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Phosphoinositide-specific phospholipase C: structural basis for catalysis and regulatory interactions

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Phosphoinositide-specific phospholipase C (PI-PLC) isozymes have an important role in cellular responses to a variety of extracellular signals. Recently, the three-dimensional structures of their isolated domains and of the multidomain core, common to all PI-PLCs, have been solved. This provided an insight into the domain organization of PI-PLCs and, together with the structure–function analysis, contributed towards an understanding of the molecular mechanisms of catalysis and regulation.

Key words: catalysis / G-proteins / phosphoinositide-specific phospholipase C (PI-PLC) / three-dimensional structure / tyrosine kinase receptors

A LARGE NUMBER of extracellular signals stimulate hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP2) by phosphoinositide-specific phospholipase C (PI-PLC) (Figure 1). For the regulation of cellular processes, the best documented consequence of this hydrolysis is the generation of two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, involved in calcium release from intracellular stores and stimulation of protein kinase C isozymes.[1,2] In addition to the second messenger production, regulation of PIP2 concentrations itself could be relevant for cell signalling since many proteins bind and/or require PIP2 to function.[3,4]

Extensive studies of PI-PLC carried out during the past 10 years revealed the existence of many PLC isozymes and multiple pathways linking these isozymes to various receptors.[5-8] Determination of PI-PLC primary structures allowed the classification of 10 mammalian isozymes into three families: PLCβ (β1–β4), PLCγ (γ1–γ2) and PLCδ (δ1–δ4). While all isozymes have similar catalytic properties, different families are characterized by distinct ways of regulation. The PLCβ isozymes are activated through interaction with the α subunits of the pertussis toxin-insensitive Gq family of heterotrimeric G-proteins. The G-protein coupled receptors that are known to utilize this $G_q\alpha$/PLCβ pathway include those for bradykinin, bombesin, angiotensin, histamine, vasopressin as well as muscarinic (m1, m2 and m3) and α1 adrenergic agonists. PLCβ isozymes are also activated by the βγ subunits of pertussis toxin-sensitive G-proteins from the Gi/o family. The m2 and m4 muscarinic acetylcholine receptors and interleukin-8 receptor seem to be linked to the Gβγ/PLCβ pathway. Members of the PLCγ family are activated by receptor and non-receptor protein tyrosine kinases. Agonists for receptor tyrosine kinases such as PDGF, EGF, FGF and NGF are known to stimulate PLCγ in a wide variety of cells. Non-receptor protein tyrosine kinases that provide a link between different receptors (e.g. multicomponent T-cell antigen receptor and some G-protein coupled receptors) and PLCγ isozymes are likely to include members of Src, Syk and Jak/Tyk families. PLCγ isozymes could also be activated independently of the tyrosine kinase stimulation, for example, in the presence of arachidonic acid and microtubule-associated tau proteins.[9] The stimulation by arachidonic acid could serve as a link between receptor activation of phospholipase A2 (PLA2) and the PI-PLC pathway. In comparison to PLCβ and PLCγ isozymes, the physiological role and regulation of members of the PLCδ family have remained poorly defined. Studies of these isozymes have focused on PLCδ1 and on recently described PLCδ4. PLCδ4, but not PLCδ1, is expressed at S-phase of the cell cycle and is found predominantly in the nucleus.[10] Studies of PLCδ1 have shown binding of this isozyme to p122 protein with Rho GAP activity[11] and a novel type of a GTP-binding protein Gh,[12] suggesting regulation through protein–protein interactions. Interaction of PLCδ1 with Gh is likely to provide a direct link with transmembrane signalling by coupling this isozyme to the α1-adrenergic receptor. Studies of PLCδ1 also revealed that molecules such as calcium[13] and IP3[14,15] could have an important regulatory role.

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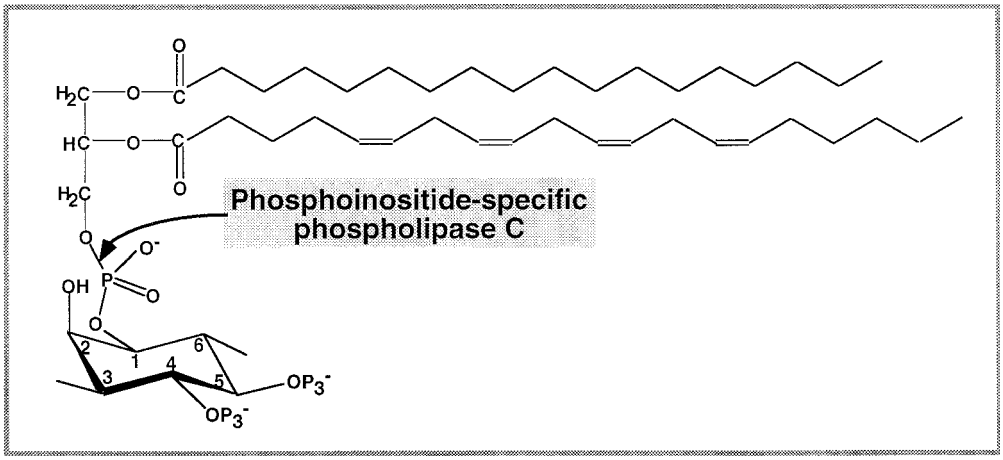

Figure 1. Hydrolysis of PIP2 by PI-PLC.

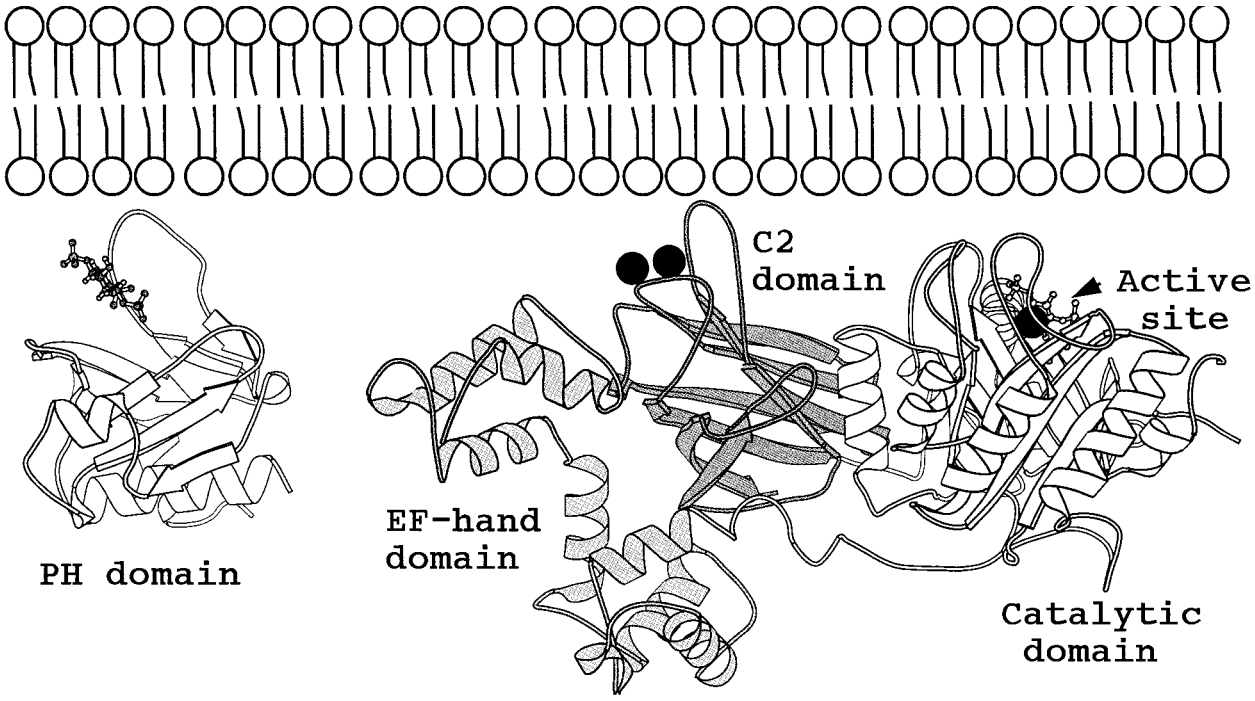

Figure 2. Structure and ligand binding sites of PLCδ1. PLCδ1 has four domains: the pleckstrin homology (PH) domain, the EF-hand domain, the catalytic domain and the C2 domain. The PH domain (white) is a seven-stranded β-sandwich formed by two antiparallel β-sheets, closed at one end with an α-helix. Loops connecting the β strands are involved in binding of IP3 (shown in a ball-and-stick representation) and phospholipid headgroup of PIP2. The EF-hand domain (light grey) consists of four EF-hand structural motifs with a characteristic helix-loop-helix topology. They are distributed in two lobes, each containing two EF-hands. The catalytic domain (white) is an α/β- or distorted TIM-barrel and consists of eight parallel β-strands connected by seven helical elements. The active site is a broad depression at the C-terminal end of the barrel shown to accommodate the substrate headgroup (ball-and-stick) and one calcium ion (large black sphere). The C2 domain (dark grey) has an antiparallel, eight-stranded β-sandwich architecture and a calcium binding region formed by flexible loops that coordinate binding of multiple calcium ions (black spheres). The lipid bilayer is not drawn to scale.

Although a large number of PI-PLC isozymes have been identified and their main regulatory pathways mapped, questions related to molecular mechanisms of catalysis and regulation remained: What is the structural basis of substrate recognition and regulatory interactions? What is the mechanism of substrate hydrolysis? What changes underlie the transition from an inactive into an active PI-PLC after stimulation? In this review we discuss how recent progress in the determination of three-dimensional structures and structure–function analysis of PI-PLCs provided some of the answers to these questions.

PI-PLC structures

Insights into the structure of PI-PLC isozymes resulted from the determination of three-dimensional structures of their isolated domains and groups of domains.[16–20] The picture is almost complete for one of the isozymes from the PLCδ family, PLCδ1, where the structure of the N-terminal part[17] and of the enzymatically active deletion mutant lacking the N-terminus[16] have been solved (Figure 2). The N-terminal part corresponds to the pleckstrin homology (PH) domain while the rest of the molecule comprises the EF-hand, catalytic and C2 domain. What remains to be clarified is how the PH domain interacts with the three-domain core structure centered on the C2 domain. Structural studies and limited proteolysis have indicated a flexible surface-exposed link between the PH and EF-hand domain.[16,21]

Based on sequence similarity and structural studies, modular PH, EF-hand and C2 domains have been described in many proteins.[22-24] PLCδ1 PH domain shares a high degree of structural similarity with other known PH domains.[17] Similarly, structures of the PLCδ1 C2 domain[16] and the C2 domain from synaptotagmin I[25] superimpose well. Four EF structural motifs within the EF-hand domain are distributed in two lobes, showing similar arrangement as described for calmodulin.[16] Each of these structural modules found in different proteins is, however, likely to perform a variety of functions that could be mediated by interactions with different ligands. An insight into ligand binding properties of PLCδ1 modular domains has been obtained from complexes with IP3 and calcium.[16,17] These studies revealed how the loop region of the PH-domain can provide the non-catalytic substrate binding site. They also provided an insight into likely calcium binding sites

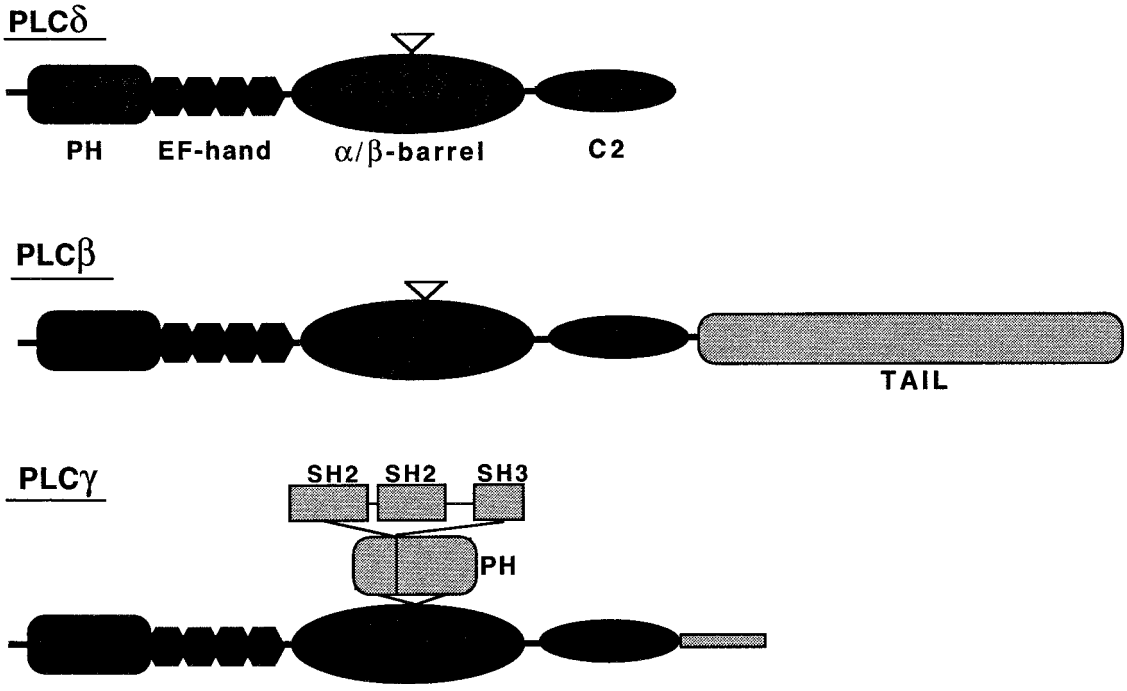

Figure 3. Domain organization of PLCδ, PLCβ and PLCγ isozymes. Domains present in PLCδ1 (black) represent a core structure of PI-PLC isozymes. PLCβ and PLCγ isozymes have additional regions unique for these PI-PLC families (grey).

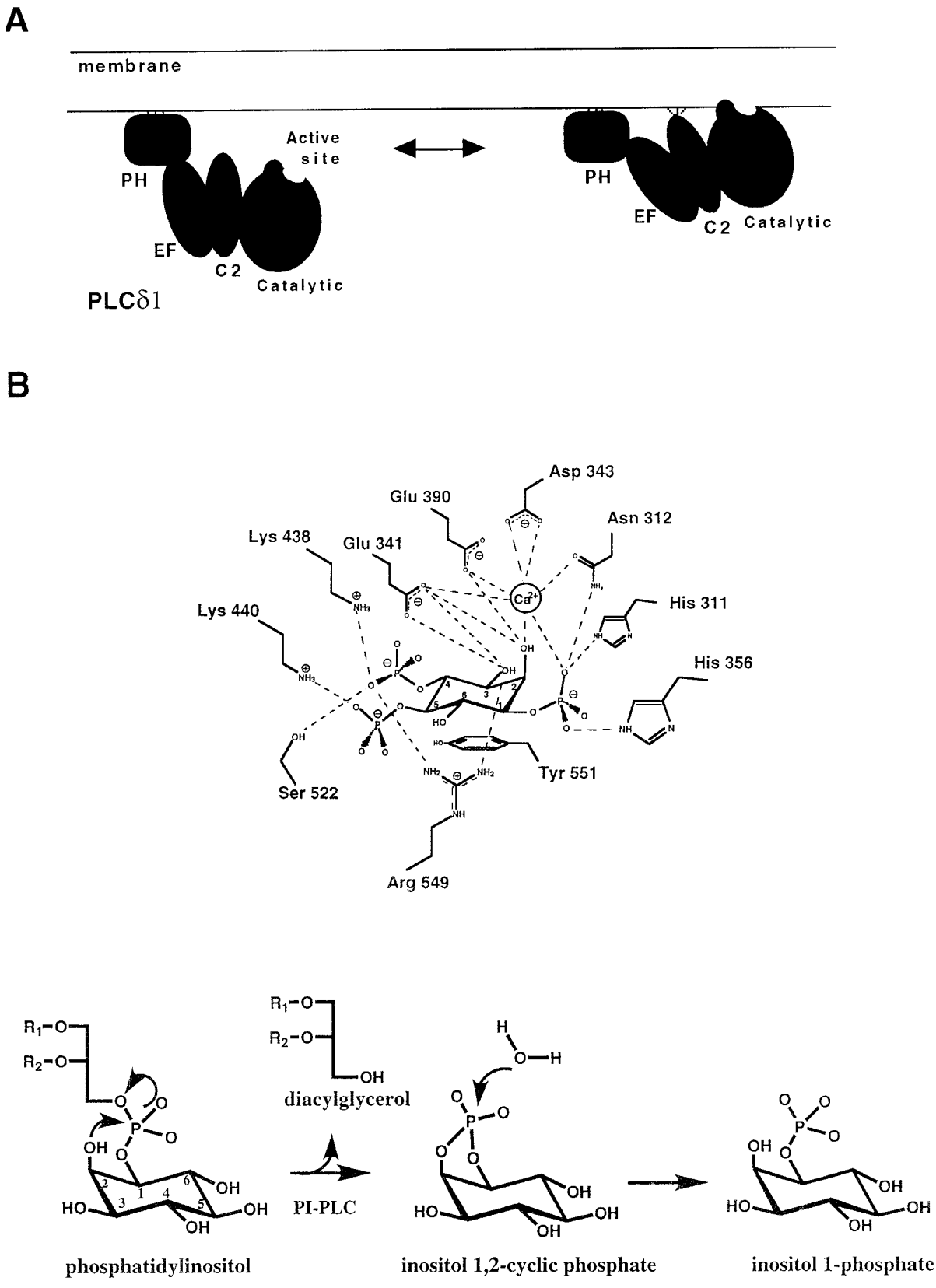
A
membrane
Active site
PH
EF
C2
Catalytic
PLCδ1
PH
EF
C2
Catalytic
B
Asp 343
Glu 390
Asn 312
Lys 438
Glu 341
Lys 440
His 311
His 356
Ca2+
Tyr 551
Ser 522
Arg 549
R1-O
R2-O
OH
diacylglycerol
PI-PLC
H
O—H
phosphatidylinositol
inositol 1,2-cyclic phosphate
inositol 1-phosphate

Figure 4.

within the C2 domain. The active site of the catalytic (α/β-barrel[26]) domain accommodates both the catalytic calcium and the substrate headgroup.

PLCδ1 shares similarity with other PI-PLC isozymes throughout its sequence.[5,27] An alignment of PI-PLC sequences has suggested that most PLCβ, PLCγ and PLCδ isozymes have all four domains found in PLCδ1[16,66] (Figure 3). Interactions between the domains are also expected to be similar. The regions of highest sequence similarity between different isozymes are contained within the catalytic domain and include all residues implicated in substrate binding and hydrolysis. Ligand binding properties of other domains (PH, EF-hand and C2 domain) are difficult to predict since, in most instances, the relevant residues present in PLCδ1 do not seem to be strictly conserved in other isozymes. In addition to the common domains, PLCβ and PLCγ isozymes have domains unique for each of these families (Figure 3). The PLCβ isozymes have a C-terminal extension of unknown structure; secondary structure prediction indicates that this region may be mostly helical. The PLCγ isozymes have an array of domains connected to the catalytic domain through a flexible region between the fourth β strand and the following α helix of the α/β-barrel. A likely arrangement of these domains could be described as a PH domain with one of its loops extended to accommodate two SH2 and SH3 domain. The three-dimensional structure is known only for the isolated C-terminal SH2 domain[19] and the SH3 domain[18] of PLCγ1.

Catalysis at an interface

PI-PLC isozymes, like many other enzymes involved in lipid signalling and metabolism, interact with their substrate at the lipid–water interface. The interfacial catalysis of mammalian PI-PLCs has been studied using phospolipid vesicles or monolayers and can be described as a two-step mechanism in which these enzymes first associate with the membrane via a site(s) distinct from the active site and then carry out substrate hydrolysis in the active site. Kinetic studies with purified PLCβ, PLCγ and PLCδ in the absence of other proteins have suggested that the membrane-associated isozymes can catalyse several cycles of PIP2 hydrolysis, functioning in a processive mode of interfacial catalysis.[28-30]

Interactions with the membrane

Interaction of PLCδ1 with the plasma membrane could involve the PH-domain, C2-domain and a hydrophobic ridge at the rim of active site opening[16] (Figure 4A). Several lines of experimental evidence support a role of the PH-domain in membrane attachment, mediated by the binding to PIP2 present in cellular membranes. *In vitro*, the high affinity binding of PLCδ1 to phospholipid vesicles occurs only if they contain PIP2.[31] Recognition of the PIP2 polar headgroup is critical and interactions with the vesicles could be inhibited by IP3.[14,15] The high-affinity, stereospecific binding to PIP2 and the processive catalysis require an intact PH domain.[29,32] The crystal structure of the PLCδ1 PH domain in a complex with IP3 revealed that the recognition of the PIP2 headgroup involves extensive interactions with the 4- and 5- phosphoryl groups and fewer interactions with the 1-phosphate.[17] Measurements of the binding affinity for PIP2 *in vitro* ($Kd \sim 2\,\mu M$)[33] suggest that it is sufficiently high to mediate the PH domain binding to the phospholipid *in vivo* at its estimated physiological concentrations (30–150 μm).[34] A role of the PH domain / PIP2 interactions *in vivo* is further supported by the studies of PLCδ1 localization in living cells; these studies have demonstrated that the PH domain is necessary for the plasma–membrane attachment.[35] It has also been suggested that changes in cellular concentrations of PIP2 and IP3 could regulate membrane binding of PLCδ1 and, consequently, its activity.[22]

At present, there is no direct evidence that the C2 domain of PLCδ1 interacts with the phospholipid membranes. However, since the similarity with the first C2 domain of synaptotagmin I is not limited to the overall structure and includes coordination of calcium analogues,[16,20] it is possible that the PLCδ1 C2 domain has the calcium-dependent lipid-binding (CaLB) function well documented for synaptotagmin I and cPLA2.[36,37] The calcium binding loops of the PLCδ1 C2 domain are on the same side of the molecule as the substrate binding site, in a position

Figure 4. Catalysis at an interface: Association with the membrane (A) and recognition and hydrolysis of the substrate within the active site (B). The PH domain is the main determinant of membrane tethering in PLCδ1. The C2 domain and the hydrophobic part of the catalytic domain could form additional interactions that fix PLCδ1 at the membrane surface (A). The residues involved in the substrate / calcium binding in the active site (B, upper panel) are highly conserved among PI-PLC isozymes. The hydrolysis of substrate is carried out by a sequential two-step mechanism (B, lower panel).

suitable for this putative calcium-dependent phospholipid headgroup binding. Studies of a PLCδ1 deletion mutant lacking the PH domain suggested that the interactions via the C2 domain may not be sufficient to independently mediate membrane interaction.[35] The function of this domain could be to, after membrane 'tethering' of PLCδ1 via the PH domain, provide additional interaction sites and 'fix' the catalytic domain into a productive orientation. In this orientation the hydrophobic ridge of the catalytic domain would presumably partially penetrate the membrane.

Studies of PLCβ and PLCγ have indicated that, like PLCδ1, these isozymes can directly bind to phospholipid vesicles. The structural basis for these interactions could be provided by the PH and C2 domains or by the domains specific for PLCβ and PLCγ isozymes. Studies limited to PLCβ1 and PLCβ2 isozymes have shown that the C-terminal extension was not required for binding to phospholipid vesicles.[38] However, it is not clear to what extent interactions observed with lipid vesicles contribute to interactions with the cellular membrane since studies of subcellular distribution of PLCβ isozymes demonstrated involvement of the C-terminal extension in binding to the particulate (membrane and cytoskeleton containing) fraction.[39]

Binding and hydrolysis of inositol lipids within the active site

The crystal structure of the PLCδ1 complex with different inositol phosphates and calcium revealed the structural basis for the substrate headgroup recognition and the calcium dependent hydrolysis[16,40] (Figure 4B). A number of amino acid residues within the active site are involved in hydrogen bonding and electrostatic interactions with the hydroxyl and phosphoryl groups of IP3 and the catalytic calcium ion. Residues interacting with 4 and 5 phosphates (Lys 438, Ser 522, Arg 549 and Lys 440) could be determinants of the substrate preference for PIP2 > PIP > > PI.[16] Consistent with this role are the data from mutational analysis of Arg 549, demonstrating the requirement of its positive charge for recognition and hydrolysis of PIP2 but not PI.[41]

Hydrolysis of the bound substrate generates acyclic inositol phosphate(s) as the main water soluble product. In addition, small amounts (5–30%) of cyclic inositol phosphates are produced by all PI-PLC isozymes.[5] To explain generation of cyclic and acyclic products two major models have been considered: a parallel mechanism in which these reaction products are formed simultaneously and a sequential mechanism which proposed formation of cyclic inositol phosphate as the reaction intermediate.[42] Recent structural and kinetic studies have provided evidence that strongly supported general acid/base catalysis in a sequential mechanism.[40] The formation of cyclic inositol involves calcium-facilitated deprotonation of the 2-hydroxyl group by a general base. The most likely candidate residues for the general base are Glu 341 and Glu 390. His 356 is suitably positioned to act as a general acid catalyst for the protonation of the diacylglycerol leaving group. The calcium ion and His 311 probably stabilize the transition state in the formation of the cyclic inositol phosphate. The enzyme-bound cyclic intermediate is subsequently hydrolysed and in this reaction His 356 would promote the nucleophilic attack of water as a general base catalyst while Glu 341 or Glu 390 would then have the role of the acid catalyst. Mutations of PLCδ1 His 356, His 311 and Glu 341 (and the corresponding histidines in PLCγ) greatly reduce or abolish enzyme activity.[43-46]

Regulation of PI-PLCs

As a part of signalling pathways PI-PLC isozymes are stringently controlled and the rate of PIP2 hydrolysis is stimulated only transiently in response to different input signals. As summarized in the introductory section, a large number of interactions between PI-PLC and other signalling components can cause this stimulation. In the cellular setting, an additional complexity results from the presentation and availability of PIP2. Many proteins with different cellular localization bind PIP2 and could affect its hydrolysis by PI-PLC.[3] For example, phospatidylinositol transfer protein greatly stimulates PI-PLC by increasing the pool of accessible PIP2.[47] Binding of PIP2 by other proteins could have an inhibitory effect by sequestering this phospholipid from PI-PLC; this function has recently been suggested for MARCKS (myristoylated alanine-rich C kinase substrate) protein.[48]

Despite this complexity, some of the regulatory interactions that mediate stimulation of PI-PLC isozymes have been extensively studied; these include interaction of PLCγ with tyrosine kinase receptors and interactions between G-protein subunits and PLCβ isozymes (Figure 5). Most of the data have been obtained from mutational analysis.

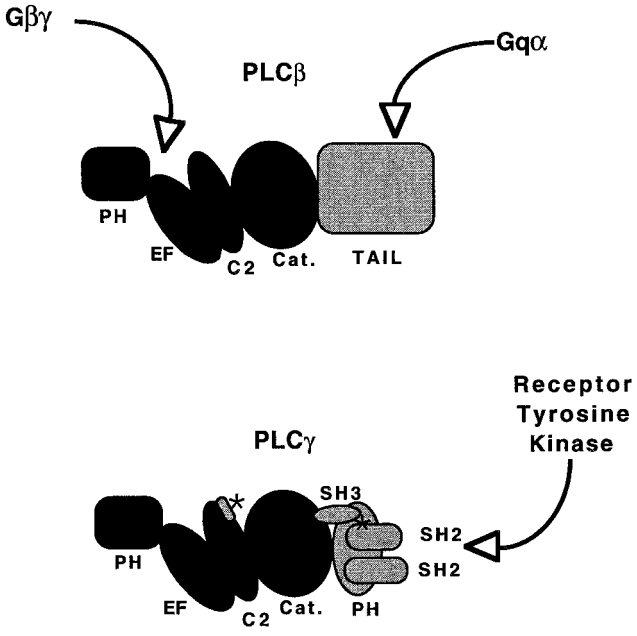

Figure 5. Interactions of PLCβ and PLCγ isozymes with their regulatory proteins. The arrows indicate interaction sites in PLCβ with the G-protein subunits, Gβγ (likely to interact with a part of the PH domain and EF-hand domain) and Gqα (binding to a 'tail' region). PLCβ isozymes differ in their sensitivity to each of these G-protein subunits. The interaction site of PLCγ with tyrosine kinase receptors is also indicated and involves binding to the SH2 domains. PLCγ is phosphorylated by receptor tyrosine kinases and two critical residues (*) are present within the region between the SH2 and SH3 domains (Tyr 783 in PLCγ1) and near the C-terminus (Tyr 1254 in PLCγ1). Colour coding is as in Figure 3.

Activation of PLCγ by tyrosine kinase receptors

Activation of PLCγ by tyrosine kinase receptors is accompanied by phosphorylation on several tyrosine residues, two of which (tyrosine residues 783 and 1254 in PLCγ1) were shown by mutational analysis to be essential for agonist-induced activation of PIP2 hydrolysis.[49] However, the phosphorylation of PLCγ is not sufficient for the activation which also requires high affinity association with the receptor, independently of the enzyme phosphorylation. This has been best documented using a mutant PDGF receptor that could readily phosphorylate PLCγ 1 but was unable to associate with or activate this isozyme.[50] The association between PLCγ and tyrosine kinase receptors involves binding by the SH2 domains of PLCγ isozymes to a subset of phosphorylated tyrosine residues within the cytoplasmic portion of a receptor. For example, the autophosphorylated Tyr1021 in the PDGF receptor is specifically recognized by the second SH2 domain of PLCγ1.[50] The recognition is accomplished by a specific fit of the acidic phosphotyrosine residue into a positively charged pocket on the SH2 domain, while several residues neighbouring the pTyr1021 in the PDGF receptor fit into an extended hydrophobic groove in the SH2 domain.[19] Studies of the PDGF phosphopeptide/PLCγ1 interaction by CD spectroscopy have indicated a conformational change in the enzyme that could be a part of the PLCγ activation mechanism by the receptor binding.[51] Phosphorylation on PLCγ by the receptor tyrosine kinase could lead to further changes where, by analogy with c-Src,[52] intramolecular interactions could be formed between SH2 domains and phosphorylated tyrosines. There are several possibilities how conformational changes resulting from the receptor stimulation could, in turn, lead to stimulation of PLCγ activity. A conformational change could either affect subcellular localization of PLCγ[53,54] or have a direct influence on the substrate access to the active site that, under specific conditions of substrate presentation, seems to be limiting for the non-stimulated enzyme.[30,55] Several lines of experimental evidence indicate that the part of PLCγ that could have a role in restricting substrate access includes the SH2/SH3 domain array.[56-58]

Activation of PLCβ by G-protein subunits

Studies of PLCβ deletion mutants and experiments based on limited proteolysis clearly demonstrated a requirement for the unique C-terminal portion of these isozymes to bring about stimulation with Gqα.[59,60] This tail region has also been shown to have a GTP-ase activating role for Gqα.[8] The interaction site with Gqα has been mapped to residues 903–1142 of PLCβ1.[59] According to secondary structure prediction, this region has several helical segments, each containing a cluster of basic residues.[39] Further analysis has shown the importance of several basic residues within this region for the stimulation by Gqα.[39] The interaction site with Gβγ is clearly different from that for Gqα[61,62] but still needs to be more precisely defined. Studies of other proteins that are regulated by Gβγ, most notably β-adrenergic receptor kinase, demonstrated that the C-terminal part of the PH domain and about 30 residues following it, participate in binding of Gβγ. It has been shown that the corresponding region in PLCβ2, present within the N-terminal part of this isozyme, was indeed required for stimulation by Gβγ.[61] However,

this region of PLCβ2 was not sufficient to confer high levels of responsiveness to Gβγ when incorporated into the much less sensitive PLCβ1 isozyme. Further analysis of PLCβ2 indicated that additional sites, present in the catalytic domain, could contribute to Gβγ stimulation.[63]

Experimental evidence needed to explain the molecular mechanism of PLCβ activation by Gqα and Gβγ is still very limited. Nevertheless, recent studies of PLCβ interaction with phospholipid vesicles in the presence and absence of Gβγ have shown that these subunits did not mediate enzyme translocation to the vesicle membrane.[64,65] In this system, saturation of membrane binding was achieved in the absence of G-protein subunits and their stimulatory effect was exerted through interaction with the membrane-bound enzyme. A function of these protein/protein interactions could be to stabilize PLCβ interactions with the membrane surface and perhaps to orient the catalytic domain more favourably with respect to the membrane-resident substrate. Although the function of G-protein subunits in the cellular context is more complex than in the system using phospholipid vesicles, protein–protein interactions between membrane-bound components are likely to be an important part of the PLCβ activation mechanism.

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