An Autoinhibitory Helix in the C-Terminal Region of Phospholipase C-β Mediates Gαq Activation

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Abstract

Phospholipase C-β (PLCβ) is a key regulator of intracellular calcium levels whose activity is controlled by heptahelical receptors that couple to Gq. We have determined atomic structures of two invertebrate homologs of PLCβ (PLC21) from cephalopod retina and identified a helix from the C-terminal regulatory region that interacts with a conserved surface of the catalytic core of the enzyme. Mutations designed to disrupt the analogous interaction in human PLCβ3 dramatically increase basal activity and diminish stimulation by Gαq. Gαq binding requires displacement of the autoinhibitory helix from the catalytic core, thus providing an allosteric mechanism for activation of PLCβ.

Phospholipase C-β (PLCβ) proteins form a highly conserved enzyme family that hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol, two key second messengers that mobilize intracellular calcium and stimulate the activity of protein kinase C1,2. PLCβ isoforms are potently activated via direct interactions with heterotrimeric G proteins of the Gαq family3,4, Gβγ heterodimers5–8, and...
small GTPases such as Rac\textsuperscript{9,10}. The interaction between \( G_\text{q} \) and PLC\( \beta \) is of particular interest because regulation of PLC\( \beta \) by \( G_\text{q} \)-coupled receptors is critical for normal cardiomyocyte function, and maladaptive changes in this pathway can result in the onset of cardiac arrhythmias, cardiac hypertrophy, and heart failure\textsuperscript{11–14}.

PLC\( \beta \) proteins and their invertebrate homologs NorpA\textsuperscript{15} and PLC21\textsuperscript{16,17} share a highly conserved catalytic core comprised of an N-terminal pleckstrin homology (PH) domain, followed by four EF hand domains, a triose-phosphate isomerase (TIM) barrel-like catalytic domain split into X and Y halves by a variable linker\textsuperscript{18,19}, and a C2 domain\textsuperscript{1,20} (Fig. 1a)\textsuperscript{21,22,23}. The X-Y linker is positioned adjacent to the active site, and its cleavage or truncation increases basal activity\textsuperscript{22,24,25}. However, such activation is independent of both heterotrimeric G proteins and small GTPases\textsuperscript{22}. The distinguishing feature of PLC\( \beta \) enzymes is a ~400 amino acid C-terminal region (CTR) that is known to be important for membrane association as well as \( G_\text{q} \) binding and activation\textsuperscript{1,26–29}. Many of these functional properties have been mapped to residues within an extended coiled-coil domain found in the C-terminus, corresponding to residues 946 to 1200 in human PLC\( \beta \)3 (Fig. 1a)\textsuperscript{1,26–29}. Recently, a structure was reported for \( G_\text{q} \) in complex with a human PLC\( \beta \)3 truncation (\( \Delta \text{887} \)) that includes a small portion of the CTR (residues 848 to 882). This region forms a helix-turn-helix motif (H\( \alpha \)1-H\( \alpha \)2) that docks with the effector-binding site of \( G_\text{q} \)\textsuperscript{23}. Although this structure revealed key interactions between PLC\( \beta \)3 and \( G_\text{q} \), the activity of the \( \Delta \text{887} \) fragment was not shown to be regulated by \( G_\text{q} \) and thus it remains unclear how \( G_\text{q} \) enhances PLC\( \beta \)3 activity and how other regions of the CTR contribute to its regulation\textsuperscript{27,30,31}.

To better understand the activation mechanism of PLC\( \beta \), we solved crystal structures of two invertebrate, endogenously expressed PLC\( \beta \) homologs from cephalopod retina. Within these structures, a helix located immediately C-terminal to the portion of the H\( \alpha \)1-H\( \alpha \)2 motif that directly interacts with \( G_\text{q} \) is observed to dock with a conserved cleft on the PLC\( \beta \) catalytic core. Perturbation of the analogous interaction in human PLC\( \beta \)3 dramatically enhances basal activity, lowers the thermostability of the enzyme, increases \( G_\text{q} \) affinity, and reduces the efficacy of \( G_\text{q} \) activation. Our results are consistent with an allosteric mechanism in which \( G_\text{q} \) binding displaces this inhibitory helix, leading to enhanced activity. Our studies also confirm that more distal regions of the CTR enhance the affinity of PLC\( \beta \)3 for \( G_\text{q} \) and facilitate PIP\textsubscript{2} hydrolysis through a mechanism independent of \( G_\text{q} \).

RESULTS

Structures of cephalopod PLC21

Crystal structures of endogenously expressed \textit{Loligo pealei} (LPLC21) and Sepia officinalis (SPLC21) were solved by molecular replacement to 3.1 and 2.0 Å resolution, respectively (Fig. 1b,c and Table 1). For this work, it was necessary to sequence the coding region for SPLC21 from its endogenous source, which we found to be ~92% identical to that of LPLC21. Although full length proteins were subjected to crystallization trials, both SPLC21 and LPLC21 crystallized as ~95 kDa proteolytic fragments (Supplementary Methods online) that contain visible density for most of the catalytic core. In the higher resolution SPLC21 structure, residues 11–474 and 485–774 of the catalytic core as well as

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26 residues from the CTR (residues 790–815) are visible. The catalytic cores of LPLC21 and SPLC21 are essentially identical, and superimpose with an r.m.s.d. value of 0.36 Å for 765 Cα atoms (out of 778 total in SPLC21). Within the catalytic core of SPLC21, only a short segment of the X–Y linker (amino acids 475–484) is missing electron density (Supplementary Fig. 1 online). There are only subtle differences in the domain arrangement of the catalytic core between cephalopod and human homologs. Most notably, the EF hand and C2 domains of SPLC21 are rotated by ~6° away from the TIM barrel and PH domains relative to their positions in the structures of PLCβ2 and PLCβ3.

The TIM barrel-like domain of SPLC21 is ~55% identical in sequence with those of human PLCβ2 and PLCβ3, and can be superimposed with r.m.s.d. values of 0.38 and 0.39 Å for 232 and 234 Cα atoms, respectively, omitting the X–Y linker (residues 473–510 of SPLC21). In SPLC21, the C-terminal end of the linker (residues 500–510, corresponding to residues 576–586 in human PLCβ3) has essentially the same structure as in other PLCβ structures. However, residues 485–499 form a helix that extends from the catalytic site in a different direction than observed in previous PLCβ structures (Supplementary Fig. 1 online) 22,23. The significance of this difference is not yet understood, but this unique conformation is also found in both unique chains of LPLC21 (Fig. 1b) suggesting that it is not dictated by the crystal packing environment.

In both PLC21 structures, strong electron density is observed for a segment of the CTR that can be modeled as a helical hairpin composed of Hα2′ and Hα3 helices (residues 790–816 of SPLC21) (Fig. 2a,b and Supplementary Fig. 2 online). The loop connecting the hairpin to the end of the C2 domain (residues 775–789) is disordered. The Hα2′ helix (residues 792–805) corresponds to the C-terminal end of the PLCβ3 Hα2 helix, just beyond the region of the Hα1-Hα2 motif in PLCβ3 that directly contacts Gq (Fig. 1d and Supplementary Fig. 3a online). Hα2′ packs against a highly conserved, hydrophobic cleft formed between the TIM barrel and C2 domains of the catalytic core, in close proximity to the active site and X–Y linker, and buries 1200 Å² of solvent accessible surface area (Fig. 2a,b). The side chains of Arg797, Leu801, and Phe804 form the principal interactions from Hα2′. The positively-charged guanidinium group of Arg797 caps the C-terminus of the To5 helix of the TIM barrel domain, and, along with Ala800 and Leu801, forms a hydrophobic pocket for the Phe639 side chain located in the linker between the TIM barrel and C2 domains of the catalytic core. The side chain of Phe804 packs in a hydrophobic cavity formed by the side chains of Val584 and Ile587 in the To6 helix of the TIM barrel domain and Pro679 and Thr682 of the C2 domain. The C-terminal end of Hα2′ is capped by the side chain of Arg684 from the C2 domain. The residues involved in these interactions are highly conserved among PLCβ enzymes, but poorly so in other PLC isoforms (Supplementary Fig. 3 online), consistent with the hypothesis that they contribute to a functionally important, PLCβ-specific intramolecular contact. Indeed, the same Hα2′-catalytic core interaction is observed in the SPLC21 structure (Fig. 1c and Fig. 2a,c) and the two independent chains of the LPLC21 structure (Fig. 1b), and a very similar interaction is formed in the Gq–PLCβ3 crystal structure as an intermolecular crystal contact (Fig. 2c,d). The Hα2′-catalytic core interaction has thus persisted over 500 million years of evolution32.
Goq binding to the Hα1-Hα2 motif is incompatible with the Hα2′ helix binding to the catalytic core in the same molecule. Indeed, the region is required to undergo a large conformational change upon Goq binding, as the Cα atom of PLCβ3-Arg872 is translated ~50 Å away from its position when Hα2′ is docked with the catalytic core, as modeled by the equivalent atom of Arg797 in the SPLC21 structure (Fig. 1c,d). Given the high sequence conservation of Hα2′ among PLCβ isoforms, its close proximity to the active site, and its juxtaposition with the primary Goq binding site, we hypothesized that the Hα2′ helix plays a role in the regulation of PLCβ by Goq. For subsequent experiments, we focused on human PLCβ3 because it is readily expressed in baculovirus-infected insect cells and is potently and efficaciously regulated by Goq3.33.

The Hα2′ helix modulates stability of the catalytic core

If the Hα2′ helix plays a regulatory role, then it should stably associate with the PLCβ catalytic core in solution. To detect this interaction, we used a ThermoFluor assay to measure the thermostability of three purified recombinant variants: PLCβ3 (which spans residues 10–1234 containing the complete CTR), PLCβ3-Δ892 (which terminates at residue 891 after the proximal CTR region observed in the PLC21 and PLCβ3 crystal structures), and PLCβ3-Δ847 (which terminates at residue 846 and lacks the entire CTR) (Fig. 1a). Whereas PLCβ3 and PLCβ3-Δ892 had a similar melting temperature (Tm) of 43 and 45 °C, respectively, PLCβ3-Δ847 had a markedly lower Tm of 38 °C, suggesting that the presence of the proximal CTR enhances the thermal stability of the catalytic core (Fig. 3a and Supplementary Table 1). We next tested if this thermostabilization could be accounted for by specific residues in the interface of Hα2′ with the catalytic core. Indeed, mutation of six individual residues in the context of the full-length protein (PLCβ3) reduced the Tm to 39–40 °C, similar to that of PLCβ3-Δ847 (Supplementary Table 1). Interface mutations in the background of PLCβ3-Δ892 similarly reduced the melting point by ~5 °C. In contrast to its effects in the context of PLCβ3 and PLCβ3-Δ892, the F715A mutation of PLCβ3-Δ847 did not significantly change thermostability, indicating that mutation of the catalytic core itself does not lead to lower stability. Thus, the higher thermostability exhibited by PLCβ3 and PLCβ3-Δ892 relative to PLCβ3-Δ847 is dependent on a specific interaction between the catalytic core and Hα2′.

The Hα2′ helix is an autoinhibitory element

Given the proximity of the Hα2′ helix to the active site and its contribution to protein stability, we hypothesized that its interactions with the core could influence catalytic activity. Because the CTR coiled-coil domain of PLCβ3 facilitates membrane recruitment of the enzyme to the substrate PIP2, the role of the Hα2′ helix in catalytic activity is most easily assessed by comparison of the truncations PLCβ3-Δ892 and PLCβ3-Δ847, or of site-directed mutations made in the full-length protein versus wild-type. PLCβ3 has 10-fold higher basal specific activity than PLCβ3-Δ892 (54 vs. 4.7 mol IP3 min⁻¹ mol⁻¹ PLCβ3, respectively), confirming that residues 892–1234 of the CTR strongly promote catalysis. The PLCβ3-Δ847 variant has 3-fold higher activity than PLCβ3-Δ892 (15 mol IP3 min⁻¹ mol⁻¹ PLCβ3), indicating that the presence of the proximal CTR inhibits the basal activity of PLCβ3 (Fig. 3b and Table 2). To confirm that specific interactions of Hα2′ with the catalytic core are responsible for the observed differences in basal activity, we measured the
activities of point mutants within the Hα2′-catalytic core interface of PLCβ3. The L663D, F715A and P755K mutations in the catalytic core enhance basal activity by over an order of magnitude, as do point mutants of interacting residues in Hα2′ (Fig. 3c and Table 2). The conservative F715Y mutation leads to a more modest increase in activity (6-fold). The complimentary results obtained from mutation of residues on either Hα2′ or its binding site on the catalytic core strongly suggest that this interaction serves to repress the basal activity of PLCβ3. Interface mutations made in the background of PLCβ3-Δ892 yield smaller but consistent increases in activity, although these results should be considered along with the fact that the F715A mutation also causes a mild increase in activity in the context of PLCβ3-Δ847 (Table 2). Thus, mutations in the catalytic core itself can increase activity, but these effects are greatly amplified in the context of the full-length protein. The basal activity data of full-length PLCβ3 and the thermostability data are consistent with the interaction of Hα2′ with the catalytic core, thereby trapping the TIM barrel-like domain in a more quiescent state.

The Hα2′ helix and distal CTR modulate the affinity of Gαq for PLCβ3

The intermolecular interaction of the Hα1-Hα2 module with Gαq and the intramolecular interaction of Hα2′ with the catalytic core of PLCβ3 are expected to be competitive. If so, then disrupting the Hα2′-catalytic core interface should enhance the affinity of Gαq for PLCβ3. We used a flow cytometry protein interaction assay (FCPIA) to measure binding between Gαq and PLCβ3, in which the binding affinities of unlabeled PLCβ3 and its variants for Gαq are determined by their ability to displace a fluorescently-labeled variant of PLCβ3 from beads (Fig. 3d and Table 3). PLCβ3-Δ847 is unable to bind Gαq at any concentration tested, consistent with the absence of the proximal CTR. Although PLCβ3-Δ892 retains this region, it has nearly two orders of magnitude lower affinity for Gαq (270 nM) than full-length PLCβ3 (6 nM), suggesting that the more distal regions of the CTR contribute key interactions that enhance binding to Gαq. The affinity of our truncation is consistent with that measured by surface plasmon resonance for the Δ887 truncation used in the crystallographic analysis of the Gαq-PLCβ3 complex. However, these same studies indicated that full-length PLCβ3 has a similar binding affinity for Gαq. The 6 nM binding constant we measure for full-length PLCβ3 is consistent with previously reported EC50 values for Gαq stimulation and the EC50 values we report in Table 4.

Mutation of residues in either Hα2′ or in its binding site on the catalytic core in PLCβ3-Δ892 enhances the apparent affinity for Gαq by 10-fold, consistent with competition between binding of the Hα1-Hα2 motif to Gαq and the binding of Hα2′ to the catalytic core. Analogous mutations in the longer PLCβ3 protein also seem to enhance affinity for Gαq, but only 2–3-fold, possibly because the more distal regions of the CTR play a compensatory role in binding to Gαq (Fig. 3d and Table 3). Our results are therefore consistent with a model in which Gαq binding to PLCβ3 displaces the proximal CTR from the catalytic core, leading to increases in both Gαq affinity and catalytic activity.

Mutations in the Hα2′-catalytic core interface reduce the efficacy of Gαq

The profound enhancement in basal activity observed for variants of PLCβ3 in which the Hα2′-catalytic core interface is disrupted is on the same scale as the activation of wild-type

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PLCβ3 by Gq. We hypothesized that if Gq activates PLCβ3 by displacing the autoinhibitory Hα2′ helix, then Gq should be less efficacious at activating variants of PLCβ3 in which the Hα2′-catalytic core interface is disrupted. However, because Gq should still be able to bind these variants (Table 3), residual Gq activation effects might be observed at the same or lower EC50 values as measured for the wild-type proteins.

PLCβ3 is activated by Gq 20-fold to 1100 mol IP3 min⁻¹ mol⁻¹ with an EC50 of 3 nM, consistent with the binding affinity we measure for these proteins by FCPIA (Table 3) and with previous studies. PLCβ3-Δ847, which lacks the proximal CTR, is unresponsive to Gq. However, PLCβ3-Δ892 is activated 7-fold by Gq to 30 mol IP3 min⁻¹ mol⁻¹ (Table 4 and Supplementary Fig. 4 online). Considered along with the crystal structure of the Gq-PLCβ3 complex, our data firmly establishes that the proximal CTR region confers regulation by Gq. However, the maximal Gq-stimulated rate of hydrolysis catalyzed by PLCβ3 is 40-fold higher than that of PLCβ3-Δ892. Moreover, Gq activates PLCβ3-Δ892 with a 30-fold higher EC50 (130 nM) than wild-type, consistent with the lower binding affinity of Gq for PLCβ3-Δ892 we measure by FCPIA (Table 3). This data provides further evidence that the more distal regions of the CTR make important contributions to the activation mechanism by enhancing both the catalytic rate and the affinity for Gq.

In the context of PLCβ3, mutations that disrupt the Hα2′-catalytic core interface are activated to a lesser extent by Gq, with only 2–3 fold effects observed. Although we anticipated a small decrease in EC50 for these mutants because of their higher affinity for Gq, this trend was only evident in the background of PLCβ3-Δ892. For PLCβ3 and its variants, the EC50 values remained in the low nM range and did not consistently decrease as we had measured in FCPIA (Table 3). Because our direct-binding assay is conducted in the presence of liposomes, these results may reflect the important role of the coiled-coil domain in mediating membrane association. However, we did observe that Gq has 2–3 higher potency when assayed against Hα2′ interface mutants in the background of PLCβ3-Δ892, which retains only the proximal CTR (Supplementary Fig. 4 online and Table 4).

DISCUSSION

Regulation of PLCβ isoforms is tightly controlled, and aberrations in this pathway or its components are associated with a number of pathophysiological processes, including heart failure. One aspect of this control is their exceptionally low level of basal activity and their profound stimulation by Gq-coupled receptors. Previous studies have shown that deletion of the X–Y linker in a variety of PLC isoforms leads to increased basal activity, presumably because acidic charges in the linker electrostatically block access of PIP2 to the active site. It has been proposed that membrane recruitment by activators of PLCβ, including Gq, helps to displace this linker. However, most PLCβ isoforms are already at least partially localized to the membrane in their basal state, and deletion or perturbation of the X–Y linker, although profoundly activating, does not eliminate the ability to be activated by heterotrimeric G proteins in simple transfection assays. This suggests that heterotrimeric G proteins can activate PLCβ isoforms through a mechanism that is not entirely reliant on the integrity of the X–Y linker.
Our structures of cephalopod PLC21 and functional studies of human PLCβ3 demonstrate that the α2′ helix in the proximal CTR interacts with a conserved cleft in the catalytic core. Disrupting the interactions of α2′ with the catalytic core dramatically increases basal activity up to ~50 fold (Table 2). The mechanism by which this occurs is as of yet unclear, but is possibly linked to the fact that α2′ is positioned on the same side of the TIM barrel as the X–Y linker, which may inhibit basal activity by sterically hindering the interaction of the active site with phospholipid bilayers. Alternatively, decreased thermal stability of the highly active mutants (Supplementary Table 1) implies greater dynamics in the catalytic core, which may lead to enhanced catalysis. A third possibility is that α2′ exerts a direct negative allosteric effect on the TIM barrel domain itself, although this seems less likely because the catalytic core has essentially the same structure with and without the bound α2′ element, as can be seen by comparing structures of PLCβ2 with that of PLCβ3 in the Gq-PLCβ3 complex. Although these structural elements are in close proximity (Fig. 2), a direct functional relationship between displacement of α2′ and inhibition imposed by the X–Y linker remains to be explored, but one hypothesis is that disruption of either element leads to a less stable yet more dynamic enzyme with an active site that has easier access to its phospholipid substrates.

We also provide substantial evidence to support an allosteric mechanism by which Gq modulates the interactions of the α2′ helix to activate PLCβ. In the absence of Gq-coupled receptor stimulation, the α2′ helix is bound to the catalytic core and suppresses basal activity (Fig. 2a,b and Fig. 3b,c). Our structures of PLC21 indicate that the residues in the α1-α2 motif that bind Gq are disordered in this basal state and thus freely accessible to Gq·GTP. Gq·GTP binding to the α1-α2 motif leads to displacement of the α2′ helix from the catalytic core (cf. Fig. 1c and Fig. 1d) and dramatically enhanced PIP2 hydrolysis. Accordingly, disruption of the α2′-catalytic core interface by truncation or by site-directed mutagenesis also leads to a marked increase in basal activity (Fig. 3b,c and Table 2) at the expense of Gq efficacy (Supplementary Fig. 4 and Table 4). However, even in these cases, Gq binding still activates by ~3-fold, perhaps by increasing the affinity of the catalytic core for phospholipid membranes, or, more generally, by stabilizing a more catalytically competent state. Because our in vitro assays used Gq isolated from the soluble fraction of cell lysates, this residual activation is unlikely to represent enhanced liposome association due to palmitoylation of the N-terminus of Gq.

Our data also strongly indicate that the more distal regions of the CTR contribute to high affinity Gq binding and to basal and Gq-stimulated activity (Fig. 3b,c,d and Table 4), as has been suggested by many other studies. The coiled-coil domain in the CTR contains conserved basic regions important for function, and plays a role in increasing the affinity of PLCβ for the cell membrane. Residues in the coiled-coil domain have also been shown to be important for Gq activation, and our studies show that the distal CTR enhances the affinity of PLCβ3 for Gq in protein binding assays conducted in the absence of phospholipid vesicles. The fact that the binding affinities of Gq for PLCβ3 and PLCβ3-Δ892 (Table 3) are consistent with their respective EC50 values measured in our liposome-based hydrolysis activity assays (Table 4) supports the idea that there is a direct
functional interaction between the distal regions of the CTR and G\textsubscript{q} which has yet to be resolved.

In summary, our PLC\textsubscript{21} structures likely represent a snapshot of a fully inhibited PLC\textsubscript{\beta} catalytic core, and when compared to the G\textsubscript{q}–PLC\textsubscript{\beta}3 crystal structure (Fig. 1c,d) it provides a molecular mechanism for PLC\textsubscript{\beta} activation in which G\textsubscript{q} effectively sequesters an autoinhibitory motif. The mechanism is reminiscent of the action of transducin on cGMP phosphodiesterase (PDE) in the visual signal transduction cascade, wherein activated G\textsubscript{t} sequesters the inhibitory PDE\textsubscript{\gamma} subunit\textsuperscript{45,46–47}. As in other canonical heterotrimeric G protein effectors, such as adenylyl cyclase\textsuperscript{48}, there are additional layers of regulation that are essential for full activity and add complexity. A better molecular understanding of how the distal regions of the CTR contribute to G\textsubscript{q} binding and enzyme activity is thus essential to composing a complete picture of how PLC\textsubscript{\beta} activity is controlled and how synergy can be achieved in response to the binding of multiple activators\textsuperscript{38}.

**METHODS**

**Cloning, expression, and purification of human PLC\textsubscript{\beta}3 proteins**

DNAs encoding N-terminally His-tagged human PLC\textsubscript{\beta}3 (amino acids 10–1234)\textsuperscript{49} and C-terminally truncated variants were cloned into pFastBac Dual (Invitrogen). Point mutations were introduced using QuikChange Site-Directed Mutagenesis (Stratagene) and confirmed by sequencing over the entire open reading frame. Baculovirus-infected High Five insect cells were resuspended in 20 mM HEPES pH 8, 200 mM NaCl, 10 mM \(\beta\)-mercaptoethanol (BME), 0.1 mM EDTA, 0.1 mM EGTA, and Roche EDTA-free protease inhibitor cocktail tablets. After sonication, the lysate was centrifuged and the supernatant was loaded on a Ni-NTA (Qiagen) column pre-equilibrated with buffer A (20 mM HEPES pH 8, 100 mM NaCl, 10 mM BME, 0.1 mM EGTA, and 0.1 mM EDTA). The column was washed with 10 column volumes of buffer A supplemented with 10 mM imidazole and 300 mM NaCl. PLC\textsubscript{\beta}3 proteins were eluted with buffer A supplemented with 200 mM imidazole, and then concentrated and purified to homogeneity on two tandem Superdex S200 columns (GE Healthcare) equilibrated with 20 mM HEPES pH 8, 200 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 0.1 mM EDTA (Supplementary Fig. 5 online).

**Expression and purification of G\textsubscript{q}**

Activity assays were conducted with protein expressed from cDNA encoding murine G\textsubscript{q} amino acids 7–359 cloned into pFastBacHTA (Invitrogen) to produce an N-terminally His\textsubscript{6}-tagged protein. FCPIA was performed with a G\textsubscript{\alpha}_q/G\textsubscript{\alpha}_i chimera in which the wild-type N-terminal helix of murine G\textsubscript{\alpha}_q was replaced with G\textsubscript{\alpha}_i, as described previously\textsuperscript{50}. The expression and purification protocols for both G\textsubscript{\alpha}_q variants are identical. Protein expression in High Five cells was increased upon co-infection of virus encoding Ric8A–GST\textsuperscript{51}. The cell pellet was resuspended in 20 mM HEPES pH 8, 100 mM NaCl, 10 mM BME, 3 mM MgCl\textsubscript{2}, 10 \(\mu\)M GDP, 0.1 mM EDTA and protease inhibitors. After sonication, the lysate was centrifuged and the supernatant was loaded on a Ni-NTA (Qiagen) column. The column was washed with 20 column volumes of buffer B (20 mM HEPES pH 8, 100 mM NaCl, 10 mM BME, 1 mM MgCl\textsubscript{2}, 10 \(\mu\)M GDP), followed by 20 column volumes of buffer B.
supplemented with 10 mM imidazole and 300 mM NaCl. Goq was eluted with buffer B supplemented with 150 mM imidazole. The sample was dialyzed to lower the NaCl concentration and applied to a MonoQ column. Goq was eluted in 20 mM HEPES pH 8, 1 mM MgCl2, 10 µM GDP, and 1 mM DTT with a gradient ranging from 0–500 mM NaCl. Peak fractions were further purified on tandem S200 columns equilibrated in 20 mM HEPES pH 8, 100 mM NaCl, 1 mM MgCl2, 10 µM GDP, and 1 mM DTT.

**Crystallization and structure determination**

Endogenous SPLC21 and LPLC21 were purified from enucleated eye cups as described previously\(^\text{16}\). Full-length protein was further purified by gel filtration in 20 mM HEPES pH 8, 200 mM NaCl, and 2 mM DTT. Protein was supplemented with 5 mM CaCl\(_2\) and crystallized by the hanging drop method using a 1:1 mixture of 11 mg mL\(^{-1}\) protein and well solution containing 100 mM Bis-Tris pH 7, 100–300 mM NaCl, and 20–35% (w/v) PEG 3350. Diffraction data was collected at the Advanced Photon Source at LS-CAT beam line 21-ID-F from crystals maintained at 110 K using a wavelength of 0.979 Å and initial phases were derived by molecular replacement using the human PLC\(\beta\)2 (PDB entry 2ZKM) structure\(^\text{22}\) as a search model.

**Thermostability measurements**

Melting temperatures (\(T_m\)) were determined by monitoring 1-anilinonaphthalene-8-sulfonic acid (ANS) binding to PLC\(\beta\)3 variants during protein unfolding\(^\text{52}\). Wild type PLC\(\beta\)3 and variants (0.3 mg mL\(^{-1}\)) were incubated with 200 µm ANS in a total volume of 5 µL in triplicate in ABgene 384-well PCR microtiter plates (Thermo-Fisher). Fluorescence was measured as the temperature was increased from 20–80 °C in 1 °C intervals using a ThermoFluor 384-well plate reader (Johnson & Johnson).

**PLC\(\beta\)3 activity assays**

PLC\(\beta\)3 basal activity and Goq-mediated activation was quantified by measuring the rate of hydrolysis of \(^{3}\text{H}\)-labeled PIP\(_2\) in a liposome-based assay as previously described\(^\text{16,53}\). Briefly, lipid vesicles containing 200 µM phosphatidylyceranolamine, 50 µM PIP\(_2\), and \(~4000–8000\) cpm \(^{3}\text{H}\)-labeled PIP\(_2\) per assay were mixed, dried under nitrogen, and resuspended by sonication in 50 mM HEPES pH 7, 80 mM KCl, 2 mM EGTA, and 1 mM DTT. PLC\(\beta\)3 activity was assayed at 30 °C in 50 mM HEPES pH 7, 80 mM KCl, 15 mM NaCl, 0.83 mM MgCl\(_2\), 3 mM DTT, 1 mg mL\(^{-1}\) BSA, 2.5 mM EGTA, 0.2 mM EDTA with \(~200\) nM free Ca\(^{2+}\). Control reactions contained the same components, but lacked free Ca\(^{2+}\). Reactions were terminated by addition of BSA and 10% (w/v) ice-cold trichloroacetic acid. After centrifugation, free \(^{3}\text{H}\)-IP\(_3\) in the supernatant was measured by scintillation counting\(^\text{16,53}\). To measure Goq-stimulated activity, purified Goq was activated with 50 mM HEPES pH 7, 80 mM KCl, 30 mM NaCl, 3 mM DTT, 10 mM NaF, 20 µM AlCl\(_3\), 50 µM GDP and 1.83 mM MgCl\(_2\) for 30 min on ice. Increasing amounts of activated Goq were added to PLC\(\beta\)3 proteins, and reactions initiated by addition of liposomes, and terminated after incubation at 30 °C for 5 min.
The (R872A L876A L879A) triple mutant of PLC\(\beta_3\)–Δ892, which was considered to be the least likely to be autoinhibited by \(H_G\), was fluorescently labeled with AlexaFluor-488 (AF488) C5-maleimide. \(G_{\alpha i/q}\) was biotinylated and linked to streptavidin coated beads as previously described\(^3\). Unlabeled PLC\(\beta_3\) variants were added at increasing concentrations to bead-bound \(G_{\alpha i/q}\) followed by addition of the AF488-labeled PLC\(\beta_3\)–Δ892 variant at its measured \(K_D\) (20.5 nM). The resulting mixtures were incubated for 1 hr, and then analyzed in duplicate with an Accuri C6 Flow Cytometer. Competition data was fit by nonlinear regression using a variable slope fit (PLC\(\beta_3\) and mutants) or standard slope (other variants) using GraphPad Prism (version 5.0a). \(K_I\) values were estimated from fitted \(IC_{50}\) values using the Cheng-Prussof equation.

### Statistical methods

Statistical analyses used ANOVA with a Dunnett’s post-test as implemented in GraphPad Prism (version 5.0a).

Additional and more detailed methods are provided in the Supplementary Methods online.

### Supplementary Material

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Figure 1.
Primary and tertiary structures of PLCβ family members, and comparison of cephalopod PLC21 with the Gαq–PLCβ3 complex. (a) Primary structure of human PLCβ3. PLCβ3 truncations used in this paper are indicated below the diagram. Numbers above the diagram correspond to amino acid positions at domain boundaries. (b) Crystal structure of LPLC21. LPLC21 crystallized as a dimer with pseudo two-fold symmetry. Domains are colored as in a. The Hα2′-Hα3 hairpin from the proximal CTR is shown in cyan, and the catalytic Ca$^{2+}$ is shown as a black sphere. Disordered loops are drawn as dashed lines, with the exception of...
the connection between the C2 domain and the beginning of Hα2′, which is ambiguous in the dimer interface. The C-terminus of the C2 domain and start of Hα2′ are marked with pink and blue asterisks, respectively. N- and C-terminal ends of the protein fragment resolved in the crystal structure are labeled N and C, respectively. (c) Crystal structure of SPLC21. Domains are colored as in b. (d) Crystal structure of the Gαq–PLCβ3 complex (PDB entry 3OHM). Hα1 and Hα2, which form the primary Gαq binding site, are shown in dark blue. Residues corresponding to Hα2′ in the PLC21 structures are shown in cyan. Activated Gαq is shown in light gray, with GDP and AlF4 colored red, and Mg2+ black.
Figure 2.
Interactions of H\textalpha{}2' with the catalytic core. (a) The SPLC21 H\textalpha{}2' helix docks in a conserved cleft formed between the TIM barrel and C2 domains, in close proximity to the active site and the X–Y linker. The shorter H\textalpha{}3 helix forms a hairpin interaction with H\textalpha{}2' stabilized by hydrophobic interactions. Domains are colored as in 1a. (b) Specific interactions of SPLC21 H\textalpha{}2' with the catalytic core. Side chains that make large contributions to the binding interface are shown as sticks with carbons colored according to their respective domains and nitrogens blue. (c) The H\textalpha{}2'-catalytic core interaction is recapitulated in a crystal contact of the G\textalpha{}q–PLC\beta{}3 structure (PDB entry 3OHM). Domains are colored as in 1d. The subunit of G\textalpha{}q shown is in complex with a different catalytic core in the crystal lattice. (d) Specific interactions between H\textalpha{}2' and the catalytic core in human PLC\beta{}3 (Fig. 1c). Residues analogous to those of SPLC21 shown in b are drawn as sticks, and site-directed mutations created in this study to perturb the interface are indicated. The SPLC21 H\textalpha{}2' helix is continuous, whereas H\textalpha{}2' in human PLC\beta{}3 is kinked at Ala877, as if to optimize the interactions of the Leu879 side chain, which is smaller than that of the corresponding Phe804 residue in SPLC21.
Figure 3.
Functional studies of PLCβ3 variants. (a) The proximal CTR stabilizes the catalytic core. ThermoFluor assays were used to measure the melting point of three PLCβ3 variants by monitoring the change in fluorescence of ANS. Representative curves are shown for PLCβ3 (circles), PLCβ3-Δ892 (squares), and PLCβ3-Δ847 (triangles). PLCβ3-Δ847 is 5–7 °C less stable (left-shifted) than PLCβ3 or PLCβ3-Δ892. See Supplementary Table 1. AU, arbitrary units. (b) Comparison of the basal activity of PLCβ3 variants. Deletion of the proximal CTR in PLCβ3-Δ847 increases basal activity relative to PLCβ3-Δ892. The higher basal activity of PLCβ3 reflects the contribution of more distal regions of the CTR to maximal activity. Activity was measured by counting free [3H]-IP3 released from liposomes containing [3H]-PIP2 at 30 °C in the presence of ~200 nM free Ca2+ at 4–5 time points. The data shown represent at least four individual experiments performed in duplicate ± SEM. (c) Mutation of PLCβ3 at positions that contribute to the Hα2′-catalytic core interface dramatically increase basal activity, indicating that this interaction is involved in autoinhibition. (d) Distal regions of the PLCβ3 CTR enhance binding to Goq. FCPIA was used to quantify the ability of PLCβ3 truncations to displace AlexaFluor488-labeled PLCβ3-Δ892 (R872A L876A L879A triple mutant) from biotinylated, AlF4-activated Goq bound to avidin beads. Representative
curves for PLCβ3 (circles), PLCβ3-Δ892 (squares) and PLCβ3-Δ847 (triangles) are shown. See Table 3 for measured inhibition constants.
Table 1

Data collection and refinement statistics

|                | Sepia PLC21 | Loligo PLC21 |
|----------------|-------------|--------------|
| **Data collection** |             |              |
| Space group     | \(P_{2121}\) | \(P_{2121}\) |
| **Cell dimensions** |             |              |
| \(a, b, c\) (Å) | 60.8, 83.4, 163.1 | 82.4, 148.9, 151.6 |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å)  | 27.4-2.00 (2.04-2.00) | 29.97-3.10 (3.15-3.10) |
| \(R_{sym}\) or \(R_{merge}\) | 0.069 (0.679) | 0.146 (0.814) |
| \(I/\sigma I\) | 17.4 (1.58) | 14.2 (2.3) |
| Completeness (%) | 97.7 (98.2) | 100 (100) |
| Redundancy      | 3.7 (3.6) | 7.2 (7.2) |
| **Refinement**  |             |              |
| Resolution (Å)  | 27.4-2.00 | 30.0-3.20 |
| No. reflections | 53335 | 29832 |
| \(R_{work}\) / \(R_{free}\) | 0.176/0.208 | 0.232/0.256 |
| No. atoms       |             |              |
| Protein         | 12668 | 24953 |
| Ligand/ion      | 126 | 2 |
| Water           | 452 | 38 |
| **B-factors**   |             |              |
| Protein         | 20.2 | 61.5 |
| Ligand/ion      | 45.9 | 60.2 |
| Water           | 28.1 | 36.3 |
| **R.m.s. deviations** |         |              |
| Bond lengths (Å) | 0.006 | 0.006 |
| Bond angles (°) | 0.946 | 0.881 |

Each data set was collected from a single crystal.

*Values in parentheses are for highest-resolution shell.
| Variant | Specific Activity ± SEM (mol IP$_3$ min$^{-1}$ mol$^{-1}$ PLCβ3) | Fold Increase Relative to Wild-Type |
|---------|-------------------------------------------------|---------------------------------|
| PLCβ3 (wt)$^b$ | 54 ± 2.7 | 1 |
| -L663D | 1150 ± 81 | 23 |
| -F715A | 2500 ± 380 | 51 |
| -F715Y | 310 ± 62 | 6 |
| -P755K | 2650 ± 216 | 53 |
| -L876A | 950 ± 69 | 19 |
| -L879A | 2000 ± 190 | 41 |
| PLCβ3-Δ892 (wt) | 4.7 ± 0.6 | 1 |
| -F715A$^b$ | 9.5 ± 0.4 | 2 |
| -R760A$^b$ | 7.2 ± 0.7 | 2 |
| -R872A$^b$ | 22 ± 2.2 | 5 |
| -L876A | 9.9 ± 1.6 | 2 |
| -L879A$^b$ | 13 ± 1.9 | 3 |
| -L876A L879A$^b$ | 14 ± 1.1 | 3 |
| -AAA$^{b,c}$ | 14 ± 1.4 | 3 |
| PLCβ3-Δ847 (wt)$^b$ | 15 ± 2.1 | 1 |
| -F715A | 39 ± 4.4 | 2.3 |

$^a$ At least five independent experiments performed in duplicate.

$^b$ Includes samples from at least two independent protein purifications.

$^c$ R872A L876A L879A
**Table 3**

Inhibition Constants of PLCβ3 Variants Measured by FCPIA

| Variant              | $K_i$ ± SEM (nM) | (n)$^a$ |
|----------------------|------------------|--------|
| PLCβ3 (wt)$^b$       | 6.0 ± 1.0        | (5)    |
| -F715A               | 2.0 ± 0.3        | (4)    |
| -L876A               | 3.1 ± 0.5        | (4)    |
| -L879A               | 1.6 ± 0.1        | (3)    |
| PLCβ3-Δ892 (wt)$^b$ | 270 ± 60         | (4)    |
| -F715A$^b$           | 31 ± 6           | (3)    |
| -AAA$^c$             | 26 ± 6           | (3)    |
| PLCβ3-Δ847 (wt)$^b$ | NB$^d$           | (3)    |

$^a$ Number (n) of independent experiments performed in duplicate.

$^b$ Includes samples from at least two independent protein purifications.

$^c$ R872A L876A L879A

$^d$ No binding detected.
Table 4

Goq Activation of PLCβ3 Variants in Vitro\(^a\)

| Variant            | EC\(_50\) (nM) | Increase in Activity (mol IP\(_3\) min\(^{-1}\) mol\(^{-1}\)PLCβ3) | Fold Max. Activity Over Basal\(^b\) |
|--------------------|----------------|---------------------------------------------------------------|-------------------------------------|
| PLCβ3 (wt)         | 3.2 ± 0.6      | 1100 ± 100                                                   | 21                                  |
| -F715A             | 9.7 ± 1.4      | 5000 ± 690                                                   | 3.0                                 |
| -P755K             | 10. ± 1.8      | 3200 ± 230                                                   | 2.2                                 |
| -L876A             | 0.7 ± 0.1      | 1360 ± 120                                                   | 2.4                                 |
| -L879A             | 9.4 ± 1.7      | 4700 ± 310                                                   | 3.3                                 |
| -AAA\(^c\)         | ND\(^d\)       | ND                                                            | ND                                  |
| PLCβ3-Δ892 (wt)    | 130 ± 19       | 30 ± 4                                                        | 7.3                                 |
| -F715A             | 54 ± 13        | 41 ± 3                                                        | 3.9                                 |
| -AAA\(^c\)         | 67 ± 13        | 33 ± 2                                                        | 3.3                                 |
| PLCβ3-Δ847 (wt)    | ---            | −9 ± 2                                                        | 0.4                                 |

\(^a\)Three to 12 independent experiments, each performed in duplicate, ± SEM. See Supplementary Figure 4 online.

\(^b\)Fold activation is calculated as the (basal activity + increase in activity) × basal activity\(^{-1}\). Due to errors in determining very low basal activities in these dose response curves, basal activities are taken from Table 2.

\(^c\)R872A L876A L879A

\(^d\)Not determined. The full-length version of this protein was highly susceptible to proteolytic cleavage after the proximal CTR, and consequently the activity could not be unambiguously measured.