha helix blocks the active site entrance preventing activity (PDB entry 6kz8). D and e. The human PLD1 and PLD2 catalytic domains contain the beta sandwich core and eukaryotic C-terminal domains. A unique beta hairpin of unknown function is found on the cytoplasmic face. (PDB entry 6u8z and 6ohm respectively). HKD1 (yellow), HKD2 (cyan), Eukaryotic specific C-terminal domain (Magenta)

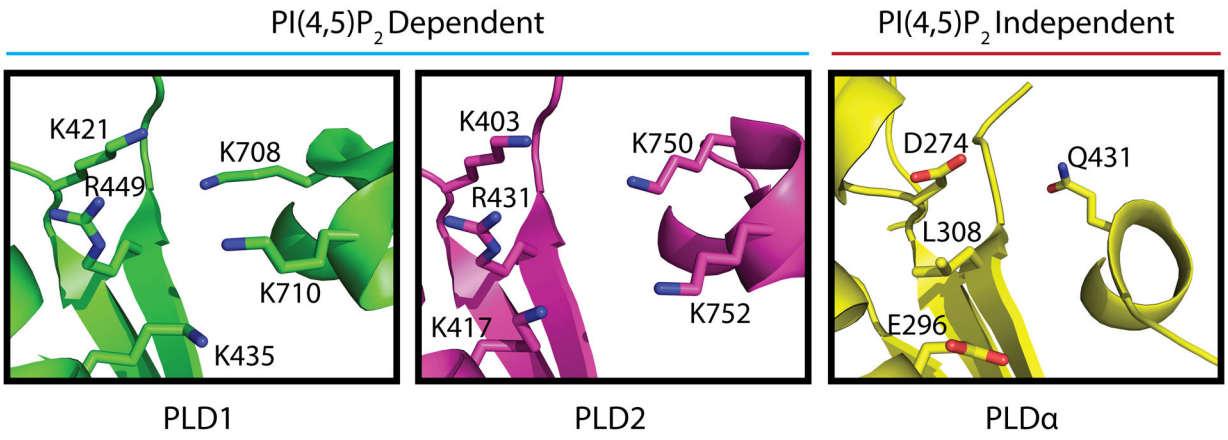

Fig. 3. Comparison of PI(4,5)P_2 binding polybasic pocket in eukaryotic PLDs.

PI(4,5)P_2 dependent PLD1/2 both contain a membrane facing polybasic pocket believed to be the PI(4,5)P_2 cofactor interaction site The same structural motif in the PI(4,5)P_2 independent PLDα lacks the same basic characteristics. The residues are replaced by neutral and acidic residues that are expected to be unable to bind the negatively charged PI(4,5)P_2 head group. Stick representations of the polybasic pocket residues in PI(4,5)P_2 dependent human PLD1 and PLD2. Superimposition of the region in PI(4,5)P_2 independent PLDα.

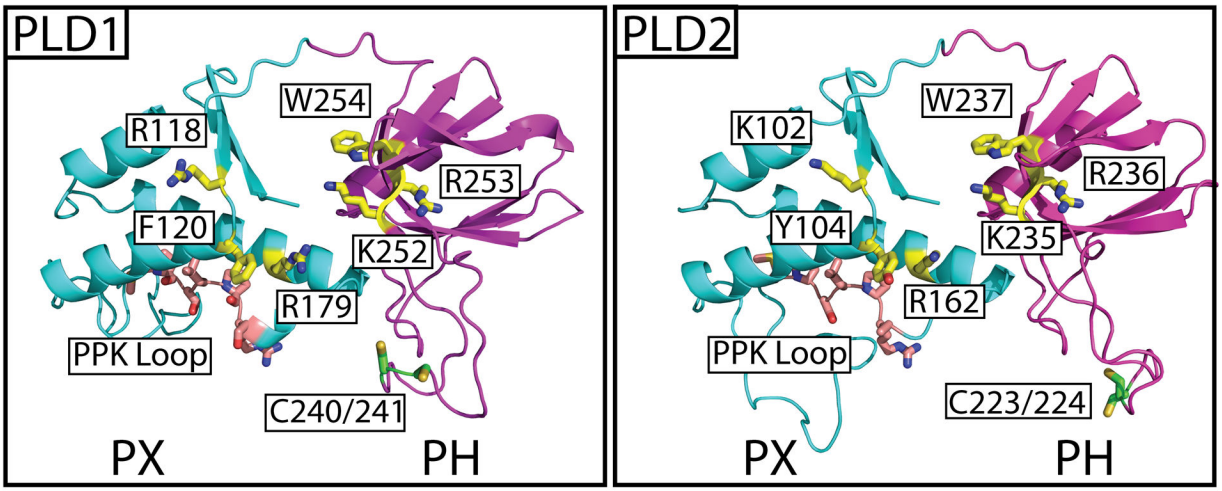

Fig 4. Homology model of PLD1 and PLD2 PX/PH domains.

The tandem PX/PH domain of yeast Bem3 protein (PDB entry 6fsf) was used as the template for homology modeling of the human PLD1/2 PX/PH domains. The generated PX domain contains two beta strands followed by three α-helices. The PX domain in PLD1/2 lacks the traditional PPK loop with the final lysine residue replaced by a serine and alanine respectively. The PH domain contains an antiparallel beta sandwich followed by a single α-helix. The residues that are critical to phosphoinositide binding cluster between the two domains. The PH domain palmitoylated cysteines are found on a long flexible loop not conserved in the Bem3 model thus the homology model may not properly represent this region. PX domain (cyan), PH domain (magenta), residues critical for phosphoinositide binding (yellow), PX domain PPK loop (salmon), palmitoylated cysteines (green).