authors suggested a complex regulation patterns between PLD, PA, and their binding partners. This paucity of intersection indicates that indeed, PLD is an enzyme that cannot be confined to the sole actions derived from its enzymatic activity and, as I will discuss later, the protein-protein interactions involving the whole PLD or parts of the PLD molecular are central to PLD signaling, particularly in cell migration.

A further interest in this PA-PLD topic has become highlighted by the discovery of PLD2 as a GEF that makes more challenging a demarcation between lipase-mediated and/or GEF-mediated functions of PLD2 (9). However, the finding that PA regulates the GEF activity of PLD2 adds a further level of sophistication in the regulation of this enzyme that is necessary considering the key role it has in cellular functions.

---

### Figure 1: Classifications of PLDs

**A** Phospholipase D activity with HKD domains

<table>
<thead>
<tr>
<th>PLD isoforms</th>
<th>Modular Architecture</th>
<th>Main features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces species (except chromofuscus)</td>
<td></td>
<td>Streptomyces species (except chromofuscus)</td>
</tr>
<tr>
<td>Plant PLD with C2 (α,β,δ,τ,τ)</td>
<td>PX</td>
<td>“Stress signal” such as drought</td>
</tr>
<tr>
<td>Plant PLD with PXPH (C)</td>
<td>PH</td>
<td>Two HKD motifs</td>
</tr>
<tr>
<td>Model organisms PLDs</td>
<td></td>
<td>Sporulation, Mitosis in yeast, Dicotystellum isoforms are PLDa, PLDb and PLDc</td>
</tr>
<tr>
<td>Spo14 (Yeast), Ce PLD</td>
<td></td>
<td>Varied in 30% homology to mPLD</td>
</tr>
<tr>
<td>DmPLD, DrPLD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLD1</td>
<td>PX</td>
<td>Mammalian PLD1, Two HKD motifs</td>
</tr>
<tr>
<td>PLD2</td>
<td>PX</td>
<td>Mammalian PLD2, Two HKD motifs</td>
</tr>
<tr>
<td>PLD6 (mito PLD)</td>
<td></td>
<td>One HKD motif, Dimerizes during mitofusion, Uses cardiolipin as substrate</td>
</tr>
<tr>
<td>Zuc is mouse isofom</td>
<td>MLS</td>
<td></td>
</tr>
</tbody>
</table>

**B** HKD-bearing proteins without phospholipase activity (normally endonucleases)

<table>
<thead>
<tr>
<th>PLD isoforms</th>
<th>Modular Architecture</th>
<th>Main features</th>
</tr>
</thead>
<tbody>
<tr>
<td>K4</td>
<td></td>
<td>Vaccinia virus PLD, Nick joining activity (endonuclease)</td>
</tr>
<tr>
<td>Nuc</td>
<td></td>
<td>Bacterial e.g. Salmonella (pyhimmureum), One HKD motif, Endonuclease activity (cleaves dsDNA non-specifically)</td>
</tr>
<tr>
<td>Bfil</td>
<td></td>
<td>Bacterial e.g. Clostridium acetobacter, Homologous to E Coli DNA helicase, Cleaves double stranded DNA specifically</td>
</tr>
<tr>
<td>PLD3 (Hu-K4)</td>
<td></td>
<td>Human homolog of K4, Consists of Transmembrane (TM) domain, Protein has endonuclease activity</td>
</tr>
<tr>
<td>PLD4</td>
<td></td>
<td>Consists of Transmembrane (TM) motif, Localization in Golgi</td>
</tr>
<tr>
<td>mPLD5</td>
<td></td>
<td>Low HKD homology, Not well described</td>
</tr>
<tr>
<td>Zuc</td>
<td></td>
<td>“Zucchini” PLD - Drosophila hologue of PLD6, One HKD motif, Essential for piRNA regeneration</td>
</tr>
</tbody>
</table>

**C** Non-HKD Phospholipases D (dependent on divalent cations)

<table>
<thead>
<tr>
<th>PLD isoforms</th>
<th>Main features</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc-PLD (Streptomyces chromofuscus)</td>
<td>Bacterial, sc-PLD is secreted as virulence factor, Ca2+ dependent</td>
</tr>
<tr>
<td>PLD-A (Arcanobacteria sps)</td>
<td></td>
</tr>
<tr>
<td>PLD-P (Corynebacterium sps)</td>
<td></td>
</tr>
<tr>
<td>Lyso-PLD</td>
<td>Spider venom PLD, secreted, Causes hemolysis</td>
</tr>
<tr>
<td>Autotaxin</td>
<td>Mammalian lyso-PLD, Uses lyso-PC as substrate and produces LPA and cLPA</td>
</tr>
<tr>
<td>GPI-PLD</td>
<td>Uses GPI as substrate instead of PC, Secreted in blood</td>
</tr>
</tbody>
</table>
Further, with the discovery of the GEF catalytic site, it is now possible to use lipase-inactive or GEF-inactive mutants to determine lipase or GEF-mediated functions (11).

**PLD Signaling as a Phosphoprotein and Its Interaction with Tyrosine Kinases**

PLD is a phosphoprotein whose phosphorylation is regulated by kinases and phosphatases (Fig. 3). Protein kinase C (PKC) interacts with both PLD1 and PLD2 and enhances lipase activity (21, 22). PKCδ phosphorylates PLD2 by direct association, thereby aiding in the localization of PLD2 at lamellipodia and promoting integrin-mediated cell spreading (23). A physical association between PLD2 and PLCγ occurs in an EGF-dependent fashion and enhances PLD activity (24). Cdk5-mediated phosphorylation and activation of PLD2 is responsible for EGF-dependent insulin secretion (25). Phosphorylated PLD2 forms a ternary complex with both PTP1b and Grb2, a critical signal transducer of EGFR, which links PLD2 to cellular proliferation and the MAPK and Ras/Erk pathways (26).
Although PLD2 can be phosphorylated by the serine/threonine kinase AKT at residue Thr-175, which serves to up-regulate DNA synthesis, more typically PLD is known as a substrate for many receptor (EGFR and PDGFR) and non-receptor tyrosine kinases (Src and JAK3). Choi et al. (27) have found that PLD2 is specifically phosphorylated on residues Tyr-11, Tyr-14, Tyr-165, and Tyr-470. Phosphorylation targets within the PLD2 molecule have been mapped that are vital to its regulation as a lipase and thus correlated in vitro to at least three different tyrosine kinases, EGFR, Src, and Janus kinase 3 (JAK3) (28), that target Tyr-296, Tyr-511, and Tyr-415, respectively, and that yield either positive or negative effects on the lipase.

Elevation of either PLD1 or PLD2 has the potential to transform rat fibroblasts and contribute to cancer progression of the malignant phenotype in cells that also have elevated levels of EGFR or Src tyrosine kinases (29). Contrarily, it has been hypothesized that PLD2 activity in certain breast cancer cell lines is comparatively low when compared with non-cancerous cells or other breast cancer cell lines because it is down-regulated by tyrosyl phosphorylation at Tyr-296 via EGFR (28). This low level of PLD activity can be increased by in vitro treatment with either JAK3 or Src. Src participates in the activation of PLD through the Ras pathway and the kinases Fyn and Fgr but not Lyn (27).

There are also protein-protein interactions between PLD2 and JAK3, as well as with another tyrosine kinase, FES, which is implicated in the proliferation of breast cancer cells (30). The PLD2-JAK-FES inter-regulation of this lipase and these kinases is implicated in the high proliferation rate of MDA-MB-231 breast cancer cells (30). Additionally, PLD interacts with type-Ia phosphatidylinositol-4-phosphate 5 (PI4P5) kinase. In turn, phosphatidylinositol 4,5-bisphosphate (PIP2) generated by phosphatidylinositol-4-phosphate 5 kinase is essential for PLD activity (31).

The Complex Interaction between Small GTPases with PLD

GTPases regulate PLD activity, and PLD in turn regulates GTPases (32). For GTPases regulating PLD, it was found initially that Arf1 and RaLA directly interact with and activate...
PLD1 (33). Several other GTPases, such as RhoA, RhoB, Rac1, Rac2, and Cdc42, activate PLD. The Switch I region of RhoA directly interacts with the C-terminal region of PLD1 (34, 35). These GTPases must be GTP-bound to stimulate/activate PLD because mutation of the Rho binding site on PLD1 abrogates PLD1/Arf interaction.

There is a dual (positive and negative) effect of Rac2 on PLD2 activity that is implicated in regulation of chemotaxis. Rac2 localizes in vivo at the leading edge of leukocyte pseudopodia, with PLD2 being physically posterior to a wave of Rac2. This impedes the membrane association of PLD2 and thereby inhibits the lipase activity (36). Rac2 has a negative effect on PLD2 gene expression as well (37). Regulation of PLD2 activity by the small GTPase Sar1p is implicated in COPII-mediated endoplasmic reticulum export (38) (39). Further, PLD2 acts as a GTPase-activating factor (GAP) for dynamin (40).

PLD2 Is a GEF

Not only is PLD2 regulated by small GTPases, as just discussed, but PLD2 also regulates GTPases; in fact, PLD2 is a GEF for small GTPases (Fig. 2). PLD2 but not PLD1 is upstream to small GTPases, such as Rac1, RhoA, and Rac2 via its GEF activity or via a PA-dependent manner (9, 10, 23). PLD2 possesses a GEF activity for the small GTPase Rac2 or RhoA (9, 10). After the discovery of the GEF activity of PLD2, PLD2-mediated functions are more challenging in terms of demarcating the lipase- or GEF-mediated functions of PLD2. By extensive mutational analysis, my laboratory discovered the essential amino acid residues for GEF catalysis: Phe-107, Phe-129, Leu-166, Arg-172, and Leu-173 (Fig. 2C) (11). This information is valuable in using either the mutant lipase-inactive PLD2 or the mutant GEF-inactive PLD2 to differentiate between varieties of PLD2-mediated functions. PLD2-GEF activity correlates with Ras activation in highly proliferative and metastatic breast cancer cells (41). This is a very important area to be pursued further, as not only mutations in Ras, but also hyperactivation of Ras, promote tumorigenesis (42).

PLD2 is a GEF with GEF and lipase activities embedded in the N- and C-terminal regions, respectively. Very interestingly, for the dual GEF/lipase activity, the products of the lipase and the GEF reactions regulate the alternate activity. This involves the dual effect of PA on PLD2-GEF activity and a temporal switch in lipase and GEF activities (20).

**WASp, Grb2, and Rac2: The Mechanism by Which PLD Acts on Cell Migration**

PLD is an important player in the regulation of actin cytoskeletal regulation and, as such, a key element for cell migration (Fig. 4). A component of this effect is due to the product of its reaction, PA, and another is through protein-protein interac-
MINIREVIEW: Phospholipase D in Cell Signaling

Phospholipase D (PLD) plays a crucial role in cell signaling by hydrolyzing phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidic acid (PA) and diacylglycerol (DAG). PA can act as a survival signal in cancer and also mediates tumorigenesis. PLD inhibitors have a negative effect on tumor growth and are thus promising candidates for cancer therapy.

PLD in Tumor and Cancer Metastasis

PLD2 overexpression leads to elevated adhesion invasion and metastasis in a lymphoma cell line (65). Further, elevated PLD activity, as well as expression, has been reported in a wide variety of cancers, such as gastric, colorectal, renal, stomach, esophagus, lung, and breast. In addition, a PLD2 gene polymorphism was shown to be prevalent in colorectal cancer, where it was demonstrated that a C→T mutation resulting in Thr→Ile is associated with colorectal cancer. However, lipase activity was not affected with this mutation (66). A clear correlation was observed between PLD2 expression and the tumor size, as well as patient survival, and it has been proposed that PLD2 might be a prognostic indicator in colon cancers (67).

PLD also acts as a survival signal for cancers, such as renal cancer cells where PLD regulates hypoxia-inducible factor 1a (HIF-1a) at the translation level, in a von Hippel-Lindau (vHL)-independent fashion, and promotes cancer cell proliferation (68). In ovarian cancer cells, PLD is shown to be essential for agonist-induced lysophosphatidic acid production and promotes motility, growth, and proliferation (69). PLD2 enhances the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL in lymphoma cells (70).

PLD signaling with other cancer regulators (Ras, PDGF, TGF, and kinases) provides survival signals, thereby promoting tumorigenesis (71). PLD2 is linked to the progression of EWS-Fli sarcoma due to its cross-talk with PDGF-mediated signaling (72). A transmodulation between PLD2 and the oncogenic kinase RET is evident in thyroid cancer cells where PLD2 enhances STAT3 phosphorylation and transcriptional activation (73). A role for kinase-mediated regulation of PLD2 was seen in cell proliferation (74).

Recent Developments in Cancer and PLD Research

Some important clues indicating a role for PLD in cancer were given by the fact that PLD was involved in cell proliferation and in cell invasion. Additionally, it has been demonstrated that active PLD enhances lymphoma cell metastasis, and inactive PLD2 inhibits metastasis (75), MMP-2 expression, and glioma cell invasion (76). PLD2, EGFR, and JAK3 are involved in common pathways that maximize cancer cell invasion (77, 78). Several PLD-specific inhibitors interfere with cancer cell invasion (79). Because of this role of PLD in cell migration, chemotaxis, and cell invasion, the role of PLD in cancer has been significantly expanded.

The last 5–6 years have witnessed an exponential growth in research in PLD and cancer. PLD inhibitors have a negative effect on tumor growth in mice (75, 80, 81). A PLD2-specific inhibitor (ML298) and a dual PLD1/PLD2 inhibitor (ML299) were both found to have a potential role in treating brain cancer (82). FES and JAK3 were found to elevate PLD2 expression, and this interaction was found to be a reason for the elevated proliferation rate of MDA-MB-231 cells (30).

Elevated levels of PA are observed in colorectal tumors, which are driven by the Wnt/β-catenin pathway. In the same study, it has been reported that PLD1 and PLD2 are targets of the Wnt/β-catenin pathway (83–85). A potential therapeutic target for osteolytic bone metastases in lung cancer patients has been proposed (86). PLD inhibitors inhibit the invasion of breast cancer cells in culture or their proliferation (87, 88).

Cell Invasion and Metastasis, Central to the Tumorigenic Potency of PA

As indicated earlier, PLD2 has a direct role in cell migration, and it is also key to cell invasion and metastasis (65, 75, 80).
Knowledge of the particular molecular mechanisms of PLD in cancer tissues now enable us to take advantage of the many new biological tools, and these mechanisms are only now coming to light. A tumorigenic role for PLD2 was established by xenotransplantation of human breast cancer cells into SCID mice (80). Primary tumors from xenotransplanted mice were larger, grew faster, and developed more lung metastases. Micro-osmotic pumps that delivered PLD-specific small-molecule inhibitors were implanted into xenotransplanted SCID mice, which inhibited primary tumor growth and lung metastases. Ablation of PLD1 in the tumor environment compromised the neovascularization and growth of tumors (81). PLD1 deficiency reduced tumor angiogenesis in a xenograft model. In addition, mice lacking PLD1 or treatment with 5-fluoro-2-indolyl deschlorohalopemide incurred fewer lung metastases than did wild-type mice.

Very recent studies have indicated that PLD1 specific inhibitors prefer (S)-configuration on the methyl carbon adjacent to the amide linkage, whereas PLD2 selective inhibitors prefer spiro ring fused with lactam. Based on these factors, 4-aminoypyrazolopyrimidines (used as kinase inhibitors) have been developed, which have IC_{50} values of 5 and 15 nM for PLD1 and PLD2, respectively (89). Although targeting PLD isoforms is the main focus for abrogating the effects of PLD on cancer growth, using indirect inhibitors of upstream regulators of PLD is another approach. Rebamipide, an antiulcer drug, has been shown to inhibit Helicobacter pylori-induced PLD1 expression and activity in gastric cancer cells (90).

Inhibition of PLD2 but not PLD1 or diacylglycerol kinase (DGK) inhibited nuclear ERK activity in a variety of cancer cells, causing a reduction in ERK-targeted gene expression. This suggests that PLD2 is upstream of ERK and that targeting PLD2 will further suppress ERK-mediated cancer cell growth factor signaling (91). Breast cancer cells expressing an oncogene FAM83B have been shown to possess high PLD1 but not PLD2 activity. In addition, PLD1 activity is an essential factor required for the transformation mediated by Ras and FAM83B (92).

One of the major problems in cancer treatment is resistance of cancer cells to chemotherapy and radiation. Radiation in combination with PLD inhibition (PLD1 and PLD2) has been shown to be an efficient way to improve radiosensitivity of the human breast cancer cell line, MDA-MB-231 (93). In agreement with the involvement of PLD in inducing resistance of cancer cells, it has been shown in laryngeal cancer cells that membrane-associated estrogen receptor α36 (ERα36) activates PKC, which in turn enhances PLD activity via estradiol (E2) (94).

Unresponsiveness of cancer cells to upstream chemokines makes them more aggressive. In this context, PLD1/Arf signaling has been demonstrated as one of the key factors that contribute to this unresponsiveness of leukemia cells (95). The activation of PLD improves chemotherapeutic sensitivity via reducing the gene expression of multidrug resistance (96).

The involvement of PLD in inhibiting multidrug resistance (99) is in contradiction with other studies that support the role of PLD in making the cancer cells resistant (98). One possibility might be that this phenomenon of PLD might be cell/tissue- or cancer-dependent mechanism rather than a general mechanism. However, it is essential to confirm the chemotherapeutic sensitivity-promoting nature of the otherwise cancer-promoting PLD2. At any rate, a more conclusive explanation awaits. This is important because a compelling case will be needed for use of PLD inhibitors in the treatment of cancer, even if such information is used to determine which cancers are likely to respond to such inhibitors in a manner that has therapeutic utility, i.e. leading to a stratified approach.

Cancer, Autophagy, and PLD

Despite its role in promoting cancer, the mechanism behind PLD-mediated cancer is not clearly understood, and some subtopics are not entirely settled yet. Take for example the role of PLD in autophagy and cancer. On the one hand, PLD appears to inhibit autophagy (97) because PLD/PA has been shown to activate mammalian target of rapamycin (mTOR), which is an inhibitor of autophagy. Therefore, PLD inhibitors increase autophagy, which in this case leads to cell death. In contrast, another group of researchers (98) has indicated that PLD activates autophagy as inhibition of PLD reduces autophagy, leading to a decrease in cell viability, whereby autophagy might be a protective cell survival mechanism. In addition, these cancers might have different dependence on AKT or mTOR for regulating the cellular outcome of the autophagic response in a particular cancer. This discrepancy might be a result of dependence on cell or cancer type. Because the research on the effects of PLD on autophagy is novel, it is very important to investigate the same in various types of cancers and determine whether it is a general phenomenon or cancer type-dependent.

Remaining Challenges

At least four challenges remain for the immediate future. First, there is no crystal structure of mammalian PLD2 currently. To understand the mechanism underlying the multiple roles of PLD2 as a lipase, GEF, and as a signaling protein by itself via protein interactions, it is essential to obtain a three-dimensional structure of PLD2. This will further facilitate the investigation of PLD2-mediated biochemical functions and develop novel PLD molecule-specific inhibitors or modulators that can be developed to regulate PLD activities/protein interactions.

Second, although PLD2 activity is shown to be necessary for cellular processes like chemotaxis and phagocytosis, deregulated PLD2 levels were reported in several cancers such as breast, colorectal, and renal cancers. All this suggests increasing demand for the understanding of the in vivo mechanisms for which there is an abundant amount of information regarding in vitro and cultured cells, but it remains to be seen which of those are applicable to in vivo cancer studies.

Third, and as studies with autophagy and ARF have amply demonstrated, PLD might be cell/tissue- or cancer-dependent mechanism rather than a general mechanism. Genome sequencing of specific cancer cells derived from patients at several stages of the disease should clarify this, and this should provide a better understanding of which PLD inhibitor (or appropriate therapy) should be followed.

Fourth, it is becoming evident that several lipid enzymes are deregulated in cancer tissues. It will probably not come as a
MINIREVIEW: Phospholipase D in Cell Signaling

The tyrosine kinase Fer is a downstream target of the PLD-PA pathway that regulates cell migration. Sci. Signal. 2, ra52


MINIREVIEW: Phospholipase D in Cell Signaling

phospholipase D2 regulation that explains both the onset and termination of chemotaxis. Mol. Cell. Biol. 31, 2227–2240


MINIREVIEW: Phospholipase D in Cell Signaling


22566 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 289 • NUMBER 33 • AUGUST 15, 2014