Regulation and Possible Role of Mammalian Phospholipase D in Cellular Functions

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Phospholipase D (PLD), an enzyme widely distributed in bacteria, fungi, plants, and animals, catalyzes the hydrolysis of phosphatidylcholine (PC) and other phospholipids to generate phosphatidic acid (PA). PLD activity can lead to the generation of phosphatidylalcohol in the presence of a primary alcohol. This reaction, referred to as transphosphatidylation, is not only a hallmark of PLD activity but has also been used to block the production of PA in functional studies. PLD activity in mammalian cells is transiently increased following the occupation of many cell surface receptors, including those of the heterotrimeric G-protein and tyrosine kinase families (1-4). The generated PA and the further metabolites, 1,2-diacylglycerol (DG) and lysoPA, are important biologically-active products that are able to recruit or modulate specific target proteins. This review will focus on recent developments in regulation of the mammalian PLD isozymes involved in cell stimulation and on the functional roles of PLD signaling.

The phospholipase D gene superfamily

Eukaryotic PC-hydrolysing PLD was first cloned from a plant, then from yeast, and finally from mammalians. Wang and his colleagues first isolated a castor-bean endosperm cDNA (PLD-α) that encoded a 92-kDa protein exhibiting both hydrolytic and transphosphatidylation Ca2+-dependent PLD activity with PC as substrate. Plant PLDs have been cloned in three forms, PLDα, PLDβ, and PLDγ (3). A yeast sporulation gene, called SPO14/PLD1 and having sequence homology to plant PLD, has been isolated from Saccharomyces cerevisiae and identified as a gene exhibiting Ca2+-independent PLD activity. On the basis of the plant and yeast genes,
a human PLD cDNA having 1,074 amino acids and a molecular mass of 124 kDa was cloned and termed PLD1a (5). A shorter splice variant of hPLD1a (hPLD1b), which lacks a 38-amino-acid region and has similar regulatory properties, has been identified (6). Another PLD (PLD2), a 106-kDa protein with 933 amino acids and 51% amino acid sequence identity with hPLD1a, has been cloned from a mouse embryonic library (7). Another fungal PLD gene has been cloned from *Candida albicans* (8). In addition, several putative PLD genes, from the nematode *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, and three bacterial PLDs from *Streptomyces antibioticus*, *Streptoverticillium cinnamoneum*, and *Yersinia pestis*, respectively, comprise the PLD family tree (Fig. 1A), which was created using the CLUSTAL W software based on BLAST analyses of the individual sequences (3). These PLD genes all belong to an extended gene superfamily that also includes phosphatidyltransferases, bacterial phospholipid synthases, endonucleases, and pox envelope proteins (1-3). Mammalian-like PLDs have also been found to be present in the protozoa *Leishmania donovani* and *Tetrahymena pyriformis*.

PLD superfamily members all share a conserved (HXKX4DX6GG/S) (HKD) motif that is involved in catalysis and that confers a similar mechanism of action (1-3). The catalytic core of all eukaryotic PLDs is comprised of domains I-IV. These four domains are also found in the bacterial PLDs. Mutagenesis and structural studies have indicated that the HKD motifs are required for catalytic activity and that they may dimerize to form an active center. In addition to domains I-IV, other domains (PH, PX) are conserved in the yeast, human, and nematode sequences, but are absent from the plant and bacterial PLDs (Fig. 1B). Plant PLDs exhibit an N-terminal C2 domain (3). Analysis of the crystal structure of an endonuclease PLD superfamily member revealed that the HKD motif acts as a dimer and nucleophile that forms a covalent phosphohistidine intermediate (3).

**Regulation of mammalian PLD1 and PLD2 in receptor signaling**

The recombinant mammalian PLD1a and 1b are activated by ADP-ribosylation factor ARF, Rho protein family (Rho, CDC42, and Rac), RaIA, and conventional protein kinase C (PKCα, β) in *vitro* assay (1-4). On the other hand, recombinant PLD2 exhibits high basal activity and is only modestly regulated by ARF. Both PLD1 and PLD2 require PIP₂ for activity. PIP₂ binds to a highly conserved region containing basic and hydrophobic amino acids (amino acids 691-712 for PLD1 and amino acids 554-575 for PLD2), but the PH domain is not necessary for PIP₂ interactions (9). The domain in PLD1 with which Rho interacts is localized within the C-terminal 362 amino acids. PKC interacts with a domain in the N-terminus (amino acids 1-325, which contain the PX and PH domains) of PLD1 (2). The site of ARF interaction has not yet been identified.

In intact cells, agonist-induced PLD activity is regulated by various protein kinases, including PKC, protein tyrosine kinase, and the MAP kinase family, in addition to ARF and Rho family proteins (1-4). Phorbol ester activates PLD in many cell lines,
and PLD activity induced by various agonists is abolished by PKC inhibitors, implying that PKC is a major factor in the regulation of PLD \textit{in vivo} \cite{1}. Some studies have shown that PKC can directly activate PLD1 in an ATP-independent manner. A PLD1 mutant unresponsive to PKC cannot be activated by G-protein coupled receptor stimulation when expressed in HEK 293 cells, suggesting that direct interaction with PKC is important for PLD1 activation \cite{10}. On the other hand, another report has suggested that a phosphorylation-dependent mechanism is important in the cells. PMA-induced phosphorylation sites of endogenous PLD1 have been identified at serine 2, threonine 147, and serine 561 in rat fibroblast 3Y1 cells, and mutation of these sites significantly decreased PMA-induced PLD1 activity \cite{11}. Many receptors also stimulate phosphatidylinositol-specific phospholipase C (PI-PLC) activity, leading to an increase in cellular Ca\textsuperscript{2+} as well as DG, which activates PKC, suggesting that PI-PLC activation is upstream of PLD activation \cite{1, 12, 13}. PKN\textalpha and PKN\textbeta, serine/threonine kinases binding to RhoA, directly interact with PLD1 at the residues 228-598 for PKN\textalpha and the residues 1-228/229-598 for PKN\textbeta when transfected in COS-7 cells \cite{14}. Significant stimulation of PLD1 activity by PKN\textalpha was observed in the presence of arachidonic acid.

Although recombinant PLD2 exhibits high basal activity, PLD2 expressed in the cells and in the membrane exhibits low activity. Therefore, it is suggested that the PLD2 activity may be inhibited \textit{in vivo} by inhibitory proteins. \textalpha- and \textbeta-synucleins, clathrin assembly protein AP180, fodrin, and synaptojanin have been identified as cytosolic inhibitory proteins \cite{2}. The regulatory mechanism of PLD2 is less understood. PLD2 expressed in HEK 293 cells and in Sf9 cells can be regulated by PMA, indicating a positive regulation of PLD2 by PKC. Co-expression of PLD2 and PKC\textalpha results in increase in PLD2 activity \cite{12, 15}. ARF also increases PLD2 activity when co-expressed in HEK 293 cells \cite{12}. PLD2 is found to be associated with cytoskeletal proteins such as gelsolin, \textalpha-actinin and \textbeta-actin, and these proteins inhibit PLD2 activity \cite{16, 17}. The inhibition by \textalpha-actinin could be reversed by ARF1 \cite{16}. These data suggest that ARF can also regulate PLD2 activity under certain conditions in the cells.

The possible involvement of protein tyrosine kinase (PTK) and other serine/threonin kinases in the PLD activation has been suggested in response to various stimuli. H\textsubscript{2}O\textsubscript{2} stimulates PLD2 activity in PC12 cells and endothelial cells, and the pretreatment with the PTK inhibitors abolished the PLD activation, suggesting that PLD2 is regulated by PTK \cite{18, 19}. PLD2, but not PLD1, forms a physical complex with the EGF receptor, and its Tyr 11 becomes phosphorylated in response to EGF stimulation \cite{20}. However, the tyrosine phosphorylation of PLD2 has no effect on the enzyme activity.

Recent evidence suggests the involvement of a MAP kinase (ERK and p38 MAP kinase) pathway in the stimulation of PLD activity in some cells. We suggested that the H\textsubscript{2}O\textsubscript{2}-induced PLD2 activation in PC12 cells was mediated through a signaling cascade of Src-type PTK, a calcium-dependent proline-rich tyrosine kinase-2 Pyk2,
ERK, and p38 MAP kinase (18, 21). A similar pathway has been proposed in glucose-induced PLD activation in muscle and adipose tissues, where PLD functions downstream of the PYK2/ERK pathway and upstream of PKCζ/λ (22). Furthermore, norepinephrine-induced PLD1 activity is mediated via the ras/ERK pathway by a phosphorylation-dependent mechanism in aortic smooth muscle cells (23). Both PLD1 and PLD2 directly associate with p38 MAPK and are phosphorylated in response to diperoxovanadate stimulation in endothelial cells (24). However, phosphorylation by ERK or p38 MAPK failed to affect either PLD isoform activity in vitro.

Biological functions of PLD

PLD activation causes changes in the physiological properties of cellular membranes by reducing PC to increase PA, which remains in the membrane and interacts with various proteins located in the membrane or cytosol. Many enzyme activities have been reported to undergo changes induced by PA in vitro. Some proteins, including coatomer, ARF, and Raf-1, have been identified as specific PA-binding proteins from brain cytosol (25). Therefore, it is hoped that elucidation of the roles of PLD in cellular regulation will allow us to identify cellular targets affected by its reaction product, PA (Fig. 2).

To understand the function of PLD activity, it is important to determine its location within cells. Numerous studies in which epitope-tagged forms of PLDs were overexpressed in several different cell types have suggested that both PLD1 and PLD2 can be found both at the plasma membrane and in intracellular compartments including the endoplasmic reticulum, Golgi, lysosomes, endosomes, and secretory granules (3, 4). It is possible that transient overexpression of PLDs leads to mislocalization of the enzyme. Recent study using antibodies of endogenous enzymes have confirmed that PLD1 is tightly associated with the Golgi complex, and also located in cell nuclei (26). PLD1 is translocated to the plasma membrane (containing caveolae) when stimulated with PMA (11, 27). Other recent studies have demonstrated that endogenous PLD2 is located in caveolar membrane and in the perinuclear region containing the Golgi (3, 27). We have found that PLD2 is present in isolated nuclei from hepatoma cells. However, it has not been clearly demonstrated that different PLD isoforms are involved in different physiological processes.

Vesicle trafficking and cytoskeletal reorganization

Several lines of evidence implicate ARF-dependent PLD1 in agonist-dependent cellular secretion and vesicle trafficking (4). Interestingly, a recent study with inactive PLD1- and PLD2-transfected epithelial cells has demonstrated that PLD1 and PLD2 regulate protein transit between the trans-Golgi network and the apical plasma membrane at different steps: in the resting cells, constitutive secretion of protein appears mainly regulated by PLD2; but the increase in secretion triggered by PMA stimulation is PLD1-dependent (27).
A possible role of PLD1 and PLD2 has been demonstrated in GLUT-4 translocation. PLD1 is associated with GLUT-4 containing intracellular membrane and acts to promote the mechanism of GLUT-4 translocation by insulin (28). Insulin-mediated activation of PLD1 is induced through the ARF pathway in adipocytes, which is consistent with the hypothesis that stimulation of PI 3-kinase by insulin may cause enhancement of ARNO, which in turn activates the ARF/PLD. On the other hand, PLD has also been implicated in glucose-induced increases in GLUT4 translocations in adipocytes and muscle cells (22). In these tissues, GLUT4 translocation appears to require activation of PKCζ/λ, which is activated by PA. The glucose-induced activation of PKCζ/λ is independent of the PI3 kinase pathway, but is mediated by a mechanism requiring PYK2, ERK, and PLD (most likely PLD2).

PLD may participate in regulating agonist-induced reorganization of the cytoskeleton, such as membrane ruffling and stress fiber formation. One possible function of PLD that would explain its role in cytoskeletal reorganization is related to the ability of PA to regulate PI(4)P 5-kinase, which is required for synthesis of PIP2. In HeLa cells, PI(4)P 5 kinase, ARF6, and PLD2 are colocalized in the ruffles upon stimulation with EGF, and PI(4)P 5 kinase is activated synergistically with ARF6 and PLD2 product PA to increase local production of PIP2 in the plasma membrane (29). RhoA-dependent-PLD1 has been found to play a role in stimulation of actin stress fiber formation. Actin stress fiber formation in response to LPA is inhibited by expression of the catalytically inactive form of PLD1 but not PLD2 (30).

**Growth and differentiation**

PLD is activated in mammalian cells in response to a variety of mitogenic stimuli involving EGF, PDGF, insulin, Src, and LPA, and primary alcohol, which inhibits PA generation by PLD, suppresses cell proliferation, suggesting that PLD activity plays a role in cell proliferation. This notion has been supported by evidence that PLD2 is involved in insulin-induced ERK activation by inducing membrane translocation of Raf-1, which is directly bound to PA (25, 31). Thus, PLD2 may be involved in mitogenic signaling via cross-talk with the ras/ERK signaling cascade.

Numerous studies have suggested that PLD might be involved in tumorigenesis. Enhanced expressions and increases of activity of two PLD isoforms have been found in cancer cells and tissues (32-34). We have observed that ARF-activated PLD activity is transiently increased in regenerating liver nuclei, and that PLD2 level is markedly elevated in nuclei of Hepatoma cells and kidney tumors (34, 35). Other evidence has shown that the PLD2 levels of caveolae are markedly elevated in oncogenic stimulated cells and multidrug-resistant cancer cells (36). More direct evidence has shown that overexpression of PLD1 and PLD2 in mouse fibroblasts induces colony formation in soft agar, and both transformants induce undifferentiated sarcoma when transplanted into nude mice (37). These studies suggest that overexpression of the two PLD isozymes may play an important role in neoplastic transformation.
Our studies have implicated PLD in the regulation of cell differentiation in several cell types. mRNA expressions of PLD1a and PLD1b, and Rho- or ARF-dependent PLD1 activity were increased during differentiation of dbcAMP-induced HL60 cells and C6 glioma cells, and of NGF-induced PC12 cells (38). Elevation of PLD1a expression and the activity is also observed in differentiation of epidermal keratinocytes in response to 1,25-dihydroxyvitamin D3 (39). On the other hand, ARF-dependent PLD activity and PLD1 protein level are concomitantly decreased during differentiation of F9 cells into pariental endoderm (40). These data suggest a role for PLD1 and PLD2 in regulation of differentiation in diverse cell types. Although the mechanism(s) of regulation by PLD of cell differentiation remains unclear, one possible explanation may lie in recent results implicating PLD1 and PLD2 activities in the P38 MAP kinase pathway, which plays a role in cell differentiation as an upstream or downstream regulator (21, 24, 41).

Apoptosis and cell survival

Accumulating evidence suggests that sphingomyelin metabolites such as ceramide and sphingosine 1-phosphate (S1P) are involved in regulation of apoptosis and survival, respectively. In several cell types, cell-permeable ceramides decreased PLD activity (42). In vitro studies indicate that ceramide-mediated decreases of the two PLD isozyme activities are due to direct effects of ceramides on PLD per se or on its activating factors. The PLD product PA is able to inhibit ceramide-induced apoptotic events, caspase activation, and PARP proteolysis via inhibition of protein phosphatase 1 (43). These findings suggest that PLD activities may be involved in apoptosis, and other evidence supports this hypothesis. In hematopoietic cells (Jurkat T cells, A20 B cells, and HL60 cells), apoptosis-inducing drugs such as actinomycin D, anti-Fas antibody, TNF-α, and anti-cancer drugs induce up-regulation of PLD activity together with apoptotic changes (42). The PLD activity in Jurkat cells is oleate-dependent, which may be PLD2, since oleate can activate PLD2 but inhibit PLD1. On the other hand, PLD activity in A20 cells and HL60 cells is regulated by PC-PLC/PKC. These PLD activations are considered to be an anti-apoptotic event. In this context, it has been demonstrated that PC12 cells overexpressing wild-type PLD2, but not the inactive mutant, show suppressive effects on H2O2- or hypoxia-induced cell death and caspase-3 activation in PC12 cells (44, 45). Accumulating evidence in support of this hypothesis includes the finding that sphingosine-1-phosphate (S1P), an important mediator in mitogenic and survival signaling, stimulates PLD activity through Edg-1, Edg-3, or Edg-5 in various cells (13, 46, 47). Further, sphingosine kinase, an S1P-producing and pro-apoptotic enzyme, is stimulated by the PLD product PA (48). Recently, we have presented evidence that PLD may participate in Edg-3 receptor-mediated activation of the Akt/PKB pathway, which plays a crucial role in anti-apoptotic or pro-survival signaling (46). These studies were based on the finding that 1-butanol, but not 2-butanol, prevented sphingosine kinase and PI3K/Akt activation in response to agonist stimulation. However, the exact mechanisms by which PLD activates these enzymes remain to be defined.
Concluding remarks

Cloning of PLD genes has provided further insight into the regulation and catalytic mechanisms of the PLD. Recent evidence suggests that PLD participates in various signal-transduction cascades, and that there is cross-talk between PLD pathways and complex signal-tranduction networks, such as the Ras/MAPK pathway, Arf/PI4P-5K, sphingomyelin metabolites, and PI3K/Akt. This cross-talk, characteristic of important cellular signal-transductions, implies that PLD also plays important roles in regulation of glucose transport, the actin cytoskeleton reorganization and membrane ruffling, secretion, cell growth and apoptosis/cell survival. In seeking deeper insight into these PLD biological functions, it will be important to fully understand the downstream events that occur in consequence of PLD activation, and to identify target proteins that are directly or indirectly regulated by PA in the cells.

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