New Developments in Phospholipase D*

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Phospholipase D (PLD) (1) is present in bacteria, fungi, plants, and animals. It is widely distributed in mammalian cells, where it is regulated by a variety of hormones, growth factors, and other extracellular signals. Its major substrate is phosphatidylcholine (PC), which is hydrolyzed to phosphatidic acid (PA) and choline, but it can also act on phosphatidylethanolamine and phosphatidylinositol in some organisms and tissues. It also catalyzes a phosphatidyl transfer reaction in which a primary alcohol acts as nucleophile in place of H2O. The resulting production of phosphatidyl alcohol represents a specific assay for PLD.

Enzymology of PLD

PLD has been partially purified from many sources (1) and has recently been cloned from yeast, bacteria, plant, and mammalian sources (2). The enzymes from Saccharomyces, Ricus (castor bean), and Streptomyces have several sequences that are conserved in the human enzyme (Fig. 1) (2), and these presumably represent components of the catalytic site. These sequences are also found in cardiopin synthase and phosphatidylserine synthase from Escherichia coli (2, 3) (Fig. 1). These enzymes also catalyze phosphatidyl transfer, suggesting that PLD is a member of a larger family of enzymes (2, 3).

The first reported mammalian PLD (hPLD1a) has 1072 amino acids and a molecular mass of 124 kDa (4). It is specific for PC and was obtained by using the yeast PLD gene (SP014) (5) to identify a human expressed sequence tag for screening a HeLa cDNA library. A shorter splice variant of hPLD1a with 1034 amino acids (hPLD1b) (Fig. 1), which has similar regulatory properties, has been identified (6), and another PLD (PLD2) (Fig. 1), which has 932 amino acids and 51% amino acid sequence identity to hPLD1a, has been cloned from a mouse embryonic library (7). In our laboratory, enzymes corresponding to hPLD1a and hPLD1b have been cloned from rat tissues. The regulation of these cloned enzymes will be discussed below. Other PLDs have been identified in human tissues and C6 glioma cells (8, 9), and their partial sequences indicate similarity, if not identity, to hPLD1.

PLD has been purified to very high degree from pig lung microsomes (10). It is specific for PC and has a molecular mass of 190 kDa and a pH optimum of 6.6. Another PLD has been substantially enriched from pig brain membranes (11). It has a molecular mass of 95 kDa, based on hydrodynamic measurements, and is markedly stimulated by PIP2, and the small G proteins ARF and RhoA. Other PLDs have been purified to a limited extent, including various forms from rat brain that differ in their pH optima and responses to Ca2+, PIP2, oleate, or detergents (1, 12), and cytosolic PLDs that are Ca2+-responsive and differ in their substrate specificity (1, 12). These differences suggest the existence of PLD isozymes, but since the enzymes are far from homogeneous, this is unclear.

The subcellular localization of PLD activity shows some interesting features. The enzyme is enriched in plasma membranes from many tissues but is also present in high activity in Golgi and nuclei (13–17). There is significant activity in cytosol but not in mitochondria. In liver, the plasma membrane enzyme responds more to RhoA than ARF, whereas the reverse is true for that in other subcellular fractions (13). Whether these differences reflect differences in the subcellular distribution of PLD isozymes (7) or other factors remains to be determined. Studies of PLD isozymes expressed in fibroblasts indicate that PLD2 localizes predominantly in the plasma membrane, whereas PLD1 is perinuclear, i.e. in endoplasmic reticulum, Golgi, and late endosomes (7).

Brown et al. (18) discovered that PLD is strongly stimulated by PIP2, and this has been observed for most but not all (10, 19, 20) preparations of the enzyme. PI-3,4,5-P3 is also effective (6), but other acidic phospholipids, including PI-3,4-P2, PI-4-P, and PI, are nearly or completely ineffective (18, 21). There is evidence that PIP2 is required for the activation of PLD in intact cells (22), but it is unclear that physiological changes in PIP2 or PIP3 levels control the enzyme in vivo. Studies with cloned, purified PLDs indicate that PIP2 and PIP3 directly activate the enzyme (6).

Regulation of PLD by Protein Kinase C

There is abundant evidence that PLD is regulated by PKC in most mammalian cells. This comes from studies of the effects of phorbol esters, PKC inhibitors, down-regulation of the enzyme, and overexpression and deletion of specific PKC isozymes (1). Although a role for PKC in the actions of many agonists on PLD in many tissues/cells has been indicated, there are also instances where the enzyme does not seem to be involved (1). Since many of the agonists that activate PLD also stimulate the hydrolysis of PIP2 by PI-phospholipase C with subsequent production of diacylglycerol and activation of PKC, PKC hydrolysis is often secondary to PIP2 breakdown (1). Activation of PKC is also associated with translocation of the enzyme to cell membranes, and this relocalization is probably required for PKC activation of PLD, which is predominantly membrane-associated (Fig. 2).

The most direct mechanism of control of PLD by PKC would be through phosphorylation of the enzyme. However, in those studies where the effects of PKC on PLD have been studied directly, activation does not involve ATP, i.e. a phosphorylation mechanism (6, 23–25). In particular, studies with cloned PLD purified from S9 fractions indicate that PKCα and PKCβ can directly activate the enzyme in an ATP-independent manner (6), but other isozymes are ineffective (24). The interaction is not affected by staurosporine and involves the regulatory do-

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The abbreviations used are: PLD, phospholipase D; PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 4,5-trisphosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PKC, protein kinase C; ARF, ADP-ribosylation factor; GTPyS, guanosine 5′-O-(3-thiotriphosphate); LPA, lysophosphatidic acid; DAG, diacylglycerol.

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2 S.-K. Park, J. I. Provost, C. D. Bae, W.-T. Ho, and J. H. Exton, submitted for publication.

3 D.-S. Min and J. H. Exton, unpublished observations.
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Regulation of PLD by ARF

Rho family proteins regulate many cellular activities including those involving the actin cytoskeleton. The proteins include Rho, which controls the formation of focal adhesions and actin stress fibers, Rac, which regulates lamellipodia formation and membrane ruffling, and Cdc42, which controls the formation of filopodia (38, 39). The first evidence that PLD could be regulated by Rho proteins came from a study by Bowman et al. (40). This showed that the stimulatory effect of GTPγS on PLD in neutrophil plasma membranes was inhibited by RhoGDI, a protein that inhibits GDP dissociation from Rho proteins and thereby blocks their activation. In subsequent studies using plasma membranes from rat liver, HL-60 cells, and neutrophils, it was found that RhoA was the most effective Rho protein to activate PLD, but Rac1 or Cdc42Hs showed some activity (41–43). RhoA also stimulates PLD in fractions from other cells and partially purified preparations of the enzyme (13, 17, 31, 35, 44). Studies with cloned PLD purified from Sf9 cells indicate that RhoA interacts directly with the enzyme and that Rac1 and Cdc42 are also active (6). Interestingly, a combination of RhoA and ARF results in synergistic activation of homogeneous or partially purified PLD (6, 25, 35, 42, 44). This suggests the presence of separate but interacting sites for Rho and ARF on PLD. As in the case of ARF, there is evidence that RhoA action on PLD is enhanced by cytosolic proteins (35, 43) and by PKCa (6, 25, 43, 45).

Several bacterial toxins that inactivate Rho proteins have been used to demonstrate the involvement of RhoA in agonist regulation of PLD in vivo. These include the C3 exoenzyme of Clostridium botulinum that ADP-ribosylates RhoA and blocks the activation of PLD in Rat1 fibroblasts by LPA (46). Likewise, Toxin B from Clostridium difficile, which glucosylates and inactivates Rho proteins, blocks the activation of PLD by carbachol in intact cells and by GTPγS and AlF4− in permeabilized cells (47). The toxin also inhibits IgE receptor-mediated activation of PLD in basophilic leukemia cells (48). While these observations support a role for RhoA in agonist stimulation of PLD, caution should be observed since the small G protein also regulates PIP2 synthesis by PI-4-P 5-kinase (49), and the

Fig. 2. Postulated mechanisms by which growth factors and agonists whose receptors are linked to heterotrimeric G proteins activate PLD. Mechanisms involving PKC isozymes and Rho family proteins are shown, but other mechanisms, e.g. involving ARF proteins, may operate.

Fig. 1. Alignment of sequences of PLDs and associated enzymes from various species. Homologous regions are boxed. aa, amino acids. Modified from Ref. 2.
changes in PLD could be secondary to alterations in PIP_2 levels (50).

Because of the location of its substrate, PLD must be or must become membrane-associated for activity. Thus its regulators must also be present in membranes or translocate there. As described above, there is evidence for agonist-induced membrane relocation of ARF (36, 37), and this is a well known phenomenon for PKC isozymes. Agonist-induced membrane translocation of Rho family proteins has also been reported recently (46, 51, 52), and this is probably an important component of the mechanism of PLD activation by certain agonists (Fig. 2).

**Other Factors Regulating PLD**

Ceramides are being increasingly recognized as important regulators of cell function (53). C_(18) and C_(24)-ceramides inhibit agonist or phorbol myristate acetate-stimulated PLD activity in several cell lines (54–57) and block the stimulation of the enzyme by GTP-S plus phorbol myristate acetate, ARF, or RhoA in cell extracts (52, 56), but dihydro derivatives are ineffective. Since the ceramides also block the membrane translocation of ARF, RhoA, and Ca^{2+}-dependent PKC isozymes (52, 55), part of the inhibition could be due to these effects. In contrast to ceramides, sphingosine and its metabolite sphingosine-1-P activate PLD (58). It is probable that their effects are partly mediated by a sphingosine-1-P receptor coupled to a G protein(s) (59, 60).

The existence of cytosolic proteins that enhance the effects of RhoA and ARF on PLD has already been described. The actin-binding protein gelsolin also stimulates PLD but only in the presence of nucleoside triphosphates (61). A role for PLD in control of the actin cytoskeleton has been proposed (62, 63), but the mechanisms involved are unclear.

Inhibitors of PLD have been identified (64–67). One of these is heat-labile with a molecular mass of 150 kDa (65). Its partial sequence (65) indicates that it is synaptotagmin, which exhibits PIP_2 phosphatase activity (68) and presumably inhibits PLD by decreasing PIP_2. Another inhibitor that has a molecular mass of 30 kDa and does not bind PIP_2 has been identified (66). Fodrin, a non-erythroid form of spectrin, has been reported to inhibit PLD (67). Although fodrin has a PH domain, it apparently does not act by binding PIP_2. Brain contains an 18-kDa inhibitor that is selective for PLD2 (7).

**Biological Significance of PLD**

PLD could exert its biological effects by several mechanisms. The first is by changing the properties of cellular membranes by altering their lipid composition. Thus, by causing local changes in PC and PA and releasing polar choline, the physical properties of the membranes could be substantially changed. A second mechanism is by generating PA. This lipid would probably remain in the membrane but could interact with proteins located in the membrane or cytosol. Many proteins have been shown to have activities changed by PA in vitro (1), but evidence that they are targets of the lipid in vivo is largely lacking. Since transphosphatidylation may be a major function of PLD in vivo, the possibility should be considered that the products of this reaction may also have signaling functions.

A third mechanism of biological action of PLD arises from the fact that PA is rapidly converted to DAG in most cells through the action of phosphatidate phosphohydrolase. Thus the late phase of activation of PKC produced by agonists in many cells is mainly attributable to DAG derived from PLD action (1, 69). A fourth function of PLD is the generation of LPA through the action of a specific phospholipase _A_2 on PA. LPA is becoming recognized as a major extracellular signal produced by activated platelets and probably other cells (70). A final possibility relates to the formation of choline. Because of its high resting cellular levels, this compound probably does not have a signaling function, but it could serve as a substrate for acetylcholine synthesis in neurons (71).

Roles for PLD have been proposed for many cellular functions, but space permits consideration of only a few. The potential function of PLD in the regulation of cell proliferation remains controversial. PA and bacterial PLD are mitogenic in several cell lines (1, 72), although some of the effects may be due to LPA contamination or formation (1, 70), and there is a lack of correlation between PLD activity and mitogenesis in some cell lines (73). Other signaling pathways are undoubtedly involved in growth control. In Saccharomyces cerevisiae, PLD was first recognized as the product of the SPO14 gene, which is essential for meiosis (5). The mechanism by which PLD could control the cell cycle are unknown. However, Raf-1 kinase, which is involved in signal transduction from several receptors, has a binding site for PA and is translocated to membranes under conditions where PLD is activated (74).

As alluded to above, PLD has been implicated in the regulation of vesicle trafficking by ARF in Golgi. In Chinese hamster ovary cell lines displaying high basal PLD activity in Golgi, ARF is not necessary for coatamer assembly (15). The formation of coated vesicles is also inhibited by ethanol, which reduces PA formation due to the production of phosphatidylethanol, and treatment of Golgi membranes with bacterial PLD promotes coatamer binding (15). Studies with ethanol and exogenous PLD in other cell types have also provided evidence that PLD is involved in vesicle transport (27). It is not known whether its action is due to the generation of PA, a membrane fusigen, or to other membrane lipid changes. Colley et al. (7) have observed that PLD2 provokes cortical reorganization mediated by actin polymerization and undergoes redistribution from the plasma membrane in serum-stimulated cells. They have postulated that PLD1 and PLD2 are involved in vesicle translocation from the perinuclear region and in receptor-mediated endocytosis, respectively. Other studies have implicated PA in the control of actin polymerization (62, 63).

PLD has been implicated in _O_2 production and degranulation in neutrophils in response to chemotactic peptide (75). The generation of the bactericidal _O_2 species, which is due to activation of NADPH oxidase, is inhibited by ethanol and mimicked by the addition of PA (75). These data, which implicate PA in the control of NADPH oxidase, are supported by _in vitro_ findings (76, 77). However, these studies also provide evidence for the involvement of DAG (77) and indicate that the two lipids synergize to activate the enzyme (77).

**Concluding Comments**

Although substantial advances have recently been made in the enzymology and regulation of PLD, major questions remain. In particular, the various isozymes need to be identified and their structure/function relationships and regulatory properties defined. Molecular biological approaches should now make this possible. The mechanisms by which growth factors, hormones, and other agonists regulate the enzyme need to be clarified, with emphasis on the roles of PKC, Rho proteins, ARF, and certain lipids. Finally, but most importantly, the physiological functions of PLD need to be defined, and molecular biological approaches should also provide major help in this area.

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