Phospholipase D in Cell Signaling: From a Myriad of Cell Functions to Cancer Growth and Metastasis*

Published, JBC Papers in Press, July 2, 2014, DOI 10.1074/jbc.R114.574152
Julian Gomez-Cambronero

From the Department of Biochemistry and Molecular Biology, Boonshoft School of Medicine, Wright State University School of Medicine, Dayton, Ohio 45435

Phospholipase D (PLD) enzymes play a double vital role in cells: they maintain the integrity of cellular membranes and they participate in cell signaling including intracellular protein trafficking, cytoskeletal dynamics, cell migration, and cell proliferation. The particular involvement of PLD in cell migration is accomplished: (a) through the actions of its enzymatic product of reaction, phosphatidic acid, and its unique shape-binding role on membrane geometry; (b) through a particular guanine nucleotide exchange factor (GEF) activity (the first of its class assigned to a phospholipase) in the case of the mammalian isoform PLD2; and (c) through protein-protein interactions with a wide network of molecules: Wiskott–Aldrich syndrome protein (WASp), Grb2, ribosomal S6 kinase (S6K), and Rac2. Further, PLD interacts with a variety of kinases (PKC, FES, EGFR receptor (EGFR), and JAK3) that are activated by it, or PLD becomes the target substrate. Out of these myriad of functions, PLD is becoming recognized as a major player in cell migration, cell invasion, and cancer metastasis. This is the story of the evolution of PLD from being involved in a large number of seemingly unrelated cellular functions to its most recent role in cancer signaling, a subfield that is expected to grow exponentially.

Phospholipase D (PLD)\(^2\) hydrolyzes phosphatidyicholine (PC) to yield phosphatidic acid (PA) and free choline (1). PLD is necessary for normal maintenance of cellular or intracellular membranes (2, 3), and it also participates in several physiological cellular functions, such as intracellular protein trafficking, cytoskeletal dynamics, membrane remodeling, and cell proliferation in mammalian cells and meiotic division and sporulation in yeast (4).

An important characteristic feature of members of the phospholipase D superfamily is the presence of two HKD motifs with the consensus amino acid sequence HX\(^{K,X}_{(6)}\)DX\(^{X}_{(6)}\)GSXN. However, there are exceptions where some of the PLDs lack these motifs and some have only one HKD motif. In Fig. 1, phospholipases are classified as follows: (a) active phospholipases with HKD motifs; (b) phospholipases with HKD motifs that lack lipase activity; and (c) non-HKD phospholipases.

Mammalian PLDs

The two best characterized mammalian isoforms are PLD1 and PLD2 (5–8). Their genes share about 50% homology including two highly conserved phosphatidyltransferase HKD catalytic motifs that are requisite for catalytic activity. PLD1 and PLD2 also have phox homology (PX) and pleckstrin homology (PH) domains (2). A unique characteristic of PLD2 is that it possesses guanine nucleotide exchange factor (GEF) activity for the small GTPases Rac2 and Rho (9–11) (Fig. 2).

The existence of isoforms PLD3, PLD4, PLD5, and PLD6 has been recently reported. All of these PLD isoforms lack PX and PH domains and, therefore, are termed as “non-classical PLDs” (Fig. 1). However, all but PLD6 do have two HKD motifs, whereas PLD6 has only one such motif. PLD3 was originally identified as viral K4L homologue and, hence, named as Hu-K4. Despite the presence of two HKD motifs, no similarity with PLD1 or PLD2 was been found. SAM9 is a murine orthologue of Hu-K4, which is expressed in brain and localized in the endoplasmic reticulum (12), as is PLD4. No activity has been assigned for the products of PLD3 or PLD4 so far (13). PLD6 (also termed mitoPLD) is localized in mitochondrial outer membranes. It is required for mitochondrial fusion during which PLD6 located on one mitochondrial dimerizes with PLD6 located on a second mitochondria and hydrolyzes cardiolipin to generate PA (14, 15).

PLD and Its Product of Reaction, PA, Affect Intracellular Signaling Dramatically

PLD enzymes are involved in a large variety of physiological cellular functions, and I will consider here three molecular mechanisms by which this occurs through their lipase action, through the GEF activity (in the case of PLD2), and through protein-protein interactions that initiate signaling independently of the enzymatic activities. PA is the catalytic product of the lipase reaction with phospholipids in the cell membrane, particularly PC. The biggest dilemma concerning the function of PLD is the lack of clarity over a PA binding site on target proteins and thus understanding of the mechanism of downstream action. This is particularly concerning given the plethora of PA-binding proteins that have been identified. Three studies can be cited where it is indicated that PA binds to the positively charged amino acid residues or surface-exposed hydrophobic residues or both in the target proteins (16–18), but clearly a specific PA binding site is lacking, and once found, the field should advance considerably. Related to this, the integration of PA and PLD has been addressed only in one review by Jang et al (19). The authors found that 9 out of 50 binding partners are common between PA and PLD. Based on this, the

*This work was supported, in whole or in part, by National Institutes of Health Grant by HL056653-14 and 13GRNT17230097 from the American Heart Association (to J. G.-C.). This is the first article in the Thematic Minireview Series “Phospholipase D and Cancer.”

1 To whom correspondence should be addressed: Wright State University School Medicine, Dept. of Biochemistry and Molecular Biology, 3640 Colonel Glenn Highway, Dayton, OH 45435. Tel.: 937-775-3601; Fax: 937-775-3730; E-mail: julian.cambronero@wright.edu.

2 The abbreviations used are: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PH, pleckstrin homology; PX, phox homology; GEF, guanine nucleotide exchange factor; EGFR, EGF receptor; LPP, lipid phosphate phosphatase; WASp, Wiskott–Aldrich syndrome protein; SH, Src homology domain; Arf, ADP ribosylation factor.

**The abbreviations used are:**
- PLD: phospholipase D
- PC: phosphatidylcholine
- PA: phosphatidic acid
- PKC: protein kinase C
- FES: focal adhesion kinase
- EGFR: epidermal growth factor receptor
- GEF: guanine nucleotide exchange factor
- SH: Src homology domain
- HX: hydroxylation
- DX: aspartate
- GSXN: glycine-serine-n-glycine
- HKD: homology domain
- WASp: Wiskott–Aldrich syndrome protein
- SH: Src homology domain
- PX: phox homology domain
- PH: pleckstrin homology domain
- Arf: ADP ribosylation factor
- LPP: lipid phosphate phosphatase
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.
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).

---

**FIGURE 2. Regulation of PLD enzymatic activities.** A, a list of specific regulation(s) of PLD1 and PLD2, the most studied mammalian isoforms. PIP2, phosphatidylinositol 4,5-bisphosphate; CRIB motif, Cdc42/Rac interactive binding motif. B, phospholipase D2 is a dual enzyme that catalyzes a lipase activity, as well as a guanine nucleotide exchange. Shown are the N-terminal PLD2-PX (where part of the GEF activity resides) and the C-terminal HKD1/2 domains (where lipase activity resides). For the GEF reaction, PLD2 causes Rac2-based GDP dissociation upon interaction with Rac2-GDP. In a second step, PLD2 stabilizes nucleotide-free Rac2 until GTP binds, after which PLD2 is released from the complex, leading to the activation of Rac2. For the lipase reaction, the catalytic HKD motifs of PLD2 fold around the substrate phosphatidylcholine (P-Cho). In the first step, a phosphatidyl-histidine intermediate is generated due to a nucleophilic attack of the histidine of the lipases on the phosphate of phosphatidylcholine. In the next step, the hydroxyl group of water attacks the phosphatidyl-histidine intermediate, leading to the formation of phosphatidic acid, at which time the enzyme is regenerated for the next cycle of PC breakdown. C–E, schematic drawings of main domains in the PLD2 structure. C, ribbon model of PLD2-PX domain noting key amino acids needed for the GEF activity. D, ribbon model of PLD2-PH domain that includes CRIB-1 and CRIB-2 needed for interaction with small GTPases (e.g. Rac2). E, ribbon model of PLD2-HKD domain. Serine residues that are mutated for inhibitor studies but that retain lipase activity are highlighted. The structures in C–E were generated by using protein prediction servers such as I-TASSER and Phyre (52). Once the structures were obtained, they were validated using biochemistry data available from both my laboratory and those of published authors in the field.
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).

**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 Rho A 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. 2) (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 dual enzyme 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 interaction.
tion with the intracellular motility machinery. PA regulates actin and leukocyte cell migration because lamellipodia structures and membrane ruffles can be hindered if PLD is inhibited (43). PLD activation plays a vital role in actin cytoskeleton formation (4). ARF6 activation by ARNO stimulates epithelial cell migration through Rac1 and PLD (44), and PLD is necessary for actin localization and actin-based motility in Dictyostelium via phosphatidylinositol 4,5-bisphosphate (45). PLD2 mediates adhesion via regulation of cell surface integrins (46) and is involved in cytoskeletal organization, macrophage phagocytosis and neutrophil recruitment (43, 47, 48). In leukocytes, PA is a chemoattractant that acts via ribosomal S6 kinase (S6K) (49) and Fer (17), and 5-fluoro-2-indolyl des-chlorohalopemide (FIP1) is a PLD inhibitor that alters cell spreading and inhibits chemotaxis (50). DOCK2 is controlled by PLD during neutrophil chemotaxis (51) and, conversely Rac2 controls PLD2 regulation during the onset and termination of chemotaxis (36).

There are at least two ways by which PLD is connected to cell migration. The first involves Rho family GTPases (10, 53). The second way is through PLD-mediated cell migration, which is also regulated by specific protein-protein interactions, such as Grb2, which is a docking protein for PLD2 that is dependent on the SH2 domain of Grb2 and involves Tyr-169 and Tyr-179 of PLD2 (26). Upon interaction, Grb2 promotes lipase activity and regulates the localization of PLD2 (54). PLD2 recruits WASp to the plasma membrane and enhances phagocytic cup formation via Grb2 (55) (Fig. 4C). PLD activity and Rac2 cooperation are increased in macrophages following binding of PLD2 to Grb2, which stimulates actin polymerization and membrane ruffling (56). PLD2/Grb2-mediated chemotaxis and phagocytosis of RAW264.7/LR5 macrophages is dependent upon Grb2 interacting with other proteins, such as Rac2, PTP1b, and especially WASp (54, 57).

**PLD Close Interaction with Other Lipid Enzymes**

PLD-derived PA binds to and regulates sphingosine kinase 1 (SK1) (58). The product of SK1, sphingosine 1-phosphate, acts as a survival signal in cancer and also mediates tumorigenesis (59, 60). More importantly sphingosine-1-phosphate is also involved in transactivation of various growth factors (61) that are upstream of PLD activity. This suggests the possibility of cross-talk between SK1/sphingosine-1-phosphate and possibly PLD/PA pathways that might play a crucial role in cancer progression.

Lipid phosphate phosphatases (LPPs) hydrolyze a variety of phospholipids including PA (62). LPPs possess an inhibitory effect on lysophosphatidic acid-mediated PLD activity (63). Although LPP expression is low, PLD levels are high in a variety of cancers (64). In addition to the PLD inhibitors, the cross-signaling between LPPs and PLD/PA thus seems to be an area of interest in cancer perspective.

**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-aminopyrazolopyrimidines (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
10. Kooijman, E. E., Tieleman, D. P., Testerink, C., Munnik, T., Rijkers, D. T., Burger, K. N., and de Kruijff, B. (2007) An electrostatic/hydrogen bond switch as the basis for the specific interaction of phosphatidic acid with proteins. J. Biol. Chem. 282, 11356–11364
11. Jang, J. H., Lee, C. S., Hwang, D., and Ryu, S. H. (2012) Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners. Prog. Lipid Res. 51, 71–81
12. Mahankali, M., Henkels, K. M., and Gomez-Cambronero, J. (2013) A GEF-to-phospholipase molecular switch caused by phosphatidic acid, Rac and JAK tyrosine kinase that enables leukocyte cell migration. J. Cell Sci. 126, 1416–1428
13. Chen, J. S., and Exton, J. H. (2004) Regulation of phospholipase D2 activity by protein kinase C. J. Biol. Chem. 279, 22076–22083
14. Oishi, K., Takahashi, M., Mukai, H., Banno, Y., Nakashima, S., Kanazato, Y., Nozawa, Y., and Ono, Y. (2001) PKN regulates phospholipase D1 through direct interaction. J. Biol. Chem. 276, 18096–18101
15. Chae, Y. C., Kim, K. L., Ha, S. H., Kim, J., Suh, P. G., and Ryu, S. H. (2010) Protein kinase Cβ-mediated phosphorylation of phospholipase D controls integrin-mediated cell spreading. Mol. Cell. Biol. 30, 5086–5098
16. Huang, H., and Frohman, M. A. (2009) Lipid signaling on the mitochondrial surface. Biochim. Biophys. Acta 1791, 839–844
17. Munck, A., Böhm, C., Seibel, N. M., Hashemol Hosseini, Z., and Hampe, W. (2005) Hu-K4 is a ubiquitously expressed type 2 transmembrane protein. Biochim. Biophys. Acta 1721, 231–235
18. Otani, Y., Yamaguchi, Y., Sato, Y., Furuchi, T., Ikemoto, K., Kitani, H., and Oishi, K. (2009) Identification of the catalytic site of phospholipase D1. Biochim. Biophys. Acta 1791, 1923–1932
19. Jeon, H., Kwak, D., Noh, J., Lee, C. S., Suh, P. G., and Ryu, S. H. (2012) Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners. Prog. Lipid Res. 51, 71–81
20. Mahankali, M., Henkels, K. M., and Gomez-Cambronero, J. (2013) A GEF-to-phospholipase molecular switch caused by phosphatidic acid, Rac and JAK tyrosine kinase that enables leukocyte cell migration. J. Cell Sci. 126, 1416–1428
21. Chen, J. S., and Exton, J. H. (2004) Regulation of phospholipase D2 activity by protein kinase C. J. Biol. Chem. 279, 22076–22083
22. Oishi, K., Takahashi, M., Mukai, H., Banno, Y., Nakashima, S., Kanazato, Y., Nozawa, Y., and Ono, Y. (2001) PKN regulates phospholipase D1 through direct interaction. J. Biol. Chem. 276, 18096–18101
23. Chae, Y. C., Kim, K. L., Ha, S. H., Kim, J., Suh, P. G., and Ryu, S. H. (2010) Protein kinase Cβ-mediated phosphorylation of phospholipase D controls integrin-mediated cell spreading. Mol. Cell. Biol. 30, 5086–5098
24. Lee, H. Y., Jung, H., Jang, I. H., Suh, P. G., and Ryu, S. H. (2008) Cdk5 phosphorylates PLD2 to mediate EGF-dependent insulin secretion. Cell. Signal. 20, 1787–1794
25. Di Fulvio, M., Lehman, N., Lin, X., Lopez, I., and Gomez-Cambronero, J. (2006) The elucidation of novel SH2 binding sites on PLD2. Oncogene 25, 3032–3040
26. Choi, W. S., Hiragun, T., Lee, J. H., Kim, Y. M., Kim, H. P., Chahdi, A., Her, E., Han, J. W., and Beaver, M. A. (2004) Activation of RBL-2H3 mast cells is dependent on tyrosine phosphorylation of phospholipase D2 by Fyn and Fgr. Mol. Cell. Biol. 24, 6980–6992
27. Henkels, K. M., Peng, H. J., Frondorf, K., and Gomez-Cambronero, J. (2010) A comprehensive model that explains the regulation of phospholipase D2 activity by phosphorylation-dephosphorylation. Mol. Cell. Biol. 30, 2251–2263
28. Xu, L., Frankel, P., Jackson, D., Rotunda, T., Boshans, R. L., D’Souza-Schoettle, C., and Foster, D. A. (2003) Elevated phospholipase D activity in H-Ras- but not K-Ras-transformed cells by the synergistic action of RaA and ARF6. Mol. Cell. Biol. 23, 645–654
29. Ye, Q., Kantonen, S., Henkels, K. M., and Gomez-Cambronero, J. (2013) A new signaling pathway (JAK-Fes-phospholipase D) that is enhanced in highly proliferative breast cancer cells. J. Cell. Biochem. 115, 9881–9891
30. Divecha, N., Roefs, M., Halstead, J. R., D’Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K. M., Wakelam, J. M., and D’Antos, C. (2000) Interaction of the type Iε PI3K kinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. EMBO J. 19, 5440–5449
31. Peng, H. J., Henkels, K. M., Mahankali, M., Dinauer, M. C., and Gomez-Cambronero, J. (2011) Evidence for two Crb1 domains in phospholipase D2 (PLD2) that the enzyme uses to specifically bind to the small GTPase Rac2. J. Biol. Chem. 286, 16308–16320
32. Kim, J. H., Lee, S. D., Han, J. M., Lee, T. G., Kim, Y., Park, J. B., Lambeth, J. D., Suh, P. G., and Ryu, S. H. (1998) Activation of phospholipase D1 by direct interaction with ARF-diphosphorylation factor 1 and RaA. FEBS Lett. 430, 231–235
33. Bae, C. D., Min, D. S., Fleming, I., and Exton, J. H. (1998) Determination of interaction sites on the small G protein RhoA for phospholipase D. J. Biol. Chem. 273, 11596–11604
34. Yamazaki, M., Zhang, Y., Watanabe, H., Yokozeki, T., Ohno, S., Kaibuchi, K., Shibata, H., Mukai, H., Ono, Y., Frohman, M. A., and Kanaho, Y. (1999) Interaction of the small G protein RhoA with the C terminus of human phospholipase D1. J. Biol. Chem. 274, 6035–6038
35. Peng, H. J., Henkels, K. M., Mahankali, M., Marchal, C., Bubulaya, P., Dinauer, M. C., Gomez-Cambronero, J. (2011) The dual effect of Rac2 on
phospholipase D2 regulation that explains both the onset and termination of chemotaxis. Mol. Cell. Biol. 31, 2227–2240
37. Speranza, F. J., Mahankali, M., and Gomez-Cambronero, J. (2013) Macrophage migration arrest due to a winning balance of Rac2/Spl repression over β-catenin-induced PLD expression. J. Leukoc. Biol. 94, 953–962
38. Pathe, R., Shome, K., Blumenthal-Perry, A., Bielli, A., Haney, C. J., Alber, S., Watkins, S. C., Romero, G., and Aridor, M. (2003) Activation of phospholipase D by the small GTPase Sar1p is required to support COPII assembly and ER export. EMBO J. 22, 4059–4069
39. Speranza, F. J., Mahankali, M., and Gomez-Cambronero, J. (2013) Macrophage migration arrest due to a winning balance of Rac2/Spl repression over β-catenin-induced PLD expression. J. Leukoc. Biol. 94, 953–962
40. Lee, C. S., Kim, I. S., Park, J. B., Lee, M. N., Lee, H. Y., Suh, P. G., and Ryu, S. H. (2006) The phox homology domain of phospholipase D activates dynamin GTPase activity and accelerates EGFR endocytosis. Nat. Cell. Biol. 8, 477–484
41. Henkels, K. M., Mahankali, M., and Gomez-Cambronero, J. (2013) Increased cell growth due to a new lipase-GEF (Phospholipase D2) fastly acting on Rac. Cell. Signal. 25, 198–205
42. Eckert, L. B., Repasky, G. A., Ulki, A. S., McFall, A., Zhou, H., Sartor, C. I., and Der, C. J. (2004) Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoxia. Cancer Res. 64, 4585–4592
43. Lehman, N. D., Fulvio, M., McCray, N., Campos, I., Tabatabaian, F., and Gomez-Cambronero, J. (2006) Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2. Blood 108, 3564–3572
44. Santy, L. C., and Casanova, J. E. (2001) Activation of ARF6 by ARNO activity is essential for actin localization and actin-based motility in RHO-GEF and the newly described PLD2-GEF.
45. Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N. T., Frohman, M. A., Vitale, N., Bader, M. F., and Grant, N. J. (2006) Dynamics and function of phospholipase D and phosphatic acid during phagocytosis. Traffic 7, 365–377
46. Ali, W. H., Chen, Q., Delgiorno, K. E., Su, W., Hall, J. C., Hongu, T., Tian, M. H., Morris, A. J., and Frohman, M. A. (2009) 5-Fluoro-2-indolyl desulfated neutrophil recruitment. J. Mol. Biol. 385, 677–686
47. Banno, Y., Akao, Y., Tanaka, M., and Nozawa, Y. (2003) Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. J. Mol. Med. 81, 126–131
48. Saito, M., Iwadate, M., Higashimoto, M., Ono, K., Takebayashi, Y., and Takenoshita, S. (2007) Expression of phospholipase D2 in human colorectal carcinoma. Oncol. Rep. 18, 1329–1334
49. Toschi, A., Eidelstein, J., Rockwell, P., Ohh, M., and Foster, D. A. (2008) HIFα expression in VHL-deficient renal cancer cells is dependent on phospholipase D2. Oncogene 27, 2746–2753
50. Luquain, C., Singh, A., Wang, L., Natarajan, V., and Morris, A. J. (2003) Role of phospholipase D2 in agonist-stimulated lysophosphatidic acid synthesis by ovarian cancer cells. J. Lipid Res. 44, 1963–1975
51. Oh, K. J., Lee, S. C., Choi, H. I., Oh, D. Y., Kim, S. C., Min do, S., Kim, J. M., Lee, K. S., and Han, J. S. (2007) Role of phospholipase D2 in anti-apoptotic signaling through increased expressions of Bcl-2 and Bcl-xL. J. Cell. Biochem. 101, 1409–1422
52. Shi, M., Zheng, Y., Garcia, A., Xu, L., and Foster, D. A. (2007) Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras. Cancer Lett. 258, 268–275
53. Nozawa, S., Ohno, T., Banno, Y., Dohjima, T., Wakahara, K., Fan, D. G., and Shimizu, K. (2005) Inhibition of platelet-derived growth factor-induced cell growth signaling by a short interfering RNA for EWS-Fli1 via down-regulation of phospholipase D2 in Ewing sarcoma cells. J. Biol. Chem. 280, 27544–27551
54. Kim, Y. R., Byun, H. S., Won, M., Park, K. A., Kim, J. M., Choi, B. L., Lee, H., Hong, I. H., Park, J., Seok, J. H., Kim, D. W., Shong, M., Park, S. K., and Hur, G. M. (2008) Modulatory role of phospholipase D in the activation of signal transducer and activator of transcription (STAT)-3 by thyroid oncogenic kinase RET/PTC. BMC Cancer 8, 144
55. Di Fulvio, M., Frondorf, K., and Gomez-Cambronero, J. (2008) Mutation of Y179 on phospholipase D2 (PLD2) upregulates DNA synthesis in a PI3K- and Akt-dependent manner. Cell. Signal. 20, 176–185
56. Knoepf, S. M., Chahal, M. S., Xie, Y., Zhang, Z., Brauner, D. J., Hallman,
MINIREVIEW: Phospholipase D in Cell Signaling

M. A., Robinson, S. A., Han, S., Imai, M., Tomlinson, S., and Meier, K. E. (2008) Effects of active and inactive phospholipase D2 on signal transduction, adhesion, migration, invasion, and metastasis in EL4 lymphoma cells. Mol. Pharmacol. 74, 574–584

76. Park, M. H., Ahn, B. H., Hong, Y. K., and Min do, S. (2009) Overexpression of phospholipase D enhances matrix metalloproteinase-2 expression and glioma cell invasion via protein kinase C and protein kinase A/NF-κB/Sp1-mediated signaling pathways. Carcinogenesis 30, 356–365

77. Henkels, K. M., Farkaly, T., Mahankali, M., Segall, J. E., and Gomez-Cambronero, J. (2011) Cell invasion of highly metastatic MTLn3 cancer cells is dependent on phospholipase D2 (PLD2) and Janus kinase 3 (JAK3). J. Mol. Biol. 408, 850–862

78. Ye, Q., Kantonen, S., and Gomez-Cambronero, J. (2013) Serum deprivation confers the MDA-MB-231 breast cancer line with an EGFR/JAK3/PLD2 system that maximizes cancer cell invasion. J. Mol. Biol. 425, 755–766

79. Lavierti, R. R., Scott, S. A., Selvy, P. E., Kim, K., Jadhav, S., Morrison, R. D., Daniels, J. S., Brown, H. A., and Lindsley, C. W. (2010) Design, synthesis, and biological evaluation of halogenated N-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decane core: discovery of M1298 and M1299 that inhibit phospholipase D1 by oncogenic FAM83B. Oncogene 32, 3531–3542

80. Henkels, K. M., Boivin, G. P., Dudley, E. S., Berberich, S. I., and Gomez-Cambronero, J. (2013) Phospholipase D (PLD) drives cell invasion, tumor growth and metastasis in a human breast cancer xenograft model. Oncogene 32, 5551–5562

81. Chen, Q., Hongu, T., Sato, T., Zhang, Y., Ali, W., Cavallo, J. A., van der Velden, A., Tian, H., Di Paolo, G., Nieswandt, B., Kanaho, Y., and Frohman, M. A. (2012) Key roles for the lipid signaling enzyme phospholipase D1 in the tumor microenvironment during tumor angiogenesis and metastasis. Sci. Signal. 5, ra79

82. O’Reilly, M. C., Scott, S. A., Brown, K. A., Oguin, T. H., 3rd, Thomas, P. G., Daniels, J. S., Morrison, R., Brown, H. A., and Lindsley, C. W. (2013) Development of dual PLD1/2 and PLD2 selective inhibitors from a common 1,3,8-triazaspiro[4.5]decane core: discovery of MI298 and MI299 that decrease invasive migration in U87-MG glioblastoma cells. J. Med. Chem. 56, 2695–2699

83. Jang, Y. H., Choi, K. Y., and Min do, S. (2010) Phospholipase D1 drives a positive feedback loop to reinforce the Wnt/β-catenin/TCF signaling axis. Cancer Res. 70, 4233–4242

84. Kang, D. W., Lee, J. Y., Oh, D. H., Park, S. Y., Yoo, T. M., Kim, M. K., Park, M. H., Jang, Y. H., and Min do, S. (2009) Tripotride-induced suppression of phospholipase D expression inhibits proliferation of MDA-MB-231 breast cancer cells. Exp. Mol. Med. 41, 678–685

85. Kang, D. W., Min, G., Park do, Y., Hong, K. W., and Min do, S. (2010) Phospholipase D1 drives a positive feedback loop to reinforce the Wnt/β-catenin/TCF signaling axis. J. Biol. Chem. 285, 1496–1504

86. Hsu, Y. L., Hung, J. Y., Ko, Y. C., Hung, C. H., Huang, M. S., and Kuo, P. L. (2010) Phospholipase D signaling pathway is involved in lung cancer-derived IL-8 increased osteoclastogenesis. Carcinogenesis 31, 587–596

87. Su, W., Chen, Q., and Frohman, M. A. (2009) Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis. Future Oncol. 5, 1477–1486

88. Kang, D. W., Lee, J. Y., Oh, D. H., Park, S. Y., Yoo, T. M., Kim, M. K., Park, M. H., Jang, Y. H., and Min do, S. (2009) Tripotride-induced suppression of phospholipase D expression inhibits proliferation of MDA-MB-231 breast cancer cells. Exp. Mol. Med. 41, 678–685

89. Kulkarni, A., Quang, P., Curry, V., Keyes, R., Zhou, W., Ho, B., Baffoe, J., Török, B., and Stieglitz, K. (2014) 1,3-Disubstituted-4-aminopyrazolo [3, 4-d] pyrimidines, a new class of potent inhibitors for Phospholipase D. J. Med. Chem. 53, 5615–5626

90. Kang, D. W., Hwang, W. C., Park, M. H., Ko, G. H., Ha, W. S., Kim, K. S., Lee, Y. C., Choi, K. Y., and Min, D. S. (2013) Rebamipide abolishes Helicobacter pylori CagA-induced phospholipase D1 expression via inhibition of NFκB and suppresses invasion of gastric cancer cells. Oncogene 32, 3531–3542

91. Zhang, F., Wang, Z., Lu, M., Yonekubo, Y., Liang, X., Zhang, Y., Wu, P., Zhou, Y., Grinstein, S., Hancock, J. F., and Du, G. (2014) Temporal production of the signaling lipid phosphatidic acid by phospholipase D2 determines the output of extracellular signal-regulated kinase signaling in cancer cells. Mol. Cell. Biol. 34, 84–95

92. Cipriano, R., Bryson, B. L., Miskimen, K. L., Bartel, C. A., Hernandez-Sanchez, W., Bruntz, R. C., Scott, S. A., Lindsley, C. W., Brown, H. A., and Jackson, M. W. (2014) Hyperactivation of EGFR and downstream effector phospholipase D1 by oncogenic FAM83B. Oncogene 33, 3298–3306

93. Cheol Son, J., Woo Kang, D., Mo Yang, K., Choi, K. Y., Gen Son, T., and Min Do, S. (2013) Phospholipase D inhibitor enhances radiosensitivity of breast cancer cells. Exp. Mol. Med. 45, e38

94. Schwartz, N., Chaudhri, R. A., Hadadi, A., Schwartz, Z., and Boyan, B. D. (2014) 17β-Estradiol promotes aggressive laryngeal cancer through membrane-associated estrogen receptor α. J. Nat. Cancer 5, 22–32

95. Pye, D. S., Rubio, L., Pusch, R., Lin, K., Pettitt, A. R., and Till, K. J. (2013) Chemokine unresponsiveness of chronic lymphocytic leukemia cells results from impaired endosomal recycling of Rap1 and is associated with a distinctive type of immunological anergy. J. Immunol. 191, 1496–1504

96. Marguerite, V., Gikopoulou, E., Alberto, J. M., Gueant, J. L., and Merten, M. (2013) Phospholipase D activation mediates cobalamin-induced downregulation of Multidrug Resistance-1 gene and increase in sensitivity to vinblastine in HepG2 cells. Int. J. Biochem. Cell Biol. 45, 213–220

97. Ang, Y. H., Choi, K. Y., and Min, D. S. (2014) Phospholipase D-mediated autophagic regulation is a potential target for cancer therapy. Cell Death Differ. 21, 533–546

98. Bruntz, R. C., Taylor, H. E., Lindsley, C. W., and Brown, H. A. (2014) Phospholipase D2 mediates survival signaling through direct regulation of Akt in glioblastoma cells. J. Biol. Chem. 289, 600–616

99. Selvy, P. E., Lavierti, R. R., Lindsley, C. W., and Brown, H. A. (2011) Phospholipase D: enzymology, functionality, and chemical modulation. Chem. Rev. 111, 6064–6119