Selective interaction of the C2 domains of phospholipase C-β_1 and -β_2 with activated Gα_q subunits: An alternative function for C2-signaling modules

TIELI WANG*, SRINIVAS PENTYALA†, JOHN T. ELLIOTT*, LOUISA DOWAL*, EKTA GUPTA†, MARIO J. REBECCHI*†, AND SUZANNE SCARLATA*‡

*Department of Physiology and Biophysics and †Department of Anesthesiology, State University of New York, Stony Brook, NY 11794-8661

ABSTRACT   Phospholipase C (PLC)-β_1 and PLC-β_2 are regulated by the G$_q$ family of heterotrimeric G proteins and contain C2 domains. These domains are Ca^{2+}-binding modules that serve as membrane-attachment motifs in a number of signal transduction proteins. To determine the role that C2 domains play in PLC-β_1 and PLC-β_2 function, we measured the binding of the isolated C2 domains to membrane bilayers. We found, unexpectedly, that these modules do not bind to membranes but they associate strongly and specifically to activated [guanosine 5′-[γ-thio]triphosphate (GTP[γS])-bound] Gα_q subunits. The C2 domain of PLC-β_1 effectively suppressed the activation of the intact isozyme by Gα_q(GTP[γS]), indicating that the C2-Gα_q interaction may be physiologically relevant. C2 affinity for Gα_q(GTP[γS]) was reduced when Gα_q was deactivated to the GDP-bound state. Binding to activated Gα_{i1} subunits or to G$\beta\gamma$ subunits was not detected. Also, Gα_q(GTP[γS]) failed to associate with the C2 domain of PLC-δ, an isozyme that is not activated by Gα_q. These results indicate that the C2 domains of PLC-β_1 and PLC-β_2 provide a surface to which Gα_q subunits can dock, leading to activation of the native protein.

Phosphatidylinositol-specific phospholipase Cs (PLCs) are Ca^{2+}-dependent enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PIns(4,5)P_2] to generate the two second messengers diacylglycerol and inositol 1,4,5-trisphosphate (for reviews see refs. 1 and 2). These messengers then promote the activation of protein kinase C and the release of Ca^{2+} from intracellular stores. There are three known families of mammalian PLCs (γ, δ, and β), which differ in their regulation. PLC-γs are regulated by tyrosine kinase receptors whereas regulators of the PLC-δ family are not well defined. The four members of the PLC-β family, which we will focus on here, are activated by the subunits from the Gα_q family of heterotrimeric G proteins as well as G$\beta\gamma$ subunits.

The mammalian PLC-βs are modular proteins containing an N-terminal pleckstrin homology (PH) domain, four elongation factor (EF) hand motifs, a catalytic X/Y domain, a C2 domain, and a long, 400-residue C-terminal extension that is unique to the PLC-β family. Although the roles of the EF hand motifs and C2 domain are unknown, the functions of the PH domain and C-terminal region have been investigated. Studies of the isolated PH domains of PLC-β_1 and PLC-β_2 show that they have a membrane-binding affinity on the order of the intact enzyme and help to anchor the protein to the membrane surface, allowing for lateral association to G protein subunits (3). The PH domains PLC-β_1 and PLC-β_2 also have a strong affinity for G$\beta\gamma$ and may play a key role in docking the G$\beta\gamma$ subunits, allowing for interactions with the catalytic domain (3).

In contrast, the C-terminal extension plays a key role in Gα_q regulation because its deletion preserves intrinsic activity but abolishes activation by Gα_q subunits (4–6). Truncation of the C terminus also reduces the affinity of the protein for activated Gα_q subunits $\approx$25-fold, but the residual affinity between the two proteins is still very strong. PLC-βs are also GAPs (GTPase-activating proteins), and proteins derived from this C-terminal region have $\approx$20% of the total GAP activity of the intact enzyme (7).

In this study, we investigate the role of the C2 domains of PLC-β_1 and PLC-β_2. C2 domains have been identified and characterized in many proteins to date (for review see refs. 8 and 9). In general, C2 domains are $\approx$130 residues in length and consist of a compact β-sandwich of two four-stranded β-sheets. C2 domains generally are found in proteins that interact with lipid membranes that have been grouped into four major classes (see ref. 8): those that mediate vesicle transport, modify lipids, regulate GTPase activity, and phosphorylate proteins. Most, but not all, C2 domains bind Ca^{2+}, and many are able to target their host protein to lipid membranes. It is notable that in some cases, such as synaptotagmins, C2 domains mediate calcium-dependent dimerization, binding to target proteins and to inositol polyphosphates.

The crystal structure of PLC-δ_1 shows that its C2 domain, which binds Ca^{2+}, is integrated with the catalytic core, and so simple deletion of this domain would be expected to produce an inactive protein. It was proposed that the function of the C2 domain of PLC-δ_1 correctly orients the catalytic site to the lipid substrate (10). However, recent biochemical studies of mutated PLC-δ_1 indicate that this may not be the case, leaving the function of the C2 domain of PLC-δ_1 unclear (11). Sequence homology maps of the C2 domains of PLC-β, which we have studied here, show nonconservative replacements of some of the Asp residues required for Ca^{2+} binding.

Here, we have expressed the C2 domains of PLC-β_1, PLC-β_2, and PLC-δ_1 and studied their ability to bind various membranes in a Ca^{2+}-dependent manner by using fluorescence methods. Although only the C2-PLC-δ_1 showed membrane binding, we found, surprisingly, that the C2 domains of PLC-β_1 and PLC-β_2 mediate specific association to Gα_q subunits, indicating that these domains may mediate G protein signal transduction.

MATERIALS AND METHODS

Protein Preparation. Preparation of recombinant PLC-β_1, PLC-β_2, Gα_q, and G$\beta_1\gamma_2$ expressed in Sf9 cells and Gα_{i1} expressed in bacteria have been described (12). The integrity of the protein products was assessed electrophoretically and by PLC-β activity and Gα_q[guanosine 5'-[γ-thio]triphosphate (GTP[γS])] activation as reported (13).

C2 domains were prepared by amplifying the coding sequences for C2-PLC-β_1 (residues 663–802), C2-PLC-β_2 (residues 688–805), and C2-PLC-δ_1 (residues 615–756) by PCR and by inserting them into the corresponding sites in the expression vector pGEX (Pharmacia) fused to glutathione *S*-transferase (GST). *Escherichia coli* BL-21 (DE3) cells, transformed with pGEX-C2s expression vectors, were grown in super broth containing ampicillin (50 μg/ml). GST-C2 domain expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside at 18°C, and the bacteria were harvested after 24 h. C2-β_1 and C2-β_2 were extracted as GST fusion proteins from the soluble fraction of the bacterial lysates by using glutathione-Sepharose 4B resin according to the manufacturer's protocol (Pharmacia). Proteins were >95% pure, as judged by SDS/PAGE analysis.

Because C2-PLC-δ_1 was found mainly in the insoluble fraction of the bacterial extracts, inclusion bodies were solubilized in 8 M urea and slowly diluted to 4 M urea before subjecting to glutathione-Sepharose chromatography. Renaturation was accomplished by using the method of Rozema and Gellman, which gave a better recovery than simple dilution methods (see ref. 14). Briefly, the solution was dialyzed into 4 M urea and diluted into 0.53 mM cetyltrimethylammonium bromide/4.8 mM β-cyclodextrin to promote refolding. The sample was left overnight and dialyzed later into appropriate buffers.

Removal of the GST tag was accomplished by incubating the GST-fused proteins with biotinylated thrombin, removing the thrombin with streptavidin-agarose, and then removing the free GST by using glutathione-Sepharose. After GST cleavage, only the C2-PLC-δ_1 required renaturation. This was accomplished by redissolving into 4 M urea and then removing the urea initially by dilution and then by dialysis. Proper folding of the GST-cleaved C2 domains was confirmed by circular dichroism as described (3).

Protein Labeling. All probes were purchased from Molecular Probes. Labeling with the amine-reactive probes, 7-methoxyl coumarin succinyl ester and dabsyl-succinyl ester, has been described (3, 13). The protein/probe labeling ratios were determined by absorption spectroscopy by using the calculated extinction coefficients for the protein and the coefficients provided by the probe manufacturer. The labeling ratio for all C2 proteins was found to be $\approx$1:1 (mol/mol).

Preparation of Membranes and Proteoliposomes. Large, unilamellar vesicles were prepared by extrusion. G protein subunits were reconstituted onto the membranes by adding the detergent-solubilized proteins to a large excess of preformed, extruded vesicles as described (13).

RESULTS

Membrane-Binding Studies. Binding of the C2 domains of PLC-β_1, PLC-β_2, and PLC-δ_1 to lipid bilayers was measured using two types of fluorescence-based assays (see refs. 3 and 13). In the first, membranes were doped with the fluorescent detergent 6-lauroyl-2-(dimethylamino)naphthalene, whose head group is highly sensitive to the polarity of its environment. As proteins bind to the membrane surface and displace water, the emission intensity increases and the center of spectral mass shifts to higher energies. In the second type of assay, C2 domains were labeled with the fluorescent probe, 7-methoxycoumarin succinyl ester, and membrane binding of

the labeled proteins to the nonfluorescent lipid was followed by the increase of coumarin fluorescence. Binding of C2-β_1 and C2-β_2 could not be detected even at lipid concentrations as high as 500 μM 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC), POPC/1-palmitoyl 2-oleoyl phosphatidylserine (POPS) (1:2), or POPC/PIns(4,5)P_2 (2%) (data not shown). Addition of 10 mM free Ca^{2+} did not influence binding. We did find that C2-δ_1 bound weakly in a Ca^{2+}-dependent manner to the anionic membranes, but at affinities that were too weak to be determined accurately by these methods (partition coefficient, defined as the ratio of the concentration of free and membrane-bound protein, K_p > 1 mM).

The C2 Domains of PLC-β_1 and PLC-β_2 Bind Specifically to Gα_q Subunits. Because the C2 domain of the PLC-β isozymes lies between the catalytic core and the C-terminal region that mediates activation by Gα_q subunits, we tested the idea that the C2 domains may serve to link Gα_q to the activation and catalytic domains of the protein. Association between the C2 domains and Gα_q subunits was determined by labeling the Gα_q with coumarin (C-) and measuring the transfer of its excited energy to a nonfluorescent energy-transfer acceptor covalently attached to the C2 domains, 4-[4-(dimethylamino)phenylazo]benzoic acid (DAB-). Because energy transfer is highly distance dependent, association of the two proteins is observed by the loss of donor fluorescence. We confirmed that activation of PLC-β_1 by Gα_q(GTP[γS]) did not change after fluorescent labeling.

In Fig. 1 we show the increase in energy transfer from C-Gα_q(GTP[γS]) reconstituted onto lipid bilayers as DAB-C2-PLC-β_1 or DAB-C2-PLC-β_2 was added. From these data, the apparent dissociation constants can be determined (Table 1). We find that both C2 domains bind with similar affinities that are independent of Ca^{2+}, which is not surprising because the PLC-β domains are not expected to bind Ca^{2+} based on
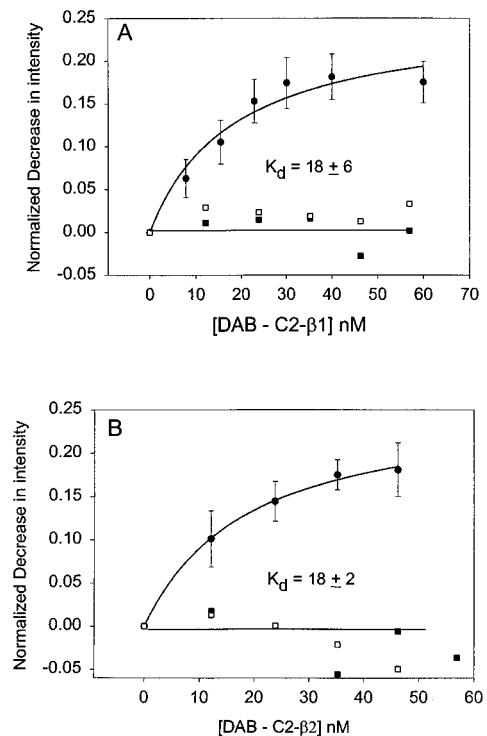

FIG. 1. Normalized decrease in the fluorescence intensity, obtained by dividing the zero-point fluorescence intensity and subtracting the background, of 2 nM C-Gα_q(GTP[γS]) reconstituted on 120–150 μM POPC/POPS/POPE (1:1:1) membranes as DAB-C2-PLC-β_1 (*A*, ●) or DAB-C2-PLC-β_2 (*B*, ●) is added. Control studies substituted C-Gα_{i1}(GTP[γS]) (□) or C-G$\beta\gamma$ (■) for C-Gα_q(GTP[γS]).

Table 1. Dissociation constants of the C2 domains of PLCs

C2 domain	$G\alpha_q(GTP[\gamma S])$	$G\alpha_q(GDP)$	$G\alpha_{i1}(GTP[\gamma S])$	$G\beta\gamma$
C2-PLC-β_1	18 ± 2	122 ± 26	>1,000	>1,000
C2-PLC-β_2	18 ± 6	64 ± 10	>1,000	>1,000
C2-PLC-δ_1	>1,000	>1,000	>1,000	>1,000

K_d values in nM were determined from the fluorescence energy-transfer titration data (e.g., Figs. 1 and 2) and fit to a bimolecular dissociation constant ($n = 2$–6).

sequence (7). The extent of energy transfer ($\approx 20\%$ loss in donor intensity) is similar to that seen for the association between C-$G\alpha_q(GTP[\gamma S])$ and DAB-PLC-β_1 and DAB-PLC-β_2 (12). Similar to the full-length proteins, we find that the affinity for $G\alpha_q$ decreases when these subunits are deactivated to their GDP-bound forms (Fig. 2 and Table 1) although the decrease is lower than that observed for the native proteins. We also note that deactivation of $G\alpha_q$ to the GDP form reduces the amount of energy transfer to DAB-C2-β domains (compare Figs. 1 and 2), implying that either a portion of the DAB-C2-β domains does not bind to $G\alpha_q(GDP)$ or that their interaction is altered so that the energy-transfer distance between the coumarin and DAB probes is increased.

Several control studies were done to convince ourselves that the decrease in C-$G\alpha_q$ fluorescence was a result of energy transfer caused by molecular association between the proteins. First, the addition of unlabeled C2 domains or dialysis buffer did not alter the intensity of C-$G\alpha_q(GTP[\gamma S])$. Second, the change in intensity could be blocked completely by preincubation of activated or deactivated C-$G\alpha_q$ with an appropriate concentration (see below) of unlabeled, full-length PLC-β_1 (Fig. 1).

Further studies were done to demonstrate that the associations between the PLC-β C2 domains and $G\alpha_q$ were specific. First, we substituted the PLC-β C2 domains with PLC-δ_1 C2
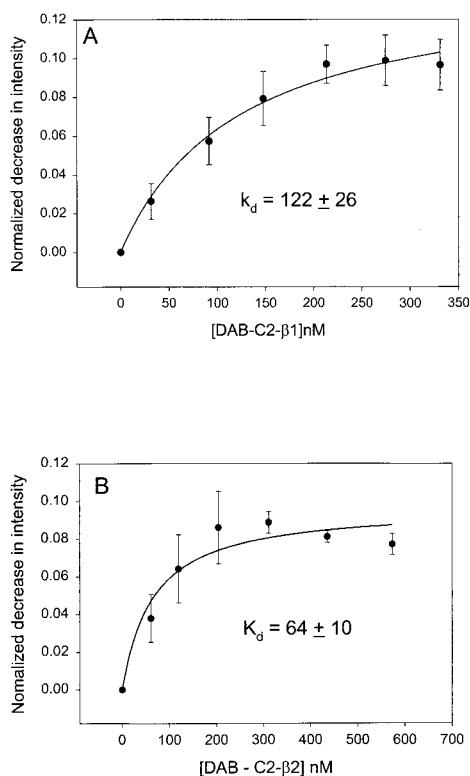
domains and did not detect association (data not shown). This result is not surprising because the intact PLC-δ_1 is not activated by $G\alpha_q(GTP[\gamma S])$. Second, we tested DAB-labeled N-terminal PH domains of PLC-β_2, which is not expected to interact with $G\alpha_q$ (15), and we could not detect association (data not shown). Third, we replaced C-$G\alpha_q$ with C-$G\beta\gamma$ subunits and could not detect a change in fluorescence when DAB-C2 domains of PLC-β_1 or PLC-β_2 were added (Figs. 1 and 2); even the intensity of C-$G\beta\gamma$ decreases $\approx 20\%$ upon association with DAB-$G\alpha_q(GDP)$, DAB-PLC-β_2, DAB-PH-PLC-β_1, or DAB-PH-β_2 (3, 13). Finally, no association could be seen when C-$G\alpha_{i1}(GTP[\gamma S])$ was substituted for C-$G\alpha_q(GTP[\gamma S])$ (Fig. 1). Taken together, we conclude that energy transfer between C-$G\alpha_q(GTP[\gamma S])$ and DAB-C2 PLC-β_1 and PLC-β_2 reflects specific protein–protein associations.

We also used a second method to confirm direct binding between the proteins. Using glutathione-Sepharose, we measured the amount of C-$G\alpha_q(GTP[\gamma S])$ that adhered to the resin in the presence and absence of bound GST-PLC-β_1-C2, with the nonbinding C-$G\alpha_{i1}(GTP[\gamma S])$ as a control. The results in Fig. 3 show that only C-$G\alpha_q(GTP[\gamma S])$ adheres to the resin only when GST-PLC-β_1-C2 is bound.

Because the data in Figs. 1 and 2 represent affinities between a protein that is bound to the membrane surface and one that is free in solution, we have calculated the affinities by using a solution-state bimolecular association constant. The interaction between the intact enzymes and G protein occurs for proteins concentrated on membrane surfaces, which must be considered when comparing the $G\alpha_q$ affinities of C2 domains and their intact proteins. The apparent K_d for the association between C2-PLC-β_1 and C2-PLC-β_2 and $G\alpha_q(GTP[\gamma S])$ is 18 nM, which is 2- and 20-fold stronger than the solution value of the intact enzymes, respectively. However, association between $G\alpha_q$ subunits and intact PLC-β_1 occurs on membrane surfaces, and their effective concentrations are higher because they are concentrated on a quasi-two-dimensional surface. Thus, at the lipid concentrations used here (i.e., 150 μM), the intact PLC-β_1 and PLC-β_2 will bind ≈ 120-fold and 10-fold more strongly to $G\alpha_q(GTP[\gamma S])$ than their isolated C2 domains. Interestingly, on this same basis we find that the C2 domains bind to deactivated $G\alpha_q(GDP)$ with an ≈ 10-fold-higher affinity than their native host proteins.

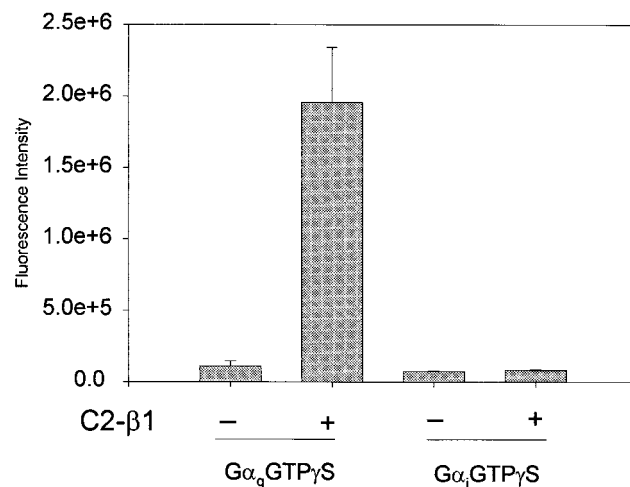FIG. 2. Normalized decrease in the fluorescence intensity of 2 nM deactivated C-$G\alpha_q(GDP)$ reconstituted on 120–150 μM POPC/POPS/POPE (1:1:1) membranes as DAB-C2-PLC-β1 (*A*, ●) or DAB-C2-PLC-β_2 (*B*, ●) is added.

FIG. 3. Interaction of C-$G\alpha_q(GTP[\gamma S])$ or C-$G\alpha_{i1}(GTP[\gamma S])$ with glutathione-Sepharose 4B resin (Amersham Pharmacia) in the presence or absence of bound GST-C2-PLC-β_1. The G protein subunits were mixed with resin and washed. The amount of G protein that adhered to the resin was assessed by fluorescence after release of the proteins by the addition of excess glutathione.
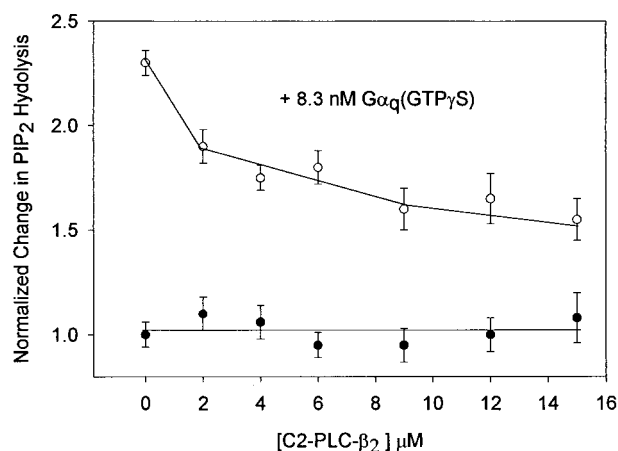
Fig. 4.   Reversal of the activation of PLC-β_1 (0.5 nM), as measured by [^{3}H]Ins(1,4,5)P_3 production, by 8 nM Gα_q(GTP[γS]) on POPC/POPS/POPE (1:1:1) vesicles with 2% PIns(4,5)P_2, where $n = 6$ (see ref. 12).

The C2 Domains of PLC-β_1 and PLC-β_2 Block Activation of PLC-β_1 by Gα_q(GTP[γS]). To determine whether the C2 domains of PLC-β_1 and PLC-β_2 are capable of competing with the native protein for activation by Gα_q(GTP[γS]), we measured the change in the level of PLC-β_1 activation by Gα_q(GTP[γS]) on POPC/POPE/POPS (1:1:1) vesicles containing 2% PIns(4,5)P_2 as C2 domains were added. The results in Fig. 4 show a systematic, but incomplete, reversal of PLC-β_1 activation. Although this concentration range is in accord with the expected range, the lack of complete reversal is most likely due to nonspecific activation or the inability of C2 to correctly place itself on the membrane surface to disrupt the complex, and/or is indicative of higher-order interactions between the proteins that have been implicated in previous studies (see ref. 7).

DISCUSSION

PLC-β isozymes are primarily membrane-resident proteins that are activated through lateral association to G protein subunits (1, 2, 16). Here, we present data showing that the C2 domains of PLC-β_1 and PLC-β_2 serve as docking sites for Gα_q subunits. The specificity of these interactions were confirmed by a number of controls that included unlabeled proteins, alternate G protein subunits, and PLC-δ C2 domains, all of which failed to bind. Competition between PLC-β_1 and its C2 domain by activity studies and fluorescence suggest that they both bind to the same site on Gα_q. Also, the affinity of the C2 domain of the PLC-βs for Gα_q is sensitive to the GTP/GDP activation state, which is similar to that of the intact protein.

Previous studies have suggested that other regions besides the C-terminal tail of PLC-βs must interact with Gα_q. Although loss of the C-terminal tail of PLC-β_1 abolishes activation by Gα_q (4–6), it reduces Gα_q affinity by only 25-fold (12, 13). Our results here imply that the residual affinity of the truncated protein is due to the C2 domain. This, coupled with the finding that the C-terminal tail accounts for 20% of the GAP activity (7), suggests that the C2 domain serves to mediate productive interactions between the Gα_q, the C-terminal tail, and the catalytic domain. The contribution of the

C-terminal tail relative to the C2 domains to Gα_q binding of the two PLC-β isozymes is indicated by the affinity of PLC-β_2, which is 10-fold-lower than that of PLC-β_1 (13), even though the Gα_q affinities of their C2 domains are identical (Table 1). Thus, the difference is expected to be due to the tail region or the disposition of the C2 domains to other regions of the protein.

Our data offer insight into the interactions that occur between Gα_q and the C2 domains of their PLC-β effectors. The absolute specificity for Gα_q subunits as compared with Gα_{i1} implicates specific interactions to nonhomologous regions of the α_q subunits, namely, residues 251–265 and 306–319. Peptides corresponding to these sequences have been shown to inhibit Gα_q activation of PLC-β_1 (16). Also, that deactivation of Gα_q reduces its C2 domain affinity only 4- to 10-fold (Table 1) indicates that the interaction site(s) involves other regions of Gα_q besides the switch region.

Here, we have found that in contrast to almost all other proteins containing this motif (see refs. 8 and 9), the C2 domains of PLC-β_1, PLC-β_2, and PLC-δ_1 do not bind with measurable affinities to membranes or inositol phosphates. Membrane binding of the C2 domains of PLC-β_1 and PLC-β_2 was not detectable under a variety of conditions. The C2 domain of PLC δ_1 bound only weakly to membranes containing anionic lipids or containing PIns(4,5)P_2. Because we have found previously that membrane binding of PLC-β_1 and PLC-β_2 is driven by the N-terminal PH domain (3), the membrane targeting function found for the C2 domains of other proteins is not required for the PLC-β isozymes. Thus, the function of C2 domains is not membrane tethering but rather to mediate Gα_q interactions between the C terminus and catalytic core, leading to allosteric activation.

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