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Minireview

Regulation of phospholipase D

John H. Exton*

Howard Hughes Medical Institute and Vanderbilt University Medical Center, Nashville, TN 38232-0295, USA

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Abstract **Structural studies of plant and bacterial members of the phospholipase D (PLD) superfamily are providing information about the role of the conserved HKD domains in the structure of the catalytic center and the catalytic mechanism of mammalian PLD isozymes (PLD1 and PLD2). Mutagenesis and sequence comparison studies have also defined the presence of pleckstrin homology and phox homology domains in the N-terminus and have demonstrated that a conserved sequence at the C-terminus is required for catalysis. The N- and C-terminal regions of PLD1 also contain interaction sites for protein kinase C, which can directly activate the enzyme through a non-phosphorylating mechanism. Small G proteins of the Rho and ADP-ribosylation factor families also directly regulate the enzyme, with RhoA binding to a sequence in the C-terminus. Certain tyrosine kinases and members of the Ras subfamily of small G proteins can activate the enzyme, but the mechanisms appear to be indirect. The mechanisms by which agonists activate PLD in vivo probably involve multiple pathways.**

Key words: Phospholipase D; Protein kinase C; Rho; ADP-ribosylation factor; Tyrosine kinase

1. General properties of phospholipase D (PLD)

PLD is a widely distributed enzyme found in bacteria, fungi, plants and animals. It has been implicated in several important cellular functions and, in mammals, is under the control of many hormones, neurotransmitters, growth factors and cytokines. Its principal substrate is phosphatidylcholine and its product, phosphatidic acid, is widely believed to have a signaling role.

PLD is a member of a superfamily, which includes phosphatidylserine synthase, cardiolipin synthase, tyrosyl-DNA phosphodiesterase, two bacterial endonucleases, a murine toxin of *Yersinia pestis* and two poxvirus envelope proteins [1]. These diverse proteins are denoted by the presence of one or more HxKxxxxD motifs abbreviated to HKD. Mutagenesis of this motif causes loss of catalytic function, and structural studies of low M_r members of the PLD superfamily indicate that the catalytic site is comprised of a dimer of two HKD domains [1]. The linear structures of some members of the PLD superfamily are shown in Fig. 1. They all show two conserved sequences designated I and IV, which contain the HKD motifs, and some have additional conserved sequences (II and III).

There are two mammalian PLD isoforms (PLD1 and PLD2) which occur as splice variants. These have four conserved sequences (I–IV) (Fig. 1) and pleckstrin homology (PH) and phox homology (PX) domains in tandem at their N-termini [2]. These domains are implicated in phospholipid and protein binding. The enzymes have an almost absolute requirement for phosphatidylinositol 4,5-bisophosphate (PIP_2) for activity. There is evidence that PIP_2 binds at the PH domain and at a conserved sequence located between conserved sequences II and III [3,4]. PLD1 and PLD2 are membrane-associated but several domains and post-translational modifications appear to be involved in membrane interaction. The C-terminal four amino acids of mammalian PLDs are completely conserved, and deletion, addition or mutation of these residues causes loss of catalytic activity [5,6]. The nature of the participation of the C-terminus in catalysis is presently unknown. Deletion of the N-terminal 168 amino acids of PLD1 results in a large increase in basal activity implying the existence of an inhibitory domain in this region [7,8]. The exact location of this domain has not been defined.

There is no general agreement about where PLD1 and PLD2 are localized in cells. This appears to be due to differences in cell type and methodology (tagging with GFP or different epitopes, immunofluorescence with different antibod-

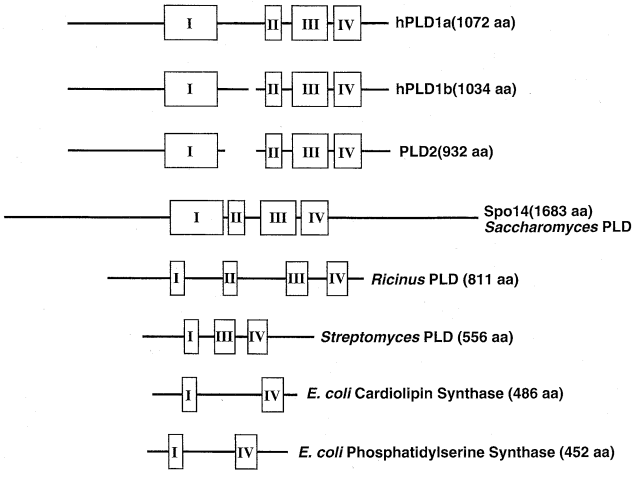

Fig. 1. Sequences of members of the PLD superfamily showing conserved sequences (I–IV).

*Fax: (1)-615-322 4381.
E-mail address: john.exton@mcmail.vanderbilt.edu (J.H. Exton).

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ies). However, PLD1 appears to be localized to the Golgi or perinuclear vesicular structures, whereas PLD2 appears to be associated with the plasma membrane [1]. The enzymes may also be present in caveolae and may co-localize with the actin cytoskeleton. Both PLD1 and PLD2 are palmitoylated and phosphorylated on Ser/Thr residues under basal conditions. These modifications are not required for catalytic activity, but play a role in membrane association [1].

2. Regulation by protein kinase C

Early studies of the activation of PLD by phorbol esters in various cell types implicated protein kinase C (PKC) in its regulation. Inhibitors of PKC reduced agonist activation of PLD activity, although the magnitude of the inhibition was variable, depending on the agonist and cell type [9]. Other approaches indicating the involvement of PKC included down-regulation of the enzyme by prolonged treatment with phorbol ester, and overexpression or antisense deletion of specific PKC isozymes [9]. In addition, overexpression of phosphoinositide phospholipase C (PLC), which generates diacylglycerol and activates PKC, resulted in increased PLD activity [10].

When the mammalian isozymes of PLD were cloned and expressed, PLD1 was shown to be stimulated by PKCα in vitro, whereas PLD2 was not affected [11,12]. More detailed studies showed that only the α- and β-isozymes of PKC could activate recombinant PLD1 in vitro [11,13]. More surprising, the activation occurred in the absence of ATP by a non-phosphorylating mechanism [11,13]. In fact, when ATP was added to the reaction, phosphorylation of PLD1 was associated with inhibition of enzyme activity [11,13]. Synergistic interaction between PKCα and other small G proteins (Rho, ADP-ribosylation factor (Arf)) that activate PLD was observed [11].

Deletional mutagenesis and binding studies have demonstrated the presence of PKC interaction sites in both the N- and C-terminal sequences of PLD1 [2,7,14]. Studies of the sequences in PKCα required for activation of PLD have shown that both the regulatory and catalytic domains of the kinase play a role [15].

Stimulation of PKC by phorbol esters and many agonists in vivo results in marked activation of PLD1 and, surprisingly, of PLD2 also [2,7,8,11,16,17]. The mechanisms involved in the activation of PLD1 and PLD2 in vivo are unclear. For example, although treatment of cells with phorbol ester results in phosphorylation of both isozymes on Ser and Thr residues, there is no clear evidence that this phosphorylation is responsible for their activation [18]. For example, mutants of PKCα that lack kinase activity do not induce phosphorylation of PLD1 or PLD2 in vivo, but they still activate the enzyme, suggesting mechanisms that do not involve phosphorylation (Tianhui Hu, Jun Song Chen and J.H.E., unpublished observations). In summary, these findings suggest that agonists that activate PLC cause membrane translocation and activation of conventional PKC isozymes which, in turn, activate PLD. The activation of PLD probably occurs because of direct interaction of the isozymes with PLD rather than direct phosphorylation.

3. Regulation by small G proteins of the Rho family

There is much evidence that RhoA, Rac1 and Cdc42Hs activate PLD1, but not PLD2 [11,12,19,20]. For example addition of GTPγS-activated forms of these proteins to recombinant PLD1 in vitro results in its activation. RhoA is the most efficacious, but Rac1 and Cdc42Hs cause significant activation [19]. The geranylgeranylated forms of the G proteins have higher activity than the unmodified forms [19]. The stimulation of PLD1 involves the activation loop (Switch I) of the G proteins, and residues in the Switch II region and the insert helix determine their relative efficacies [19,21]. The interaction site for RhoA on PLD1 has been localized to sequences in the C-terminus by binding and activity measurements [22–24]. In the absence of information about the three-dimensional structure of PLD1, it is not known how binding of Rho proteins to these sequences results in activation of the enzyme or in synergistic interaction with PKCα.

An in vivo role for RhoA in the activation of PLD1 has been demonstrated for agonists whose receptors couple to the heterotrimeric G protein G_{13} [17,24,25]. This role seems limited in comparison to the many agonists whose receptors activate phosphoinositide PLC and PKC. Studies of mutant forms of PLD1 in which the RhoA interaction sites have been mutated have indicated that the enzyme can be activated in vivo by direct binding of RhoA [17,24]. However, RhoA may also indirectly regulate the enzyme because of its effects on phosphatidylinositol 4-P 5-kinase, which is the enzyme that synthesizes PIP_2 [26–28]. The in vivo effects of RhoA on PLD activity have been explored through the use of clostridial toxins (C3 exoenzyme and toxin B) [29,30] and also by transfection of dominant negative and constitutively active forms of RhoA and Rac1 [1]. However, these agents do not distinguish between direct and indirect effects of these small G proteins on PLD.

4. Regulation by small G proteins of the Arf family

Arf was the first direct regulator of PLD1 to be recognized. All mammalian isoforms of Arf can activate the enzyme in vitro and the myristoylated forms are more active than the unmodified forms [31–33]. PLD2 shows little or no response to Arf [12,20]. The interaction site on ARF for PLD has been localized to the N-terminus [34,35], but it is not known where ARF binds on PLD. Interestingly, truncation of the first 308 amino acids of PLD2 renders it capable of responding to Arf [20]. In contrast to the many studies showing that PLD can be activated by Arf1 in vitro, there is less information on the role of this small G protein in the activation of the enzyme in vivo. On the other hand Arf 6 is principally localized to the plasma membrane [36–39] where it probably regulates PLD. For example, stimulation of chromaffin cells results in translocation of Arf6 to the plasma membrane and a concurrent increase in PLD activity [39]. Furthermore, the activation of PLD is blocked by addition of a myristoylated peptide corresponding to the N-terminus of Arf6 [39]. The use of brefeldin A to explore the role of Arf in agonist activation of PLD in vivo has given variable results. This is probably because this compound inhibits some, but not all, of the guanine nucleotide exchange factors for Arfs [40]. As for RhoA, Arfs 1 and 6 have been reported to activate phosphatidylinositol 4-P 5-kinase and this could also control the activity of PLD through increases in PIP_2 [41,42].

5. Regulation by tyrosine phosphorylation

Agents that increase reactive oxygen species can activate PLD through tyrosine phosphorylation. Thus H_2O_2 in the presence of vanadate induces both activation and tyrosine phosphorylation of PLD1 in several cell types [43–46]. Tyrosine kinase inhibitors also inhibit the activation of PLD induced by many agonists whose receptors couple to heterotrimeric G proteins [1]. However, the tyrosine kinases involved and the exact relationship between tyrosine phosphorylation of the enzyme and its increased catalytic activity remain unclear. As described below, v-Src can activate PLD [47], but the effect is mediated by members of the Ras subfamily of small G proteins.

6. Regulation by small G proteins of the Ras family

Ras does not directly activate PLD, but there is evidence that Ras mediates the activation of PLD induced by v-Src in vivo [48]. Further work has shown that RalA, a member of the Ras subfamily, is also required [49]. The precise interactions between Ras, RalA and PLD1 that lead to activation of the enzyme are not clear, but may involve Arf [50,51]. A role for Ras and Ral in PLD activation has also been indicated through the use of clostridial toxins that inactivate Ral, and by transfection with dominant negative forms of Ras and RalA [52,53].

7. Conclusions

Some information about the three-dimensional structure of mammalian PLDs is emerging from reports of the crystal structure of plant and bacterial members of the PLD superfamily. These reports have provided insights into the structure of the catalytic center and the catalytic mechanism. However, these proteins are small and do not possess the N- and C-terminal domains of mammalian PLDs at which regulatory proteins interact. The intracellular localizations of PLD1 and PLD2 need better definition to help clarify their mechanisms of regulation. PKC is a major regulator of PLD activity in vivo and in vitro, and in vitro evidence indicates that it can act through a direct non-phosphorylating mechanism. There is also evidence for a non-phosphorylating mechanism in vivo. However, PLD1 and PLD2 can be phosphorylated on Ser, Thr and Tyr residues in vivo and the significance of this in terms of their activities and intracellular localization needs to be clarified. Rho family G proteins activate PLD1 in vitro and are involved in the regulation of this enzyme by several agonists in vivo. However, the extent to which the enzyme is activated by direct interaction with Rho proteins versus changes in PIP_2 levels needs to be defined. Although all Arf family G proteins activate PLD1 in vitro, the evidence for an in vivo role is strongest for Arf 6. With respect to tyrosine kinases, studies with inhibitors of these kinases suggest a role for tyrosine phosphorylation in the activation of PLD by many agonists. Ras and Ral appear to play a role in the regulation of PLD by tyrosine kinases. However, the mechanisms involved appear to be complex and further work is needed to clarify these.

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