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Structural Insights into Phospholipase C-β Function

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Abbreviations: PLC, phospholipase C; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; GPCR, G protein-coupled receptor; PH, pleckstrin homology; TIM, triose phosphate isomerase; CTD, C-terminal domain; BAR, Bin-Amphiphysin-RVS; PDZ, post synaptic density protein/Drosophila disc large tumor suppressor/zona occludens-1 protein; GAP, GTPase activating protein

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Abstract

Phospholipase C (PLC) enzymes convert phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messengers diacylglyercol (DAG) and inositol-1,4,5-triphosphate (IP_3). The production of these molecules promotes the release of intracellular calcium and activation of PKC, which results in profound cellular changes. The PLCβ subfamily is of particular interest given its prominent role in cardiovascular and neuronal signaling and its regulation by G protein-coupled receptors, as PLCβ is the canonical downstream target of the heterotrimeric G protein $G\alpha_q$. However, this is not the only mechanism regulating PLCβ activity. Extensive structural and biochemical evidence has revealed regulatory roles for autoinhibitory elements within PLCβ, Gβγ, small molecular weight G proteins, and the lipid membrane itself. Such complex regulation highlights the central role this enzyme plays in cell signaling. A better understanding of the molecular mechanisms underlying the control of its activity will greatly facilitate the search for selective small molecule modulators of PLCβ.

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Introduction

Phospholipase C (PLC) enzymes are responsible for the hydrolysis of the inner membrane component phosphatidylinositol-4,5-bisphosphate (PIP_2), generating the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is freely diffusible and binds to IP_3-specific receptors, leading to the release of intracellular Ca^{2+}. DAG remains membrane associated and, together with increasing Ca^{2+}, activates protein kinase C (PKC). These events are associated with the regulation of numerous physiological processes, including muscle contraction (Berridge, 2003; Woodcock, 2009), chemotaxis (Jiang et al., 1997; Li et al., 2000), opioid sensitivity (Mathews et al., 2008; Murthy and Makhlouf, 1996; Wu et al., 1998), and cell proliferation and survival (Braz et al., 2004; Newton, 2010; Palaniyandi et al., 2009).

There are six subfamilies of PLC in higher eukaryotes (Gresset et al., 2012; Kadamur and Ross, 2012). Of these, the PLCβ subfamily is among the most intensively studied. These enzymes are the canonical downstream targets of the G_q subfamily of G protein-coupled receptors (GPCRs) and play prominent roles in cardiovascular function, chemotaxis, and neuronal signaling. In the absence of extracellular stimuli, PLCβ exhibits very low intrinsic PIP_2 hydrolysis, but is robustly activated upon direct interactions with $G\alpha_q$. GPCR-mediated activation of PLCβ also occurs through release of the Gβγ heterodimer, which is thought to be mediated by activation of G_i-coupled GPCRs (Camps et al., 1992; Katz et al., 1992; Wu et al., 1998; Xie et al., 1999). Members of the Rho family of small molecular weight G proteins, such as the Rac isoforms, also directly bind and activate PLCβ, linking PLCβ activity to GPCR-independent signaling cascades (Gresset et al., 2012; Kadamur and Ross, 2012). It is also increasingly recognized that the membrane itself plays a role in the regulation of PLCβ, as may interactions with scaffolding proteins (Cartier et al., 2011; Grubb et al., 2011; Grubb et al., 2012;

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Sun et al., 2013). In this review, we highlight the current understanding of the molecular basis of regulation of mammalian PLCβ enzymes and their modulation by small molecules, with an emphasis on recent structural discoveries.

Structure of the PLCβ catalytic core and its C-terminal extension.

As in most other PLC enzymes, PLCβ proteins share a highly conserved catalytic core comprised of an N-terminal pleckstrin homology (PH) domain, four tandem EF hand repeats, a triose phosphate isomerase (TIM)-like barrel domain split into X and Y halves and which houses the active site, and a C2 domain (**Figures 1, 2**). With the exception of the TIM barrel, the domains have somewhat unconventional roles. Unlike the PLCδ PH domain, which binds PIP_2 with high specificity and affinity, the PLCβ PH domain only weakly contributes to membrane association (Tall et al., 1997; Wang et al., 1999b) and is intimately associated with the rest of the catalytic core. Instead, its most significant role is arguably its contribution to regulatory protein–protein interactions. The EF hands, contrary to their role in other well-known proteins such as calmodulin, do not bind Ca^{2+}. In PLCβ, they serve as a scaffold and contain the loop responsible for stimulating GTP hydrolysis when $G\alpha_q$ is bound. Finally, unlike many other C2 domains, the PLCβ C2 domain does not participate in Ca^{2+}-mediated interactions with the membrane, but instead contributes to intra- and intermolecular regulatory binding sites.

The mechanism by which PLC enzymes hydrolyze PIP_2 to generate DAG and IP_3 was determined with the help of crystal structures of PLCδ1 (Ellis et al., 1998; Essen et al., 1996; Essen et al., 1997) and is described in greater detail elsewhere (Gresset et al., 2012). Briefly, the catalytic Ca^{2+} is proposed to decrease the pK_a of the inositol 2-hydroxyl group, and with the assistance of the putative catalytic base (Glu341 in PLCβ3), promotes the formation of a 1,2-

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cyclic monophosphate intermediate and DAG. This cyclic intermediate is stabilized via the 1-phosphate by a histidine (His332 in PLCβ3) and Ca^{2+}. In the next step, another histidine (His379 in PLCβ3) abstracts a proton from water, which attacks the intermediate to release IP_3 (Ellis et al., 1998; Essen et al., 1997). A ridge of hydrophobic residues adjacent to the active site also facilitates catalysis (**Figure 3**) (Essen et al., 1997). Mutation of these residues within PLCδ (Ellis et al., 1998) or in PLCβ3 (Lyon et al., 2013) decreases basal activity and/or protein expression. Studies of PLCβ1 and PLCβ2 found that increasing surface pressure diminishes catalytic activity, suggesting membrane insertion contributes to activity (James et al., 1997). Taken together, these observations are consistent with the idea that insertion of the hydrophobic ridge into the membrane is required for efficient catalysis.

Although all the catalytic machinery is in place, the PLCβ active site cannot readily bind PIP_2 in any reported crystal structure. The two halves of the PLCβ catalytic TIM barrel-like domain are separated by a poorly conserved X–Y linker that typically bears a stretch of highly acidic residues (**Figure 1, 2**). The C-terminus of this linker is ordered in all reported crystal structures (**Table 1**) and interacts with residues adjacent to the active site cavity in a manner that would sterically prevent the binding of PIP_2 (**Figure 3**). As discussed below, perturbation of the X–Y linker region may play an important role in regulation of PLCβ isozymes (Ellis et al., 1993; Hicks et al., 2008; Schnabel and Camps, 1998; Zhang and Neer, 2001).

The defining element of the PLCβ subfamily is a ~400 amino acid C-terminal extension that contains highly conserved segments at its N-terminus (the proximal CTD) and an elongated ~300 amino acid coiled-coil domain (the distal CTD) separated by a 28–61 residue flexible linker region (the CTD linker). Numerous studies have shown the C-terminal extension is required for maximum basal and $G\alpha_q$-stimulated activity, $G\alpha_q$ binding and association with the

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particulate fraction of cells and/or membranes (Adjobo-Hermans et al., 2013; Adjobo-Hermans et al., 2008; Jenco et al., 1997; Kim et al., 1996; Park et al., 1993; Schnabel et al., 1993), yet it is dispensable for Rac and Gβγ activation (Illenberger et al., 2003a; Lee et al., 1993b; Waldo et al., 2010; Wu et al., 1993a). The proximal CTD is comprised of the first ~40 amino acids immediately following the C2 domain and contains the primary $G\alpha_q$ binding site (Waldo et al., 2010), followed by an autoinhibitory helix designated Hα2' (Lyon et al., 2011) (**Figures 2, 5)**. The role of these structural elements in regulation of activity is discussed in later sections.

The distal CTD is believed to be the primary membrane binding determinant in PLCβ isozymes and is required for maximal basal and stimulated activity (Kim et al., 1996; Lee et al., 1993b; Lyon et al., 2013; Lyon et al., 2011; Waldo et al., 2010). One mystery concerning the distal CTD is its lack of strong sequence conservation (~30–35% identity across PLCβ isoforms) despite its importance in activity and regulation by $G\alpha_q$. Structural insights into the distal CTD were first obtained from a crystal structure of an isolated engineered domain derived from turkey PLCβ (Singer et al., 2002), revealing an unusual ~140 Å long helical bundle comprised primarily of three long, kinked helical spans and several shorter, bridging helices (**Figures 2, 4**). The "core" of the domain, which contains some of the most highly conserved residues, is found where the Dα2 helix crosses one face of the helical bundle. The entire distal CTD is stabilized primarily through coiled-coil interactions, which may have relatively low stringency for amino acid side chains, and thus could account for the low sequence conservation (Lyon et al., 2013; Singer et al., 2002; Zhang et al., 2006). The tertiary structure of the distal CTD was confirmed in the crystal structure of full length PLCβ3 in complex with $G\alpha_q$ (**Figures 2, 4**) (Lyon et al., 2013). The PLCβ3 distal CTD has a greater degree of curvature compared to the turkey structure, likely due to the inherent flexibility of the domain and to differences in sequence and crystal contacts.

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Comparisons of the PLCβ distal CTD to other structures identified the Bin-Amphiphysin-Rvs (BAR) domains as structural homologs. These domains are also extended helical bundles that interact with negatively charged phospholipids (Peter et al., 2004; Qualmann et al., 2011). Although an intriguing possibility, it is unknown whether the PLCβ distal CTD can sense and/or induce membrane curvature, as do some BAR domains.

The turkey distal CTD crystallized as a dimer, burying ~3100 $Å^2$ of accessible surface area. Mutation of conserved hydrophobic residues within the analogous dimer interface of PLCβ1 were shown to impair activation by $Gα_q$ (Ilkaeva et al., 2002), and size exclusion analysis of both purified PLCβ proteins and isolated distal CTDs, as well as cell-based studies suggested the existence of dimers (Singer et al., 2002; Zhang et al., 2006). Conversely, studies of full-length human PLCβ3 found no evidence of oligomerization as assessed by size exclusion chromatography, multi-angle light scattering, cryo-electron microscopy, or X-ray crystallography (Lyon et al., 2013). Instead, many of the conserved residues that contributed to the dimer interface in the turkey distal CTD structure instead form an intermolecular contact with the N-terminus of $Gα_q$ (**Figures 2, 4**).

PLCβ isoforms, splice variants, and function.

There are four PLCβ isoforms (PLCβ1–4), three of which are expressed as splice variants (**Figure 1**). The sites of variation are typically localized within the C-terminal extension and alter the total length of the enzyme, potentially effecting membrane association and/or the ability to interact with scaffolding proteins or activators (Suh et al., 2008). Now that a full length PLCβ enzyme has been structurally characterized, the sites of variation can be more accurately mapped and functional differences resulting from these changes can be considered.

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PLCβ1 is expressed in the cerebral cortex and hippocampus, where the enzyme regulates neuronal activity (Bohm et al., 2002; Kim et al., 1997), and in the cardiovascular system (Arthur et al., 2001; Descorbeth and Anand-Srivastava, 2010; Mende et al., 1999; Ushio-Fukai et al., 1998). In vascular smooth muscles cells exposed to high glucose concentrations, $G\alpha_q$ and PLCβ1 expression increases, resulting in higher intracellular Ca^{2+}. This Ca^{2+} increase is thought to be an underlying mechanism in vascular complications of diabetes (Descorbeth and Anand-Srivastava, 2010). There are two PLCβ1 splice variants, each of which has been assigned a specific role (Faenza et al., 2000; Filtz et al., 2009; Grubb et al., 2008; Woodcock et al., 2009). PLCβ1a and PLCβ1b differ at the extreme C-terminus, beyond the last residue observed in the reported crystal structures. PLCβ1a is longer and contains a consensus PDZ motif at its C-terminus, whereas PLCβ1b contains a proline-rich region (Bahk et al., 1994; Bahk et al., 1998; Grubb et al., 2008). Both variants are reported to interact with the membrane, suggesting full function of the distal CTD, although there may be variation between cells (Adjobo-Hermans et al., 2013; Adjobo-Hermans et al., 2008; Grubb et al., 2008). PLCβ1a and PLCβ1b have been detected in the nucleus where they contribute to the regulation of cell cycle progression, in particular the G_1/S transition (Bahk et al., 1998; Faenza et al., 2000; Fiume et al., 2012; O'Carroll et al., 2009). The PLCβ1 variants have been reported to have unique functions with the cardiac sarcolemma. PLCβ1b is membrane-associated and interacts with the scaffold proteins Homer1b/c and Shank3, enabling its rapid activation upon G_q-coupled receptor stimulation (Grubb et al., 2011; Grubb et al., 2012; Grubb et al., 2008; Shin et al., 2003). In contrast, PLCβ1a is cytosolic and does not interact with these scaffold proteins. Upregulation of the $G\alpha_q$-PLCβ1b pathway results in increased cell size and expression of hypertrophic markers (Descorbeth and Anand-Srivastava, 2010; Filtz et al., 2009).

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PLCβ2 is expressed in hematopoietic cells and platelets, where it is involved in chemotaxis (Mao et al., 2000; Suh et al., 2008; Sun et al., 2007; Tang et al., 2011). Paradoxically, loss of PLCβ2 in neutrophils increased their sensitivity to inflammatory agents and chemoattractants, despite a requirement for Ca^{2+} and IP_3 during the early stages of chemotaxis. It may be that in later stages of chemotaxis, PLCβ2 has an inhibitory role (Jiang et al., 1997; Li et al., 2000). PLCβ2 is also required for thrombin-induced Ca^{2+} release in platelets through a $G\alpha_q$-dependent mechanism (Vaidyula and Rao, 2003). PLCβ2 is found as two splice variants, PLCβ2a and PLCβ2b, where PLCβ2b is missing 19 internal residues that span the C-terminus of the CTD linker and the Dα1 helix of the distal CTD (human PLCβ3 residues 930-948). Based on the structure of full length PLCβ3, this deletion is expected to unmask a hydrophobic patch on the surface of the PLCβ2b distal CTD, but it is unclear whether this would significantly alter known functions of the domain (**Figure 4**).

Only one variant of PLCβ3 has been characterized in humans, where it is expressed in the brain, liver, parotid gland (Bianchi et al., 2009; Han et al., 2006; Jhon et al., 1993), hematopoietic cells (Cai et al., 2005; Li et al., 2000; Xiao et al., 2009) and the cardiovascular system (Arthur et al., 2001; Mende et al., 1999). Within the nervous system, PLCβ3 is required for opioid-induced Ca^{2+} release through a Gβγ-dependent pathway, and it also mediates Ca^{2+} release in response to noxious stimuli (Bianchi et al., 2009; Han et al., 2006; Mathews et al., 2008; Xie et al., 1999). In the hematopoietic system, PLCβ3 inhibits proliferation by preventing differentiation through interactions with the transcription factor Stat5 and its regulator SHP1 (Xiao et al., 2009), and it also contributes to regulation of chemotaxis in neutrophils (Li et al., 2000). Lastly, in mouse models of G_q-mediated cardiac hypertrophy, increased PLCβ3 expression and activity have been reported (Mende et al., 1998; Mende et al., 1999).

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PLCβ4 is most similar to NorpA, the invertebrate PLCβ homolog required for phototransduction, and is highly expressed in the retina and the cerebellum (Adamski et al., 1999; Jiang et al., 1996; Lee et al., 1993a; Suh et al., 2008). Within the retina, PLCβ4 is required for visual processing events following phototransduction (Jiang et al., 1996), and loss of PLCβ4 in mice also results in motor defects (Kim et al., 1997). Two splice variants PLCβ4 have been identified in humans. PLCβ4a is the full-length protein, whereas PLCβ4b is truncated at the extreme C-terminus after the end of the structurally characterized distal CTD in PLCβ3 (**Figure 1**) (Adamski et al., 1999), which has been proposed to alter the efficacy of $G\alpha_q$-dependent activation. An interesting splice variant of PLCβ4 has been identified in rat retina, in which the protein is truncated at the beginning of helix Dα4 (human PLCβ3 residue 1040) (Kim et al., 1998). This variant would clearly disrupt the fold of the distal CTD, likely explaining its loss of membrane association and $G\alpha_q$ responsiveness. As Kim *et al.* conjectured, even though the remaining portion of the distal CTD in this variant contain some of the most significant stretches of basic charge, their spatial localization, as dictated by a properly folded domain, seems to be essential for association with the particulate fraction of cells.

Regulation of PLCβ Basal Activity.

PLCβ Membrane Binding Determinants. To prevent aberrant signaling and retain sensitivity to extracellular signals, PLCβ isozymes must have very low intrinsic activity. Because they interact with phospholipid bilayers to hydrolyze PIP_2, control of membrane localization provides a straightforward mechanism for regulation of basal enzymatic activity (Jenco et al., 1997; Romoser et al., 1996; Runnels et al., 1996; Scarlata, 2002). Each PLCβ isoform has a unique

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subcellular distribution, despite sharing a conserved structure and membrane binding determinants. Therefore, relatively subtle differences in their amino acid sequences, and potentially their interactions with scaffolding proteins, likely dictate their cellular location. In general, PLCβ1 and PLCβ4 variants seem to be primarily membrane associated, whereas PLCβ2 and PLCβ3 seem primarily cytosolic (Adjobo-Hermans et al., 2013; Adjobo-Hermans et al., 2008; Grubb et al., 2008; Illenberger et al., 2003b), although it is likely that all of these isoforms are in equilibrium between the membrane and cytoplasm.

The PLCβ PH domain has been proposed to contribute to membrane binding, in part because some PH domains have high specificity and affinity for certain phospholipids (Lemmon, 2004; Philip et al., 2002). The PLCδ PH domain specifically binds PIP_2 and flexibly tethers the rest of enzyme to the membrane (Cifuentes et al., 1993; Essen et al., 1996; Ferguson et al., 1995; Garcia et al., 1995). However, most of the residues that coordinate the inositol head group in PLCδ are absent in PLCβ, and the PLCβ PH domain has micromolar affinity and little specificity for negatively charged phospholipids (Tall et al., 1997; Wang et al., 1999b). In addition, in all reported structures, the PLCβ PH domain forms an extended interface with the EF hands and the X domain of the TIM barrel, burying ~3000 $Å^2$ surface area (Hicks et al., 2008; Jezyk et al., 2006; Lyon et al., 2013; Lyon et al., 2011; Waldo et al., 2010). Therefore, lipid interactions with the PH domain could, in principle, directly influence the orientation of the entire catalytic core at the membrane.

However, the primary membrane binding element within PLCβ enzymes seems to be the distal CTD. Truncation of the C-terminal extension, internal deletions, and mutations within the distal CTD are sufficient to abrogate association with the particulate fraction of cells, membranes and liposomes (Jenco et al., 1997; Kim et al., 1996; Kim et al., 1998; Lee et al., 1993b; Schnabel

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et al., 1993; Wu et al., 1993a), and the overexpressed C-terminal extensions of PLCβ associate with membranes in cells (Adjobo-Hermans et al., 2013). Initial studies led to the identification of the "P box", a 127 residue region (human PLCβ3 residues 947–1057) essential for membrane association (Wu et al., 1993a). Additional studies of the C-terminal extension identified three highly conserved basic clusters whose mutation significantly decreased particulate association and lowered basal activity. However, only deletion of the entire C-terminal extension completely eliminated particulate association (Ilkaeva et al., 2002; Kim et al., 1996; Lee et al., 1993b; Wu et al., 1993a). Crystallographic studies confirmed these basic residue clusters fall on the same face of the distal CTD formed by the Dα3 and Dα4 helices, generating a long and highly polarized electrostatic surface that likely forms the primary membrane interaction site (**Figure 4**) (Lyon et al., 2013; Singer et al., 2002). Sequence variation among the PLCβ isoforms in the distal CTD may result in different degrees of membrane association, or lead to distinct modes of auto-inhibition via protein-protein interactions *in cis*. For example, in both the crystal structure and single particle cryo-electron microscopy 3D reconstruction of full-length PLCβ3, the distal CTD interacts with the hydrophobic ridge of the catalytic core, sequestering the basic surface of the distal CTD and preventing the hydrophobic ridge from accessing the membrane (Lyon et al., 2013). These observations may help partially explain the cytosolic localization of PLCβ3 and its lower basal activity compared to that of the other isoforms (Adjobo-Hermans et al., 2013; Philip et al., 2010; Smrcka and Sternweis, 1993). The PLCβ2 C-terminal extension has also been shown to influence the equilibrium between membrane-bound and cytosolic populations of this enzyme (Illenberger et al., 2003b).

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Autoinhibition by the X–Y linker. In PLCβ, the TIM barrel-like domain is split into X and Y halves connected by a poorly conserved linker, which contains highly acidic stretches in mammalian enzymes. An autoinhibitory role for this X–Y linker was identified in reconstitution studies of PLCβ2, wherein fragments containing the PH, EF hands, and X domain were combined with fragments containing the Y and C2 domains, and exhibited a ~10-fold increase in basal activity relative to the intact protein (Zhang and Neer, 2001). Treatment of the PLCβ2 catalytic core with trypsin or the V8 protease, both of which cleave the linker, also increased basal activity compared to the intact PLCβ2 catalytic core (Schnabel and Camps, 1998).

In the six reported structures of PLCβ enzymes (**Table 1**), the X–Y linker varies in length and degree of order, from 28 observed residues (out of 38) in cuttlefish PLC21, to 13 (out of 116) in the structure of PLCβ3 in complex with $G\alpha_q$. However, in each structure the C-terminal 12 amino acids of the linker adopt a similar structure, and based on ligand-bound structures of PLCδ (Essen et al., 1996; Essen et al., 1997), would block access of the phosphoinositide head group to the active site, thereby providing a molecular basis for autoinhibition by the linker (**Figure 3**). Selective deletions in the PLCβ2 X–Y linker or single amino acid point mutations to disrupt its interaction with the TIM barrel increased basal activity up to 20-fold over wild type PLCβ2 (Hicks et al., 2008). Confusingly, the consistently ordered C-terminal portion of the linker in PLCβ enzymes is not conserved in other PLC families, and was disordered in the PLCδ structures, which allowed co-crystallization with various ligands (Essen et al., 1996; Essen et al., 1997). Nonetheless, deletion of this linker in PLCδ increased basal activity 10-fold (Hicks et al., 2008). The disordered regions of the X–Y linker contain highly acidic stretches in many PLC enzymes, and this may be a general mechanism to hinder basal interactions between the catalytic core and the negatively charged inner leaflet of the plasma membrane (Hicks et al., 2008; Waldo

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et al., 2010). PLC21, an invertebrate homolog of PLCβ, does not contain an acidic stretch, and instead features a well-ordered helix, which is stabilized by an internal series of i, i+4 salt bridges (Lyon et al., 2011). Therefore, the mechanisms underlying autoinhibition by the X–Y linker may be different for each PLC enzyme, and may include electrostatic repulsion with the membrane, well-ordered structural elements that occlude the active site, or both, as could be the case for mammalian PLCβ enzymes.

Autoinhibition by the proximal CTD. Crystal structures of invertebrate PLC21 revealed the C-terminal ~25 amino acids of the proximal CTD form a helical hairpin. The Hα2' helix of the hairpin binds to a cleft on the catalytic core formed at the interface of the TIM barrel and C2 domains. The cleft contains residues that are uniquely conserved in the PLCβ subfamily, and places the helical hairpin in close proximity to the active site and the X–Y linker, suggesting a role for this region in regulating PLCβ activity (Lyon et al., 2011). This interaction is recapitulated in two unique structures of human PLCβ3, albeit via *in trans* crystal contacts (Lyon et al., 2013; Waldo et al., 2010), suggesting the interaction is evolutionarily conserved (Koyanagi et al., 1998). The role of the Hα2'-catalytic core interaction was assessed in human PLCβ3, where point mutations in Hα2' or its binding site on the catalytic core decreased the thermal stability of the enzyme and increased basal activity up to 50-fold over wild type PLCβ3 (Lyon et al., 2011). Although other mechanisms are possible, one possible model based on these observations is that in the inactive state, Hα2' binds to and stabilizes the PLCβ catalytic core in a catalytically quiescent state that could hinder displacement of the ordered portion of the X–Y linker. Differences in the affinity of the Hα2' interaction may contribute to differences in basal activity among PLCβ isoforms.

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Mechanisms of Activation

Multiple mechanisms of autoinhibition can beget multiple modes of activation. Based on biochemical and structural data, it is clear $G\alpha_q$ has a distinct binding site and activation mechanism from $G\beta\gamma$ and the Rho GTPases. Indeed, for some isoforms, regulation by these molecules has been shown to be synergistic (Philip et al., 2010; Rebres et al., 2011; Roach et al., 2008). Below we discuss the current state of knowledge regarding the molecular basis of PLCβ activation by four key regulators: the phospholipid bilayer, $G\alpha_q$, the $G\beta\gamma$ heterodimer, and Rho GTPases.

Interfacial Activation. All known activating proteins for PLCβ are lipid modified, and these groups are required for maximum efficacy of PLCβ activation (Dietrich et al., 1996; Dietrich et al., 1994; Hepler et al., 1996; Illenberger et al., 1998; Lyon et al., 2013). Although this might imply that membrane recruitment serves as the dominant activation mechanism, it has been shown these activators do not dramatically alter the membrane or particulate association of full length PLCβ *in vitro* (Jenco et al., 1997; Romoser et al., 1996; Runnels et al., 1996; Scarlata, 2002). Cell-based assays have shown co-localization between PLCβ isoforms and activators, but it is not clear whether these interactions also lead to increased membrane affinity (Adjobo-Hermans et al., 2013; Illenberger et al., 2003b). These observations led to the hypothesis that in their basal state, PLCβ enzymes are transiently associated with the membrane via the distal CTD, and the catalytic core is flexibly attached to the distal CTD but not in itself contacting the membrane. In this model, activating proteins are required to properly orient and juxtapose the catalytic core with the phospholipid bilayer. Because deletions within the X–Y linker increase

basal activity (Hicks et al., 2008; Schnabel and Camps, 1998; Zhang and Neer, 2001), it has been proposed that the negatively charged membrane would electrostatically repel the acidic regions of the X–Y linker, which in turn would destabilize the ordered region of the PLCβ linker and allow free access of substrate into the active site (Hicks et al., 2008; Waldo et al., 2010). However, interfacial activation is clearly not the entire story, as $G\alpha_q$, Gβγ and Rac still significantly activate PLCβ proteins when the ordered or acidic portions of the X–Y linker are deleted (Hicks et al., 2008). This additional increase in activity may reflect either the contribution of optimizing the orientation of the catalytic core (e.g. facilitating insertion of the hydrophobic ridge) or other allosteric effects, as discussed below.

Regulation by Activated $G\alpha_q$. $G\alpha_q$ activates each PLCβ enzyme is activated to a different extent. PLCβ3 is the most sensitive, with reported ~20–80 fold increases over basal activity upon interactions with $G\alpha_q$. PLCβ1 is activated to a similar extent, whereas PLCβ2 and PLCβ4 are typically activated ~2–10 fold over basal, depending on the experimental method (Biddlecome et al., 1996; Jiang et al., 1994; Lee et al., 1994; Paterson et al., 1995; Philip et al., 2010; Smrcka and Sternweis, 1993). The $G\alpha_q$ interaction is also of high affinity, with EC_{50} values of 1–400 nM depending on the experimental approach (Lyon et al., 2013; Lyon et al., 2011; Runnels and Scarlata, 1999; Smrcka et al., 1991; Waldo et al., 2010). PLCβ enzymes are also able to rapidly terminate their own activation by $G\alpha_q$ by serving as a GTPase activating protein (GAP). PLCβ3 and PLCβ1 increase the rate of GTP hydrolysis by $G\alpha_q$ ~100 to 1000 fold, respectively (Berstein et al., 1992; Chidiac and Ross, 1999; Waldo et al., 2010), providing an additional level of temporal control in downstream signaling events (Berstein et al., 1992; Chidiac and Ross, 1999; Cook et al., 2000; Waldo et al., 2010).

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$G\alpha_q$ binding, activation, and GAP activity were long attributed to various regions within the C-terminal extension, as its presence increases basal and G_q-saturated PLCβ3 activity by ~3-fold and ~40-fold, respectively (Ilkaeva et al., 2002; Jenco et al., 1997; Kim et al., 1996; Lee et al., 1993b; Lyon et al., 2013; Lyon et al., 2011; Park et al., 1993; Paulssen et al., 1996; Wu et al., 1993a). However, it was not entirely clear if these results were due to defects in $G\alpha_q$ binding, or structural changes within the C-terminal extension that altered its ability to interact with membranes, which would also lower activity by decreasing membrane association. $G\alpha_q$ does not seem to alter the subcellular distribution of PLCβ or increase its affinity for membranes (Gutman et al., 2010; Jenco et al., 1997; Runnels et al., 1996; Scarlata, 2002), supporting a mechanism of $G\alpha_q$ activation that is independent of increased membrane association, despite being palmitoylated at its amino terminus (Hepler et al., 1996).

The structure of a C-terminal truncation of human PLCβ3 (PLCβ3-Δ887) in complex with activated $G\alpha_q$ provided the first glimpse into the molecular basis for recognition of activated $G\alpha_q$ and for GAP activity (**Figure 5a**). The interface between $G\alpha_q$ and PLCβ3-Δ887 interface buries ~3100 $Å^2$ of accessible surface area and involves multiple domains of PLCβ. The most important interaction is formed by a helix-turn-helix (Hα1/Hα2) in the first 25 residues of the proximal CTD (Waldo et al., 2010). This region is disordered in the absence of $G\alpha_q$ and precedes the autoinhibitory Hα2' helix (Lyon et al., 2011). Hα1/Hα2 binds to the canonical effector binding site on $G\alpha_q$, burying ~1650 $Å^2$ of accessible surface area, in a manner highly analogous to the interaction made by a helix-turn-helix in p63RhoGEF (Lutz et al., 2007). Both employ an ALXXPI binding motif (residues 858–863 in human PLCβ3). Single amino acid substitutions (e.g. L859A, **Figure 5a**) are sufficient to abolish $G\alpha_q$ binding and activation

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(Adjobo-Hermans et al., 2013; Waldo et al., 2010). Furthermore, fusing the PLCβ3 Hα1/Hα2 element to the C-terminus of PLCδ conferred some $G\alpha_q$-dependent activation on this otherwise insensitive enzyme (Waldo et al., 2010). The C2 domain and the loop connecting it to the TIM barrel also contribute to the $G\alpha_q$–PLCβ3 interface through interactions with the switch 1 and 2 regions of $G\alpha_q$, burying ~1100 $Å^2$ of accessible surface area. Mutations within this interface decreased $G\alpha_q$-dependent activation, but did not eliminate it, further confirming Hα1/Hα2 as the primary $G\alpha_q$ binding site (Waldo et al., 2010). The isolated C2 domains from PLCβ1 and PLCβ2 were previously reported to bind $G\alpha_q$ (Wang et al., 1999a), but the interface between the C2 domain alone and $G\alpha_q$ only buries ~400 $Å^2$. Thus, it is unclear if this interaction would persist in the absence of the other PLCβ binding surfaces. A third set of interactions between $G\alpha_q$ and the PLCβ3 core is mediated by residues 260–264 in the loop between the third and fourth EF hands, which buries ~900 $Å^2$ of accessible surface area (**Figure 5a**). This loop is highly conserved and unique to PLCβ isozymes. Asn260 forms a hydrogen bond with the side chain of $G\alpha_q$-Gln209, whose side chain in turn coordinates the hydrolytic water during GTP hydrolysis, an interaction essentially identical to that observed in $G\alpha_{i/q}$ subunits in complex with regulators of G protein signaling (RGS) proteins (Nance et al., 2013; Slep et al., 2001; Tesmer et al., 1997), indicating a conserved GAP mechanism. Mutation of Asn260 eliminated GAP activity, as did replacement of the EF3–EF4 loop with that of PLCδ (Cook et al., 2000; Waldo et al., 2010).

The structure of full length human PLCβ3 in complex with activated $G\alpha_q$ provided additional insights regarding the distal CTD, previously reported to be required for maximum activity and high affinity binding to $G\alpha_q$ (Kim et al., 1996; Lee et al., 1993b; Lyon et al., 2011; Park et al., 1993; Schnabel et al., 1993; Wu et al., 1993a). In this structure, a conserved

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hydrophobic patch of the distal CTD interacts the N-terminal helix of $G\alpha_q$, burying ~850 $Å^2$ of accessible surface area (**Figure 2, 4**). Cryo-electron microscopy 3D reconstructions of the $G\alpha_q$–PLCβ3 complex confirmed this interaction also occurs in solution. Mutation of residues in the hydrophobic patch or deletion of the N-terminal helix of $G\alpha_q$ decreased the efficacy of $G\alpha_q$ activation ~2-fold, but had no effect on basal activity or affinity for $G\alpha_q$. Loss or mutation of the palmitoyl groups of $G\alpha_q$ (Hepler et al., 1996) also decreased maximum $G\alpha_q$-stimulated activity, but only in the context of full length PLCβ3. Thus, the N-terminus of $G\alpha_q$ appears to play a role in activation, likely by virtue of its coordinate interaction with the distal CTD and with the membrane via its palmitoyl groups (Lyon et al., 2013). However, the relative importance of this interaction in a physiological context remains to be determined.

Unexpectedly, the CTD linker, which is disordered in the $G\alpha_q$–PLCβ3 complex, also seems important for $G\alpha_q$ activation. Deletion of the linker in PLCβ3 eliminated $G\alpha_q$-dependent activation at all concentrations tested, modestly increased basal activity, but did not alter the binding affinity of $G\alpha_q$. Thus, the length and conformational flexibility of the linker may be essential for activation by $G\alpha_q$ (Lyon et al., 2013). Whether the relative length of the CTD linker is a determinant of isoform sensitivity to $G\alpha_q$-dependent activation is unknown. However, of the human PLCβs, PLCβ2 has the shortest linker (28 residues) and is most weakly activated by $G\alpha_q$, whereas PLCβ1 and PLCβ3 have longer linker regions (61 and 56 residues, respectively) and are robustly activated by $G\alpha_q$ (Biddlecome et al., 1996; Philip et al., 2010; Smrcka and Sternweis, 1993).

In light of the two $G\alpha_q$–PLCβ3 crystal structures and associated biochemical data, we propose the following molecular mechanism for PLCβ3 activation by $G\alpha_q$ (**Figure 5a,b**). In the

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resting cell, the Hα2' helix of the proximal CTD is bound to the catalytic core, inhibiting basal activity, and the preceding Hα1/Hα2 element is disordered and freely accessible to $G\alpha_q$. The X–Y linker and the interactions between the distal CTD and the ridge of the catalytic core also likely repress basal activity. Upon G_q-coupled receptor activation, $G\alpha_q$ binds to Hα1/Hα2 and displaces the Hα2' element away from the catalytic core by ~50 Å, leading to allosteric activation of PLCβ. The interactions between the membrane, the palmitoylated N-terminus of $G\alpha_q$, and the distal CTD help bring the catalytic core into close proximity with the membrane. The conformational flexibility provided by the CTD linker is required for this optimization. The repulsion between the negatively charged residues in the X–Y linker and the membrane facilitates ejection of the ordered portion of the linker through interfacial activation. Displacement of the linker facilitates substrate binding, and the interactions between $G\alpha_q$ and PLCβ with the membrane optimize the orientation of the active site (Hicks et al., 2008; Lyon et al., 2013; Lyon et al., 2011; Waldo et al., 2010).

There are several outstanding questions regarding $G\alpha_q$ activation of PLCβ that remain to be addressed. The first is that we can only conjecture what a PLCβ enzyme looks like in a fully activated state. Neither of the two $G\alpha_q$-PLCβ3 crystal structures likely represent the "fully activated" conformation of PLCβ3, as they both preserve the Hα2'-catalytic core interaction via *in trans* crystal contacts. However, crystal structures of PLCβ2, which are truncated immediately after the C2 domain, do not exhibit large conformational differences compared to the PLCβ3 structures, implying an allosteric change that occurs upon displacement of Hα2' may be subtle. Secondly, the mechanism by which the Hα2'-catalytic core interaction regulates activity remains unknown. Finally, the relative importance of allosteric versus interfacial activation is not

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understood. In fact, they could be intimately linked: displacement of the X–Y linker upon interaction with the plasma membrane may promote displacement of the Hα2' helix, or vice versa.

Regulation by the Gβγ Heterodimer. As in $G\alpha_q$ activation, each PLCβ isoform is differentially activated upon binding to Gβγ. PLCβ3 and PLCβ1 shows the greatest increase in activity (~10 fold over basal), whereas PLCβ2 is activated ~5–20 fold over basal and PLCβ4 is unresponsive (Lee et al., 1994). However, PLCβ2 is most sensitive to Gβγ, with an EC_{50} of ~30 nM, compared to the ~90–200 nM EC_{50} values reported for PLCβ1 and PLCβ3 (Camps et al., 1992; Hicks et al., 2008; Katz et al., 1992; Lee et al., 1994; Smrcka and Sternweis, 1993). The source of Gβγ in cells is thought to be generated by G_i-coupled receptors, such as the δ and μ opioid receptors, as activation by Gβγ can be inhibited by treatment with pertussis toxin (Camps et al., 1992; Katz et al., 1992; Wu et al., 1998; Xie et al., 1999) and because G_i-coupled receptors are more abundant than G_q-coupled receptors in cells where Gβγ-dependent activation occurs (Kadamur and Ross, 2012).

Gβγ activation of PLCβ does not require the proximal and distal CTDs (Kim et al., 1996; Lee et al., 1993b; Waldo et al., 2010), and Gβγ only activates PLCβ when the Gγ subunit is prenylated (Dietrich et al., 1996; Dietrich et al., 1994; Katz et al., 1992). These observations led to the hypothesis that Gβγ simply recruits PLCβ enzymes to the membrane. However, as reported for $G\alpha_q$, there is no evidence that Gβγ changes the affinity for membranes or liposomes or the cellular distribution of PLCβ (Jenco et al., 1997; Romoser et al., 1996; Runnels et al., 1996; Scarlata, 2002; Schnabel et al., 1993; Wang et al., 1999b). If this is so, then Gβγ must

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impart an allosteric change or help orient the PLCβ catalytic core in a manner that optimizes its function.

Although there are currently no reported structures of a Gβγ–PLCβ complex that could help shed light on the molecular basis for their interaction and for activation, many studies have sought to map their protein–protein interface. GDP-bound $G\alpha_i$ subunits can inhibit PLCβ activation, suggesting a common protein interaction surface on Gβγ, which was confirmed by mutagenesis studies (Buck et al., 1999; Ford et al., 1998; Li et al., 1998; Panchenko et al., 1998; Scott et al., 2001). The outer strands of $G\beta_1$ blades 2, 6, and 7 (Panchenko et al., 1998) and the N-terminus of $G\beta_1$ (Bonacci et al., 2005; Friedman et al., 2009) have also been implicated in PLCβ binding. These regions of Gβγ may contribute to differences in the sensitivity of PLCβ isoforms to activation (Chen et al., 2005; Friedman et al., 2009; Li et al., 1998; Panchenko et al., 1998). It has also been hypothesized that Gγ or its prenyl group may be directly involved (Dietrich et al., 1996; Dietrich et al., 1994; Katz et al., 1992). Loss of prenylation eliminated interactions between PLCβ2 and PLCβ3 with Gβγ (Fogg et al., 2001), but these defects could simply reflect impaired targeting of Gβγ to the membrane. Interestingly, movement of the prenyl group with respect to the Gβ subunit via deletions at the C-terminus of Gγ also reduced PLCβ activation (Akgoz et al., 2002). This could imply either the C-terminus of Gγ is part of the interface, or that shortening this loop effects the orientation of Gβγ at the membrane such that its ability to productively interact with PLCβ or to orient the catalytic core of the enzyme at the membrane is impaired.

The location of the Gβγ binding site on PLCβ is less well-defined, although the PH domain has emerged as a strong candidate (Drin et al., 2006; Feng et al., 2005; Han et al., 2011),

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and PH domains in other proteins, such as G protein coupled receptor kinase 2 (GRK2), interact with Gβγ (Lodowski et al., 2003). One approach suggesting the PLCβ PH domain is the site of Gβγ binding takes advantage of the similarity between PLCβ and PLCδ enzymes. A PLCδ chimera, in which its PH domain was replaced with that of PLCβ2, could interact with and be stimulated by Gβγ (Drin et al., 2006; Guo et al., 2003; Runnels and Scarlata, 1999; Wang et al., 2000), whereas the reverse chimera lost responsiveness as determined through activity and FRET-based assays (Guo et al., 2003). Furthermore, the isolated PH domains of PLCβ2 and PLCβ3 have been shown to directly bind to Gβγ by FRET methods (Wang et al., 1999b).

Another candidate Gβγ binding site lies within the Y domain of the TIM barrel. A chimera in which the PLCβ2 PH, EF hands, and TIM barrel were fused to the PLCβ1 C2 domain and C-terminal extension retained the ability to be activated by Gβγ. Replacing the PLCβ2 PH and EF hands with those of PLCβ1 had no effect on Gβγ activation (Wu et al., 1993b). Subsequently, 20 amino acid peptides corresponding to PLCβ2 Y domain (residues 564-583 and 575-594) were identified that inhibited Gβγ-dependent activation of PLCβ2 and PLCβ3, impaired association between Gβγ and inactive $G\alpha_i$, and directly interacted with Gβγ in crosslinking studies (Bonacci et al., 2005; Kuang et al., 1996; Sankaran et al., 1998). These peptides correspond to the Tβ5-Tβ6 loop, Tβ6, Tα5 and Tα5', with the area of overlap between the peptides centered on the Tα5 helix. Point mutants within Tα5 decreased Gβγ-dependent activation (Bonacci et al., 2005; Rebres et al., 2011). Interestingly, this helix interacts with both the X–Y linker and the Hα2' in the PLCβ structures, raising the possibility it could contribute to regulation.

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The Tα5 helix and the PH domain reside on opposite faces of the catalytic core, and a single Gβγ molecule cannot simultaneously interact with both sites (**Figure 6a)**. Thus, clarification of the Gβγ binding site on PLCβ awaits further structural and biochemical characterization. Different activation mechanisms can be envisioned for each putative binding site. If Gβγ binds to the PH domain in a manner overlapping or adjacent to the Rac1 binding site on PLCβ2, then their activation mechanism are likely very similar: they may simply interact with catalytic core of PLCβ at the membrane and optimize its orientation (Dietrich et al., 1996; Dietrich et al., 1994; Drin et al., 2006; Han et al., 2011; Romoser et al., 1996), which could promote interfacial activation by ejection of the X–Y linker. If Gβγ binds to the Tα5 helix of the TIM barrel-like domain, the same orientation effects could occur, but there may also be a significant allosteric component to activation because this helix contacts two autoinhibitory elements, the X–Y linker and Hα2' helix (**Figure 6b**), and thus could contribute to their displacement.

Synergistic activation by $G\alpha_q$ and Gβγ. Based on current evidence, the $G\alpha_q$ and Gβγ binding sites within PLCβ are likely spatially separated and involve some independent steps leading to activation. Early evidence for synergistic activation of PLCβ enzymes came from macrophages, where treatment with G_i- and G_q-coupled receptor agonists resulted in superadditive Ca^{2+} increases over what either agonist could induce alone. This synergistic Ca^{2+} release required the activity of PLCβ3 (Roach et al., 2008). In the presence of excess activated $G\alpha_q$ and Gβγ, PLCβ3 activity is stimulated ~19-fold over what either $G\alpha_q$ or Gβγ can induce, which appears to be due to the very low basal activity of PLCβ3 (Philip et al., 2010). PLCβ2 can also be synergistically activated, but over a much narrower range of $G\alpha_q$ and Gβγ concentrations and to a lesser degree

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than PLCβ3 (Rebres et al., 2011). At this time, it is unclear how widespread synergistic activation of PLCβ enzymes is and how robust synergistic activation is in other cell types.

Regulation by small G proteins. Rho-dependent activation of PLCβ was first identified in cytosolic preparations from granulocytes, where treatment with the nonhydrolyzable GTP analog GTPγS resulted in increased rates of PIP_2 hydrolysis (Camps et al., 1990). Subsequent studies identified Cdc42, Rac1 and Rac2, but not RhoA, as the activators. These G proteins were subsequently shown to directly bind and stimulate PLCβ2 and PLCβ3, but not PLCβ1 or PLCβ4 (Illenberger et al., 1997; Illenberger et al., 2003a). As with $G\alpha_q$, only the GTP-bound conformation of the small G proteins can productively engage PLCβ (Illenberger et al., 1998; Snyder et al., 2003), and as with Gβγ, the C-terminus of the GTPase must be prenylated for activation and does not require the PLCβ C-terminal extension (Illenberger et al., 1997; Illenberger et al., 1998; Illenberger et al., 2003a).

The binding site for small G proteins on PLCβ was first identified through chimeras between PLCβ1 and PLCβ2. Replacement of the PLCβ2 PH domain with that of PLCβ1 eliminated GTPase binding and activation (Illenberger et al., 2003a; Illenberger et al., 2003b). The G protein binding site is localized entirely within the PH domain, as the isolated domains from PLCβ2 and PLCβ3 were able to bind activated Rac1 with affinity comparable to the full-length enzymes (K_d ~25 μM) (Snyder et al., 2003). This interaction is relatively weak compared to the affinities measured for $G\alpha_q$ and Gβγ, suggesting co-localization at the membrane is essential for Rac-dependent activation. In support of this mechanism, Rac1 has been shown to

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increase the membrane association time of PLCβ2 (Gutman et al., 2010; Illenberger et al., 2003b).

The crystal structure of the Rac1–PLCβ2 catalytic core complex confirmed the PH domain as the sole Rac1 binding site, burying ~1200 $Å^2$ of total accessible surface area (**Figure 6a**) (Jezyk et al., 2006). Rac1 contacts PLCβ2 via its switch 1 and 2 regions, which undergo conformational changes upon GTP binding. Accordingly, point mutations within the switch regions of Cdc42 or Rac1 eliminated its ability to activate PLCβ (Illenberger et al., 1998; Jezyk et al., 2006). Point mutations within the PLCβ2 PH domain decreased Rac1-dependent activation, but had little to no effect on Gβγ-mediated activation. Thus, if the PH domain is the binding site for both Rac1 and Gβγ, they interact with distinct sites, or the residues involved have different degrees of importance for each activator. Indeed, in one instance Gβγ and Rac2 have been reported to additively increase PLCβ2 activity (Illenberger et al., 2003a). Comparison of Rac1–PLCβ2 structure with the apo-PLCβ2 structure did not reveal any large conformational changes occurring upon complex formation (Hicks et al., 2008; Jezyk et al., 2006), suggesting the mechanism of activation does not have an allosteric component. However, as all the PLCβ crystal structures have been determined in the absence of phospholipid bilayers, it remains possible that such conformational changes are dependent on a membrane environment.

Overall, Rac1-dependent activation likely shares similarities with Gβγ activation in that both activators must be prenylated in order to activate PLCβ through interactions with the catalytic core of the enzyme, the PLCβ C-terminal extension is not required, and their activation mechanisms do not appear to involve allostery or significant conformational changes. The prenylated C-terminus of activated Rac1 restricts the orientation of the protein at the membrane and may promote higher affinity binding to the PLCβ PH domain. As a result, the Rac1–catalytic

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core complex is brought in close proximity to the membrane, possibly promoting interfacial activation (**Figure 6b**) (Hicks et al., 2008; Illenberger et al., 2003b). An interesting question is whether the Hα2' helix remains associated with the PLCβ catalytic core during Rac-dependent activation. If the X–Y linker and Hα2' are allosterically coupled, then displacement of one element could influence the other.

Small Molecule Modulators of PLCβ Activity

Selective small molecule probes can aid in elucidating the roles of specific proteins in cells and whole organisms, and, importantly, serve as leads for future therapeutic agents. Development of PIP_2-based chemical probes has been difficult, as modification of the inositol group and/or the acyl chains decreases PLC binding, hydrolysis and catalytic efficiency (Bruzik and Tsai, 1994; Essen et al., 1997; Wu et al., 1997). The lipid analog edelfosine (*1-0*-octadecyl-*2-0*-methyl-glycerol-3-phosphocholine or ET-18-OCH3) was one of the first molecules identified that selectively decreased Ca^{2+} release and inositol phosphate (IP) accumulation in tumor cells (Berkovic, 1998). Its lipid-like structure allows for incorporation into cell membranes, where it can disrupt membrane integrity, protein–membrane interactions and the catalytic activity of membrane-associated enzymes, such as the PLCs. As such, it is difficult to directly associate the effects of edelfosine treatment strictly with PLC inhibition (Arthur and Bittman, 1998; Powis et al., 1995; Seewald et al., 1990). Another PLC inhibitor is the aminosteroid U73122 (Bleasdale et al., 1989; Horowitz et al., 2005; Hou et al., 2004; Kobrinsky et al., 2000; Oh et al., 2004; Suire et al., 2012; Tanski et al., 2004; Ward et al., 2003; Wu et al., 1998). Accumulating reports of off-target effects (Burgdorf et al., 2010; Hughes et al., 2000; Klose et al., 2008; Macmillan and McCarron, 2010) prompted efforts to identify its mechanism of action. Purified PLC isoforms

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treated with U73122 and assayed for activity *in vitro* showed diverse effects, including increased activity for some PLC isoforms. The maleimide group in U73122, which is required for its inhibitory action, reacts with exposed sulfhydryl groups on the protein surface. For PLCβ, several of the modified sulfhydryl groups are on the same face of the catalytic core as the active site, and these hydrophobic adducts are proposed to increase membrane association and activity (Klein et al., 2011).

A renewed effort to develop PIP_2-based chemical probes for PLC is underway. The C6 hydroxyl group of the inositol head group was found to be amenable to chemical modifications, with little effect on PLC activity (Wang et al., 2012). A soluble PIP_2 analog with a cleavable fluorescent tag (WH-15) has also been synthesized, and is hydrolyzed at a rate comparable to that of PIP_2 (Huang et al., 2011). Although high selectivity is unlikely to be found in the active site region among PLC isozymes, such soluble analogs will greatly facilitate high-throughput screening efforts to identify more potent and selective PLC probes (Huang et al., 2013).

In light of the recent structural and functional findings, is there rational approach to developing PLCβ-specific modulators? Selectivity would arguably best be achieved by targeting known allosteric and/or regulatory sites. An interesting possibility is the Hα2' binding site on the catalytic core. This cleft contains residues unique to the PLCβ family, thus small molecules that target this site would likely be PLCβ-specific. However, the effect of chemical probes that would bind at this site is not clear. Such molecules would likely displace the Hα2' helix, but if they do not fully reproduce the autoinhibition mediated by Hα2' they would serve as activators. On the other hand, if they did repress activity, such molecules could likely inhibit PLCβ even in the face of persistent $G\alpha_q$ activation. Intermolecular protein–protein interaction sites within PLCβ are also potential targets. For example, molecules that target the Rac1 binding surface of the PH

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domain would enable the selective study of PLCβ function downstream of pathways that activate small molecular weight GTPases. A similar strategy was recently proposed as a treatment in PLCβ-mediated cardiac hypertrophy and heart failure, as peptides or small molecules that disrupt membrane association of PLCβ1b in the sarcolemma are of therapeutic interest (Woodcock et al., 2010). Despite the fact that protein–protein interfaces can be very difficult to "drug", there are proofs of principle that compounds disrupting the interactions between PLCβ and its protein regulators can be identified (Bonacci et al., 2006). The small molecule M119 (Bonacci et al., 2006) has already been used to demonstrate the involvement of Gβγ-activated PLCβ in antinociception induced by opioid receptor activation (Mathews et al., 2008).

Future Directions

Recent structural studies of PLCβ enzymes and their activation complexes have provided atomic-level insight into mechanisms of PLCβ regulation and activation. In particular, tremendous progress has been made in elucidating the molecular mechanisms of $G\alpha_q$ and Rac-dependent activation. Structural insights into how Gβγ interacts with and stimulates PLCβ remain lacking, and a molecular mechanism for Gβγ-dependent regulation of PLCβ is vital for developing a comprehensive understanding of PLCβ activity within the cell. An unexpected consequence of the most recent structural studies is recognition that the membrane itself is an active player in PLCβ regulation. How the membrane facilitates protein–protein interactions and whether its local structure changes upon PLCβ or activator–PLCβ binding is unknown. Such interactions could also alter the conformation of the PLCβ core in subtle ways, leading to increased activity. An intriguing possibility is that the distal CTD could also influence the

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membrane association of the catalytic core by inducing differences in local membrane curvature. An additional layer of regulatory complexity arises from the observation that PLCβ isozymes interact with numerous scaffolding proteins to form signaling complexes (Cai et al., 2005; Cartier et al., 2011; Sun et al., 2013). How these higher order complexes contribute to PLCβ regulation, and whether they alter activation by $G\alpha_q$, Gβγ, and small GTPases are not understood, and likely represents the next frontier for structure/function analyses of PLCβ enzymes.

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Footnotes

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Figure Legends

Figure 1. Primary structure of PLCβ isozymes and splice variants. Numbers above the diagram correspond to domain boundaries in human PLCβ3, and all domain diagrams correspond to human isoforms, with the exception of PLCβ4b, which is from *R. norvegicus*. All identified PLCβ variants share the same catalytic core, which is the minimal fragment of PLCβ that hydrolyzes PIP_2, defined as the N-terminus through the end of the C2 domain. The PLCβ isoforms differ most significantly in the length of the X–Y linker, whereas the splice variants reported for each isoform primarily vary the length and sequence of the CTD linker and extreme C-terminus. Regions with sequences unique to the PLCβ1b and PLCβ4a splice variants are shown in pink.

Figure 2. Structure of full length PLCβ3 in complex with activated $G\alpha_q$. The structure shown is derived from PDB entry 4GNK. The PLCβ3 domains are colored as in **Figure 1**, and activated $G\alpha_q$ is colored gray. The hydrophobic surface of the distal CTD that binds the $G\alpha_q$ N-terminal helix is shown in yellow. The observed ends of the proximal and distal CTD are marked with asterisks, and the N and C termini of PLCβ3 and $G\alpha_q$ are labeled N and C or N' and C', respectively. The $G\alpha_q$-bound GDP-AlF_4^- is shown in orange sticks, and Ca^{2+} and Mg^{2+} as black spheres. Disordered regions are shown as dashed lines.

Figure 3. The PLCβ X–Y linker blocks the active site. A model of IP_3 (derived from PDB ID 1DJX) bound to the PLCβ3 active site reveals a possible mechanism for autoinhibition by the X–Y linker. As observed in six independent structures of PLCβ enzymes, the ordered region of the

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X–Y linker (PLCβ3 residues 575-586) docks in a position that would prevent PIP_2 from entering the enzyme active site. Displacement of this region of the X–Y linker would therefore appear to be a prerequisite for PIP_2 binding. The catalytic residues H332, H379, and E362 are shown as sticks, and the active site Ca^{2+} as a black sphere. Dashed lines indicate the disordered region of the PLCβ3 linker, which contains a span of acidic residues. Side chains of residues that constitute the hydrophobic ridge, which is thought to help anchor the active site within the membrane, are also shown.

Figure 4. The structure and surface of the distal CTD are conserved. The isolated turkey distal CTD (PDB ID 1JAD) (**A**) and human PLCβ3 distal CTD (PDB ID 4GNK) (**B**) have the same fold and similar conserved surfaces. Basic residues within Dα3 and Dα4 (blue spheres) form an extended conserved surface along one face of the domain, which likely functions as a membrane binding site. The conserved hydrophobic patch on Dα5, which interacts with the N-terminal helix of $G\alpha_q$ in the 4GNK structure (**Figure 2**), is shown as yellow spheres. The turkey PLCβ CTD was engineered to facilitate crystallization by deletion of 32 residues from the Dα3–Dα4 loop.

Figure 5. The proximal CTD is an allosteric site for $G\alpha_q$ activation. (**A**) PLCβ3 is colored as in **Figure 1**, and activated $G\alpha_q$ is shown as a gray surface with the switch regions colored orange. In the absence of $G\alpha_q$, the Hα2' helix (cyan) is bound to the PLCβ catalytic core (**right**), and is connected to the C-terminus of the C2 domain by a ~25 amino acid disordered loop (dashed line). $G\alpha_q$ binds the disordered loop via its switch regions, ordering the Hα1/Hα2 element (dark blue). Additional interactions between switch regions of $G\alpha_q$, the EF hands, and the C2 domain

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displace Hα2' from the catalytic core (**left**). The interactions between $G\alpha_q$ and the Hα1/Hα2 element are largely hydrophobic, and mutation of Leu859 eliminates $G\alpha_q$ binding and activation. The intrinsic GAP activity of PLCβ relies on Asn260, positioned in a loop between two EF hand domains, which interacts with the catalytic glutamine of $G\alpha_q$. (**B**) In the resting cell (**left**), PLCβ is in an autoinhibited state, wherein the Hα2' and the X–Y linker are bound to the catalytic core. The distal CTD interacts with the cell membrane or the hydrophobic ridge of the catalytic core, which may help dictate the distribution of the enzyme between the membrane and cytosol. $G\alpha_q$ binding leads to allosteric activation through displacement of Hα2' and recruitment of the PLCβ catalytic core to the membrane surface. Anionic phospholipids in the inner leaflet eject the acidic X–Y linker. The orientation of the active site at the membrane is further optimized by interactions between the membrane, the palmitoylated N-terminus of $G\alpha_q$ and the distal CTD (**right**).

Figure 6. Gβγ and Rac1 bind the PLCβ catalytic core. (**A**) Rac1 (grey surface) binds exclusively to the PH domain via its switch regions (orange surface), which enables PLCβ to detect the activation state of Rac1. PLCβ domains are colored as in **Figure 1**. Current biochemical data predicts that Gβγ binds to either the PH domain or a helix within the Y domain of the TIM barrel (Tα5, in blue), which is in close proximity to the X–Y linker and the Hα2' helix. The active site Ca^{2+} is shown as a black sphere, disordered regions as dashed lines, and GTPγS bound to Rac1 as ball and sticks. (**B**) Rac1/Gβγ likely share similar PLCβ activation mechanisms. In the resting state (**center**) PLCβ is in an autoinhibited state, as described in **Figure 5b**. Rac1/Gβγ binding to the PLCβ catalytic core is dictated in part by the geometry

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imposed by the cell membrane, which likely increases the affinity between these activators and PLCβ. The interaction between Rac1/Gβγ and the PH domain (**left**) or between Gβγ and the catalytic core (**right**) likely optimize the orientation of the active site at the membrane surface, overcoming repulsion between the membrane and the acidic region in the X–Y linker, and thereby opening access to the active site.

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Table 1. Crystal Structures of PLCβ Domains and Complexes.

Structure	PDB ID	Residue Range(s) Used	Species	Resolution (Å)
distal CTD	1JAD	878-1158	turkey	2.4
PLCβ2	2ZKM	1-799	human	1.6
Sepia PLC21	3QR0	1-816	cuttlefish	2.0
Loligo PLC21	3QR1	1-813	squid	3.2
Rac1-PLCβ2	2FJU	Rac1:1-189 PLCβ2: 1-799	human human	2.2
$G\alpha_q$-PLCβ3	3OHM	$G\alpha_q$: 35-359 PLCβ3: 1-887	mouse human	2.7
$G\alpha_q$-PLCβ3	4GNK	$G\alpha_q$: 7-359 PLCβ3: 1-1234	mouse human	4.0

Figure 1

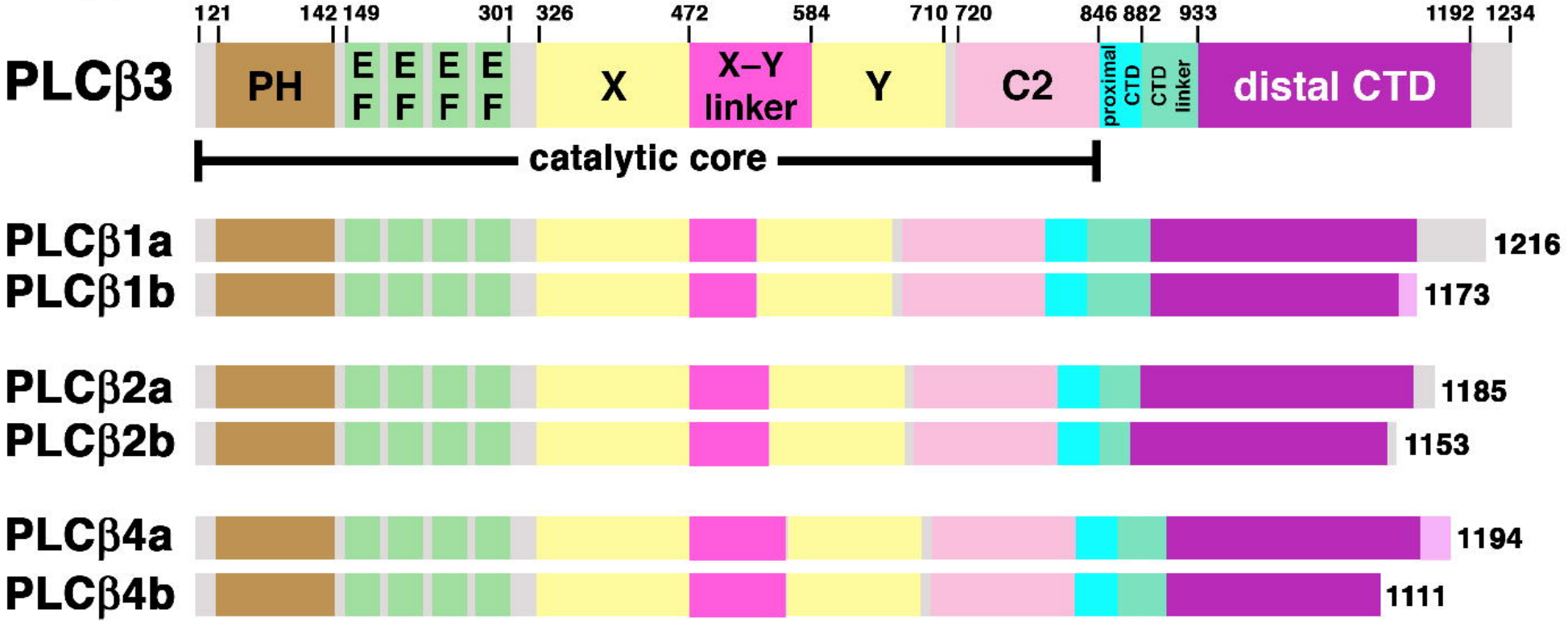
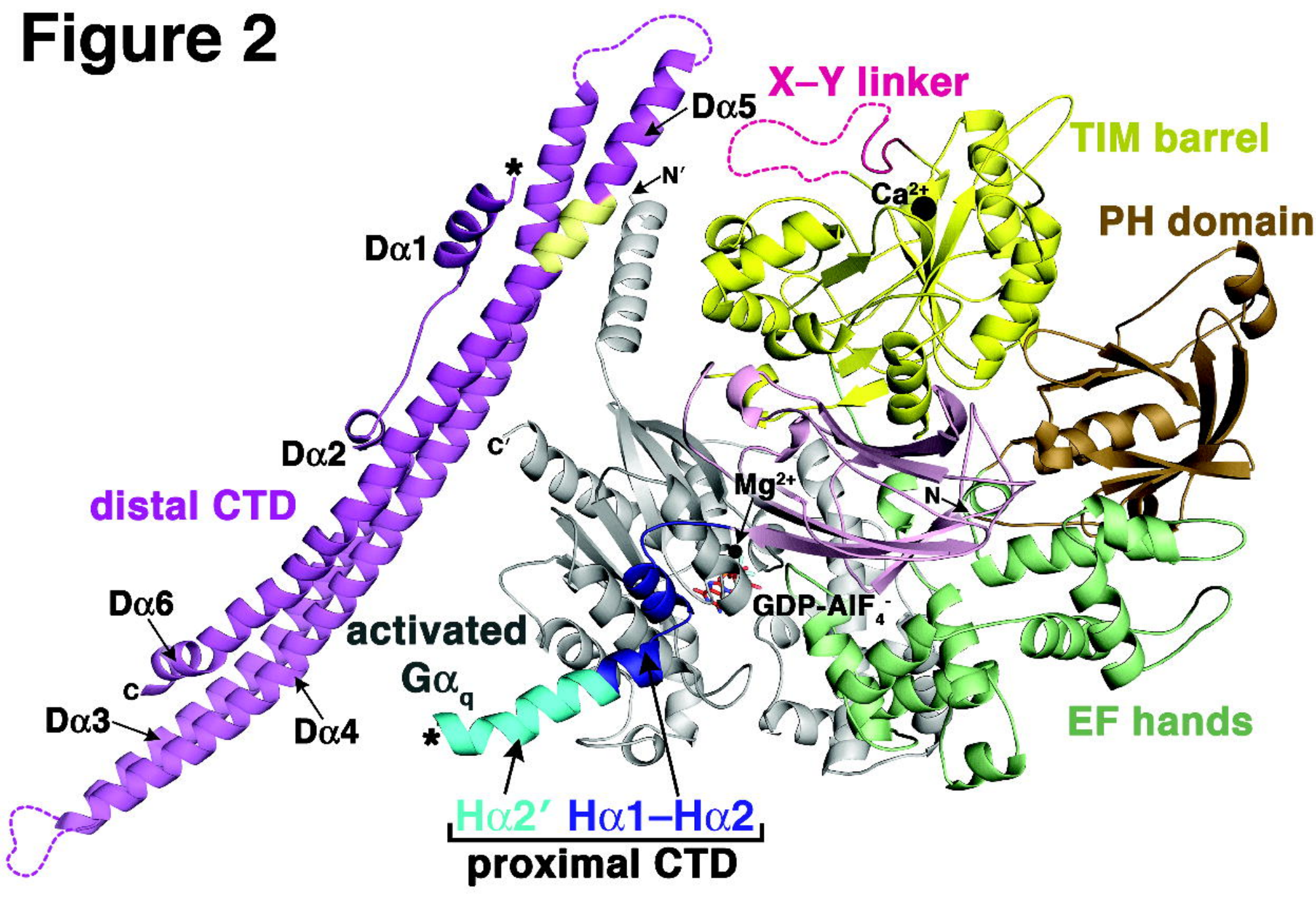

121
142
149
301
326
472
584
710
720
846
882
933
1192
1234
PLCβ3
PH
EF
EF
EF
EF
X
X–Y linker
Y
C2
proximal CTD
CTD linker
distal CTD
catalytic core
PLCβ1a
1216
PLCβ1b
1173
PLCβ2a
1185
PLCβ2b
1153
PLCβ4a
1194
PLCβ4b
1111

Figure 2


X–Y linker
Dα5
TIM barrel
Ca2+
PH domain
N′
Dα1
*
Dα2
C′
Mg2+
N
distal CTD
GDP-AlF4-
Dα6
activated
Gαq
C
Dα3
Dα4
EF hands
*
Hα2′ Hα1–Hα2
proximal CTD

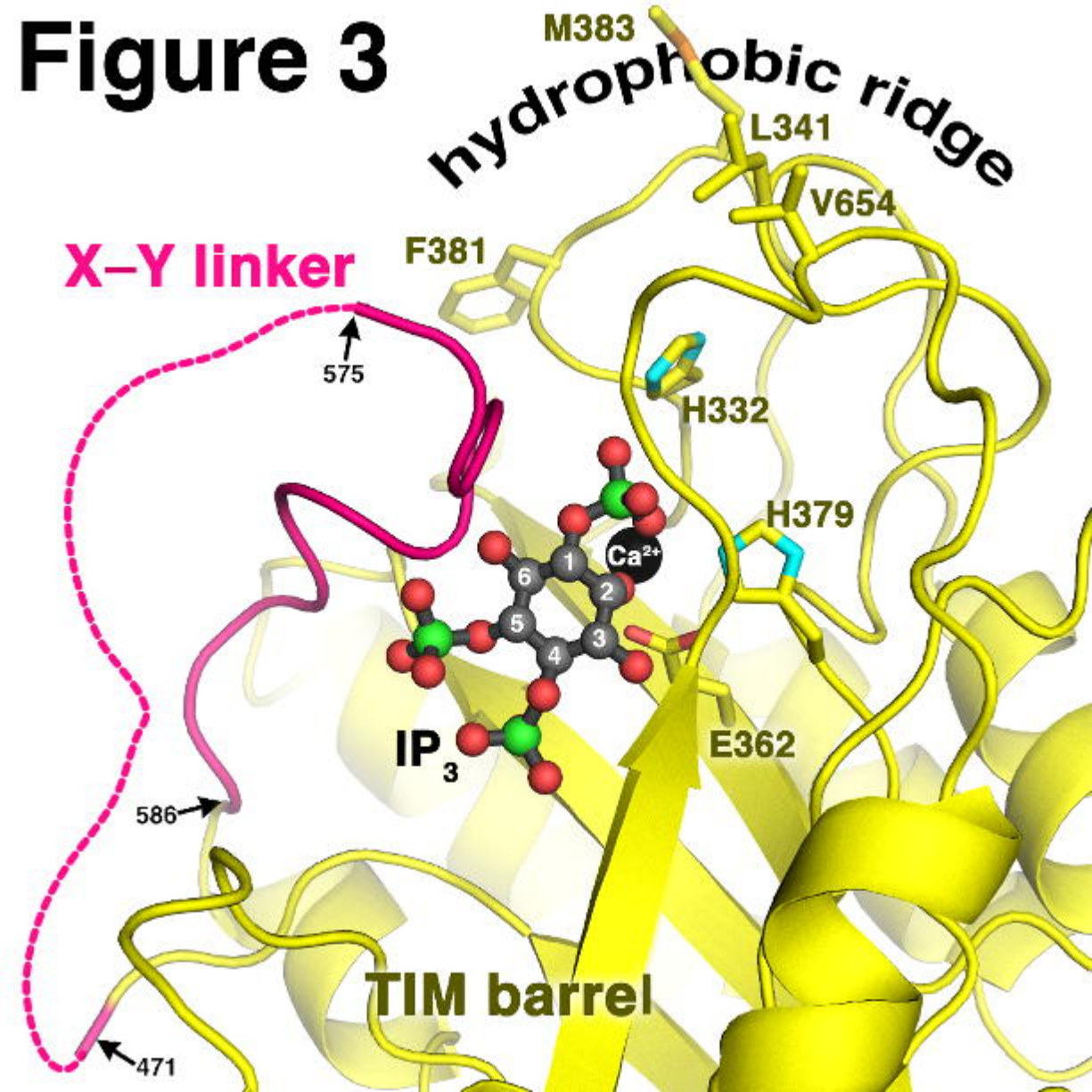


Figure 3
M383
hydrophobic ridge
L341
V654
F381
X–Y linker
575
H332
H379
Ca2+
1
2
3
4
5
6
IP3
E362
586
TIM barrel
471

Figure 4

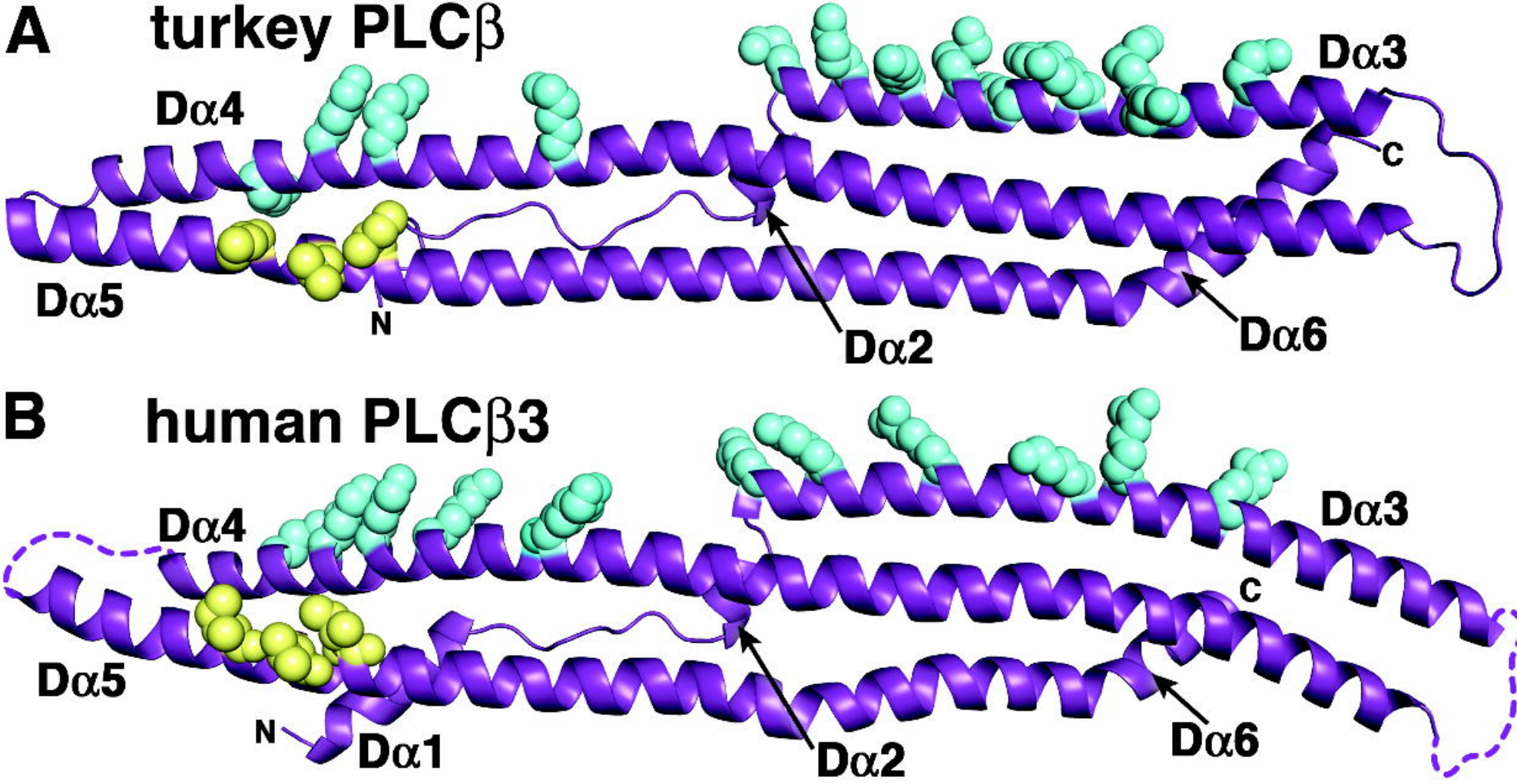

A
turkey PLCβ
Dα4
Dα3
C
Dα5
N
Dα2
Dα6
B
human PLCβ3
Dα4
Dα3
C
Dα5
N
Dα1
Dα2
Dα6

Figure 5

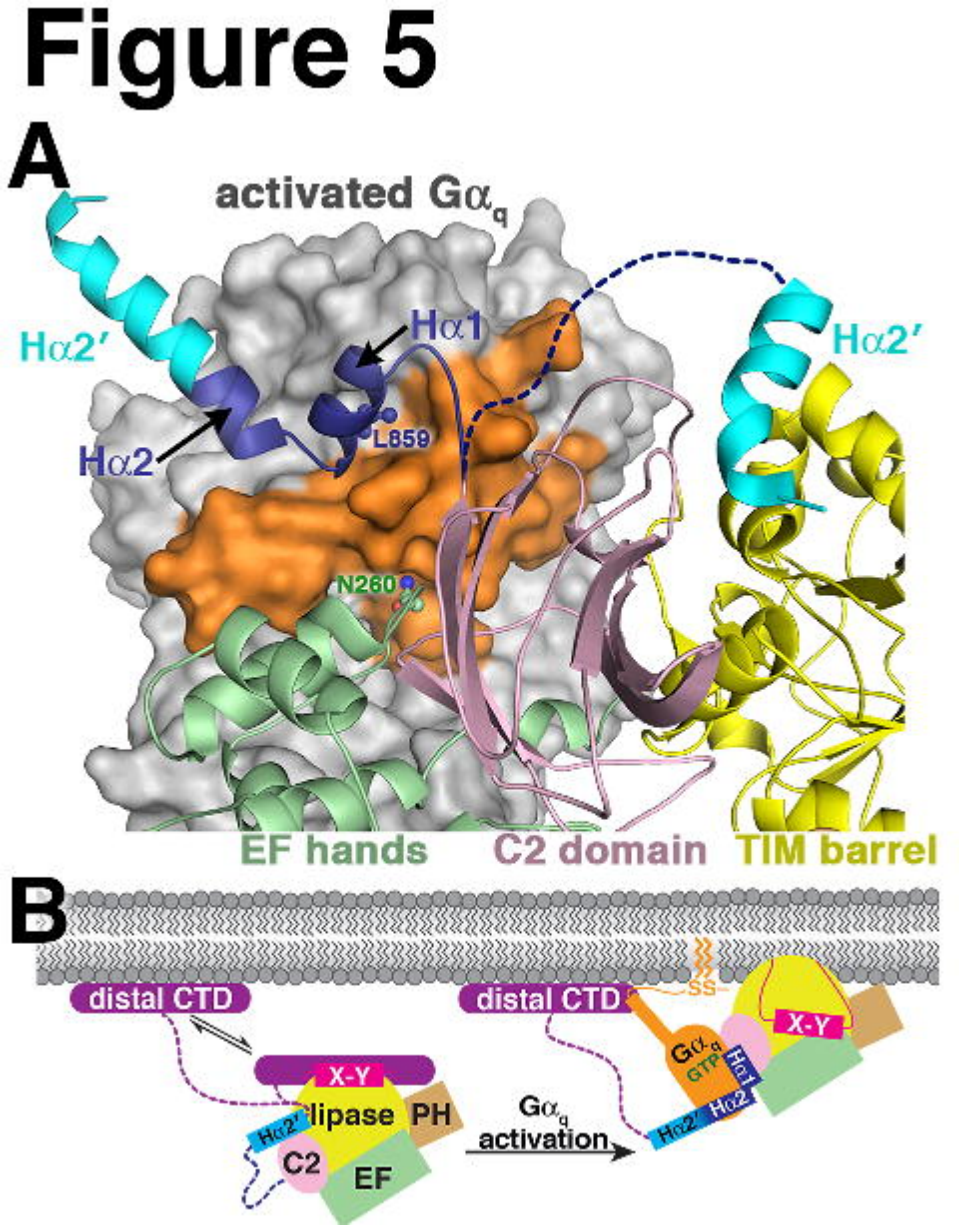
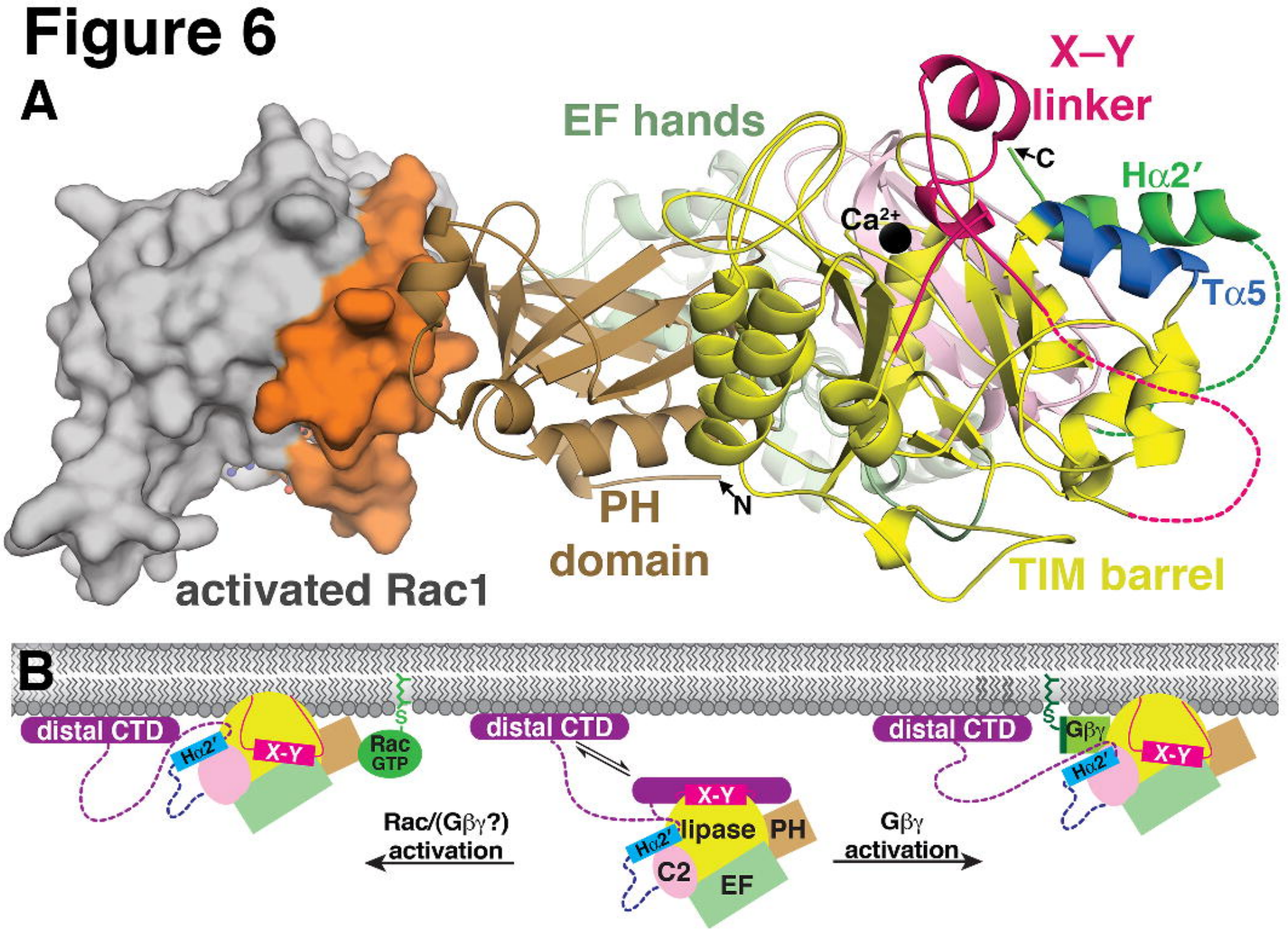

A
activated Gαq
Hα2′
Hα1
Hα2′
Hα2
L859
N260
EF hands
C2 domain
TIM barrel
B
distal CTD
distal CTD
SS
X-Y
X-Y
Gαq
GTP
Hα1
Hα2′
Hα2
lipase
PH
C2
EF
Gαq
activation

Figure 6


A
X–Y linker
EF hands
C
Hα2′
Ca2+
Tα5
N
PH domain
activated Rac1
TIM barrel
B
distal CTD
Hα2′
X-Y
Rac GTP
S
distal CTD
X-Y
Hα2′
lipase
PH
C2
EF
distal CTD
Gβγ
S
X-Y
Hα2′
Rac/(Gβγ?) activation
Gβγ activation