Identification of a Region at the N-Terminus of Phospholipase C-β3 That Interacts with G Protein $\beta\gamma$ Subunits†

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ABSTRACT: Members of the phospholipase C-β (PLC-β) family of proteins are activated either by Gα or G$\beta\gamma$ subunits of heterotrimeric G proteins. To define specific regions of PLC-β3 that are involved in binding and activation by G$\beta\gamma$, a series of fragments of PLC-β3 as glutathione-*S*-transferase (GST) fusion proteins were produced. A fragment encompassing the N-terminal pleckstrin homology (PH) domain and downstream sequence (GST-N) bound to G protein $\beta_1\gamma_2$ in an in vitro binding assay, and binding was inhibited by G protein α subunit, Gα_{i1}. This PLC-β3 fragment also inhibited G$\beta\gamma$-stimulated PLC-β activity in a reconstitution system, while having no significant effect on Gαq-stimulated PLC-β3 activity. The N-terminal G$\beta\gamma$ binding region was delineated further to the first 180 amino acids, and the sequence Asn^{150}−Ser^{180}, just distal to the PH domain, was found to be required for the interaction. Mutation of basic residues ^{154}Arg, ^{155}Lys, ^{159}Lys, and ^{161}Lys to Glu within this region reduced G$\beta\gamma$ binding affinity and specifically reduced the EC_{50} for G$\beta\gamma$-dependent activation of the mutant enzyme 3-fold. Basal activity and Gαq-dependent activation of the enzyme were unaffected by the mutations. While these basic residues may not directly mediate the interaction with G$\beta\gamma$, the data provide evidence for an N-terminal G$\beta\gamma$ binding region of PLC-β3 that is involved in activation of the enzyme.

A wide range of hormones, neurotransmitters, and chemoattractants initiate cellular responses by binding to transmembrane receptors coupled to heterotrimeric ($\alpha\beta\gamma$) G proteins, which in turn regulate effector molecules, including phosphatidylinositol-specific phospholipase C (PLC)[1] (*1*−*3*). Either activated Gα or the G$\beta\gamma$ dimer can independently activate PLC, leading to the generation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol. While the α subunits of the Gq family G proteins mediate receptor activation of PLC that is insensitive to inhibition by pertussis toxin (PTX), it is G$\beta\gamma$ subunits liberated from the Gi family of G proteins that activate this enzyme in a PTX-sensitive manner (*4*, *5*). Mammalian phosphoinositide-specific PLCs have been grouped into three classes, β, γ, and δ, based on sequence homology with multiple isoforms in each class (*3*). Four isoforms of PLC-β (-β1, -β2, -β3, and -β4) have been described that are stimulated to varying extents by either Gαq or G$\beta\gamma$. PLC-γ is activated by tyrosine phosphorylation, and it is not clear how PLC-δ is regulated (*3*).

Comparison of the deduced amino acid sequence of different PLCs reveals a number of distinct structural features. At the amino terminus, all PLC isozymes contain an ∼120 amino acid protein module known as the pleckstrin homology (PH) domain (*6*), which has been identified in numerous signaling and cytoskeletal proteins (*7*−*9*). PH domains interact with various partners including G$\beta\gamma$ (*10*, *11*), phospholipids (*12*, *13*), protein kinase C (*14*), and both lipid and G$\beta\gamma$ coordinately (*15*). The general architecture of PLC-βs is likely to reflect the known three-dimensional crystal structure of a deletion variant of PLC-δ (lacking the N-terminal PH domain) (*16*). The PLC-δ structure suggests that, following the PH domain, PLC-βs contain four putative EF-hand motifs, commonly thought to be Ca^{2+} binding sites, and two highly conserved regions, the X and Y boxes, comprising the catalytic domain. The last region common to all PLCs is the C2 domain, which is a putative calcium-dependent/independent phospholipid-binding site. PLC-β isozymes have an additional C-terminal sequence of ∼400 amino acids that is not present in PLC-δ.

Identification of the PLC-β regions involved in activation by G proteins (either Gq class or G$\beta\gamma$) has been the subject of much investigation. The C-terminal region of PLC-β following the C2 domain has been identified as important for activation by Gαq, and this region has been further subdivided into sequences important for membrane associa-

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[1] Abbreviations: PLC, phosphatidylinositol-specific phospholipase C; G$\beta\gamma$, $\beta\gamma$ subunits of heterotrimeric G proteins; GST, glutathione-*S*-transferase; PH, Pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate, $C_{12}E_{10}$, polyoxyethylene 10 lauryl ether.

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tion (P-box) and Gαq activation (G-box) (17). In contrast, the Gβγ binding region of PLC-β2 has been localized to a sequence from the Y domain (Leu^{580}−Val^{641}) (*18*, *19*). Yan and Gautam (*20*) also reported that this region interacts with the N-terminus of Gβ in the yeast two-hybrid system. By use of synthetic peptides derived from the same region, the Gβγ binding domain was further localized to amino acids Glu^{574}−Lys^{583} of PLC-β2, which overlaps the originally defined region by four amino acids (*21*). The Gβγ binding region of PLC-β3 has not been delineated to date.

Members of this laboratory[2] have observed differential chemoattractant receptor regulation of PLC-β3 activity via Gβγ- versus Gαq-dependent mechanisms (*22*, *23*; reviewed in ref *24*). Elucidation of the mechanism of G protein regulation of this enzyme will facilitate understanding of this phenomenon. Thus we sought to identify the regions of the enzyme that interact with Gβγ by measuring $Gβ_1γ_2$ binding to fragments of PLC-β3 expressed as GST fusion proteins and by testing the ability of the fragments to inhibit G protein-dependent activation of PLC in a reconstitution system. In addition, point mutations of amino acids in an N-terminal Gβγ binding region were introduced in order to confirm involvement of this region in the interaction with Gβγ.

EXPERIMENTAL PROCEDURES

Construction of Mutant Phospholipase C-β3. His-tagged (6×) PLC-β3 in pFastBac vector was used as a template for generating the RKKK → EEEE mutant (i.e., mutation of PLC-β3 residues ^{154}Arg, ^{155}Lys, ^{159}Lys, and ^{161}Lys to Glu) (*21*). The mutations were generated by overlap-extension polymerase chain reaction using mismatched primers. The sequence of the forward and reverse primers used was 5′-aggagacctccagtc-3′ and 5′-tgtggtcatggtgaagc-3′, respectively. Internal overlapping mutagenic primers were 5′-tc cgt gta tgc ttc ttc cag gaa ggt gtt ccg-3′ and 5′-gaa gaa gca tac acg gag ctg gag ctg cag gtg aac cag g-3′ and the codons for glutamic acid instead of arginine or lysine are underlined. The PCR product was digested with *Bst*EII and *Bsu*36I and ligated into pFastBac-PLC-β3 to generate the construct. The presence of the mutations was verified by DNA sequencing and a recombinant baculovirus was generated according to the instructions of the Bac-to-Bac baculovirus system (Life Technologies).

Expression and Purification of Phospholipase C and G Proteins. His-tagged (6×) PLC-β2 and PLC-β3 (wild type and mutant) were purified from Sf9 cells as described previously (*21*, *25*). Recombinant myristoylated $α_{i1}$ was purified from *Escherichia coli* coexpressing $α_{i1}$ and *N*-myristoyltransferase according to published procedures (*26*). Gαq and $β_1γ_2$ subunits were purified from Sf9 cells as described previously (*27*, *28*).

Construction of GST Fusion Proteins. cDNAs corresponding to PLC-β3 fragments were amplified by the polymerase chain reaction (PCR) using human PLC-β3 as a template (PLC-β3 cDNA was provided by Dr. G. Weber, Karolinska Institutet). For the generation of the GST−N(RKKK → EEEE) mutant, the mutant PLC-β3 in pFastBac was used as a template. 5′-Primers used for PCR contained an *Eco*RI site, and 3′-primers contained a stop codon followed by an *Xho*I site. For generation of the fragment GST-C a 3′-primer was used containing a hexahistidine tag sequence after the last amino acid of PLC-β3, followed by a stop codon, and *Bgl*II and *Xho*I sites. Amplified cDNA fragments were ligated into the open reading frame of the GST gene fusion vector PGEX-4T2 (AmershamPharmacia Biotech). The constructs were sequenced in both directions on an automated sequencer by the Duke DNA Analysis Facility.

Expression and Purification of GST Proteins. For expression of fusion proteins, each fusion protein construct was transformed into the *E. coli* strain BLR (Novagen) and a single colony was selected. Overnight cultures grown in Luria broth containing ampicillin (100 μg/mL) were diluted to an A_{600} equal to 0.5−0.6 and induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside for 5 h at 22 °C in a shaker. The cells were pelleted by centrifugation, frozen, and stored at −80 °C. The cell pellet from a 1 L culture was resuspended in 20 mL of D-PBS (2.7 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, and 8.1 mM Na_2HPO_4, pH 7.1) containing 1 mg/mL lysozyme and a 1:500 dilution of protease inhibitory cocktail (Sigma) and incubated for 20 min at 4 °C. The suspension was lysed by mild sonication with a Branson probe sonicator and cell debris was removed by centrifugation at 20000*g* for 30 min. Fusion proteins were purified by the batch method with 500 μL of a 50% slurry of glutathione−Sepahrose 4B and the gel was washed with PBS containing 0.1% Triton X-100. Proteins were eluted with 10 mM reduced glutathione in Tris-HCl (pH 8.0). GST-C was then purified on nickel−NTA−agarose resin according to the manufacturer's instructions (Qiagen). Proteins were dialyzed against PBS (2 × 500 mL) at 4 °C overnight and concentrated in a Centricon-30 or -50 (Millipore). Protein concentrations were determined by the Bio-Rad protein assay and proteins were stored at −80 °C.

Detection of Gβγ Binding to GST Fusion Proteins in Vitro. In vitro Gβγ binding assays were carried out as in refs *10* and *29*. Purified GST fusion proteins (500 nM) were incubated with glutathione−Sepharose 4B (80 μL of a 50% slurry) in a total volume of 250 μL of D-PBS at 4 °C for 10 min with constant mixing. The pelleted beads were washed twice with 250 μL of D-PBS to remove free fusion protein. $Gβ_1γ_2$ (200 nM) was added to the beads in 250 μL of D-PBS containing 0.01% $C_{12}E_{10}$ and mixed on ice for 20 min. Beads were washed 3 times with 250 μL of D-PBS containing 0.01% $C_{12}E_{10}$ to remove nonspecifically bound protein and free Gβγ subunits. Gβγ bound to GST fusion proteins was detected after SDS−12% PAGE and western blotting with the Gβ antibody SW1 at a 1:5000 dilution (NEN). Blots were developed with an HRP-linked donkey anti-rabbit antibody at a 1:5000 dilution (AmershamPharmacia Biotech) coupled with detection by ECL+ (NEN). Competition experiments with recombinant myristoylated Gαi were carried out in the presence of GDP (5 μM). Laser densitometry was used to quantitate the relative amounts of bound Gβγ.

Phospholipase C Activity Assays. PLC activity assays were conducted as described previously using sonicated vesicles containing 200 μM phosphatidylethanolamine, 50 μM PIP_2, and 6000 cpm of [^{3}H]-PIP_2 per assay (*21*, *25*). Free Ca^{2+} was buffered at ∼100 nM and reactions were carried out for 30 min at 30 °C. PLC-β3 (5 ng) and 1 ng of PLC-β2, respectively, were used per assay. Curve fitting and calcula-

[2] A.J.B., H.A., B.H., and R.S.

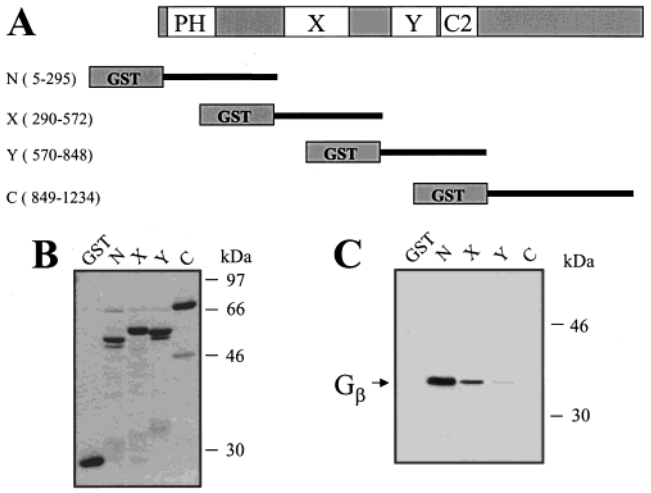

FIGURE 1: Analysis of G$\beta_1\gamma_2$ binding to GST fusion proteins from PLC-β3. (A) Domain structure of PLC-β3 (1234 amino acids), showing regions encompassed by the GST fusion proteins generated. (B) Coomassie blue-stained SDS−10% PAGE of GST and fragments of PLC-β3 expressed as GST fusion proteins (5 μg of protein/lane). (C) Western blot for Gβ in order to assess the G$\beta\gamma$ binding ability of the indicated GST fusion proteins. GST was used as a negative control. In vitro binding assays were carried out as described under Experimental Procedures. The data shown is representative of at least three separate experiments.

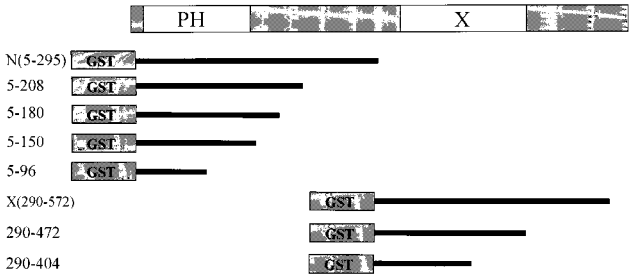

FIGURE 2: GST fusion proteins from the N-terminus and X-box of PLC-β3. Schematic representation of a further series of GST fusion proteins from the N-terminus and X-box of PLC-β3.

tion of IC_{50} values was carried out with GraphPad Prism (ISI Software).

RESULTS

G$\beta\gamma$ Binding of GST Fusion Proteins. To determine regions of PLC-β3 that interact with G protein $\beta\gamma$ subunits, fragments of PLC-β3 as glutathione-*S*-transferase (GST) fusion proteins were generated (Figure 1A). Figure 1B shows the purifed GST fusion proteins. The ability of the four GST fusion proteins to bind recombinant G$\beta_1\gamma_2$ was assessed by direct binding followed by precipitation with glutathione−Sepharose 4B and detection of Gβ by western blotting. GST alone was used to monitor nonspecific binding. Both GST-N and GST-X bound G$\beta_1\gamma_2$, although to different extents. Densitometric analysis indicated G$\beta\gamma$ binding to GST-X was ~13% of that observed with GST-N, while GST alone and GST-C did not bind. GST-Y bound G$\beta\gamma$, albeit at barely detectable levels (Figure 1C).

To define the minimal regions involved in binding G$\beta\gamma$, a series of truncated GST fusion proteins derived from GST-N and GST-X were constructed (Figure 2). Some of the purified fusion proteins from the N-terminus were proteolyzed or copurified with contaminating proteins that could not be removed (Figure 3A). Thus, the amount of purified protein used in subsequent binding experiments was adjusted visually to take account of both the contaminants and proteolytic products. Comparison of G$\beta_1\gamma_2$ binding to the series of N-terminal GST fusion proteins indicated that a fragment encompassing amino acids 5−180 of PLC-β3 retained a level of binding similar to that of GST-N. Truncation of a further 30 amino acids (GST-5−150) led to a marked loss of G$\beta\gamma$ binding. Similarly, GST-5−96 from PLC-β3 lacked G$\beta\gamma$ binding (Figure 3B).

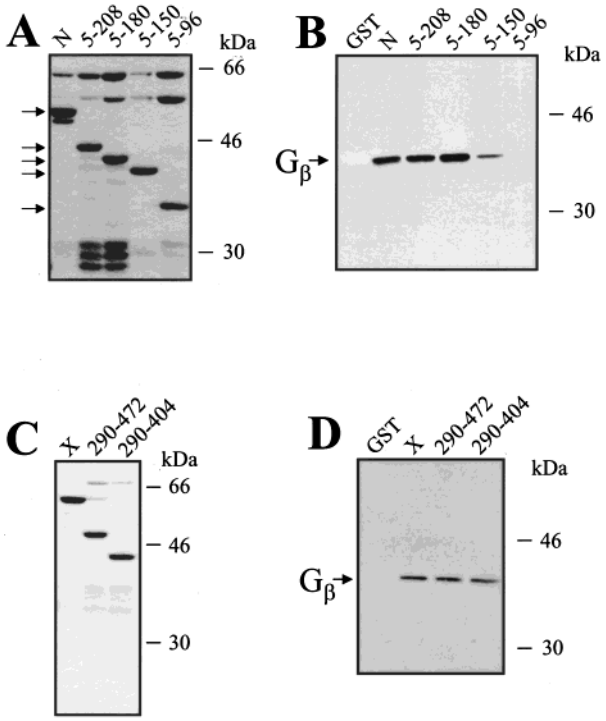

FIGURE 3: Analysis of G$\beta_1\gamma_2$ binding to GST fusion proteins from the N-terminus and X-box of PLC-β3. (A, C) Coomassie blue-stained SDS−10% PAGE of the GST fusion proteins indicated in Figure 2. N-Terminal fragments indicated by an arrow co-purified with contaminating proteins and were subject to proteolysis. The amount of protein used in G$\beta\gamma$ binding assays was adjusted to take account of this. (B, D) Western blots for Gβ in order to assess the G$\beta\gamma$ binding ability of the indicated GST fusion proteins. The data shown are representative of at least three separate experiments.

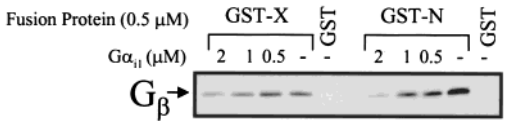

FIGURE 4: Effect of Gα_{i1} on G$\beta\gamma$ binding to GST fusion proteins. Western blot for Gβ showing effect of increasing concentrations of recombinant myristoylated Gα_{i1}·GDP on binding of G$\beta_1\gamma_2$ to GST-N and GST-X (0.5 μM). GST (0.5 μM) was used as a control. Gα_{i1} (0.5, 1, and 2 μM) was used in the presence of GDP (5 μM).

Fusion proteins GST-290−472 and GST-290−404 (Figures 2 and 3) derived from the X-box region were also tested for their ability to bind G$\beta_1\gamma_2$ in comparison with GST-X. All three proteins displayed similar levels of G$\beta_1\gamma_2$ binding, suggesting that the G$\beta\gamma$ binding region within the X-box is contained within the shortest fusion protein GST-290−404 (Figure 3D).

Competition of G$\beta\gamma$ Binding by Gα_{i1}. The effect of 1, 2, and 4 mol equiv of Gα_{i1} in the presence of GDP (Gα_{i1}·GDP) on G$\beta_1\gamma_2$ binding to GST-N and GST-X was examined

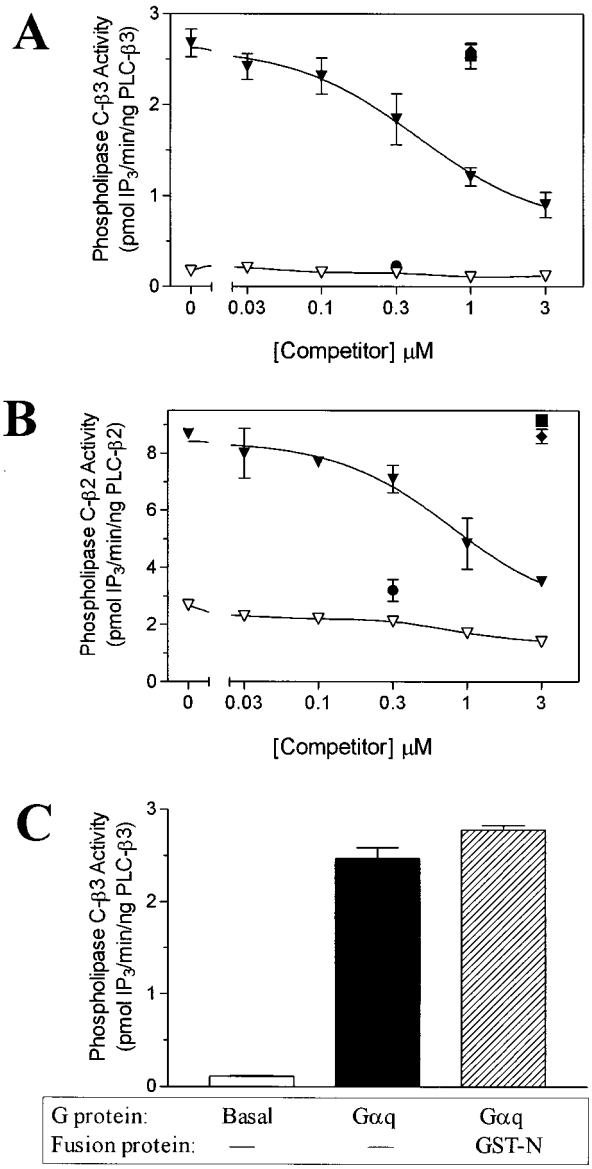

FIGURE 5: Effect of fusion proteins and $G\alpha_{i1}$ on $G\beta\gamma$-stimulated and Gαq-stimulated PLC activity. (A) PLC-β3 activity was measured in the absence (open symbols) or presence (solid symbols) of $G\beta_1\gamma_2$ (20 nM) for 30 min at 30 °C. The effect of the indicated concentration of competitors [GST-N (▼, ▽), GST-X (◆); GST-Y (■); $G\alpha_{i1}$ (●)] on the level of PLC activity in the presence or absence of $G\beta\gamma$ was examined. (B) PLC-β2 activity was measured in the absence or presence of $G\beta_1\gamma_2$ (20 nM). Symbols are as above. (C) PLC-β3 activity was measured in the absence or presence of Gαq (10 nM) together with AlF_4^- (10 mM NaF and 10 μM $AlCl_3$). The effect of GST-N (3 μM) on the Gαq-stimulated response was examined over a 30 min incubation at 30 °C. Each point represents duplicate determinations and the data are representative of three separate experiments.

(Figure 4). Binding of GST-N to $G\beta_1\gamma_2$ was attenuated by 1 mol equiv of $G\alpha_{i1}$·GDP (~66% inhibition with 1 mol equiv), while the same concentration did not reduce $G\beta\gamma$ binding to GST-X. Binding to GST-X was inhibited only weakly by higher concentrations of $G\alpha_{i1}$·GDP.

Effects of GST Fusion Proteins on PLC Activity. The fusion proteins were tested for their ability to inhibit either $G\beta\gamma$-stimulated PLC-β2 or -β3 activity (Figures 5A and 5B). A submaximal concentration of $G\beta\gamma$ (20 nM) stimulated PLC-β3 approximately 10-fold over the basal Ca^{2+}-dependent activity. Basal activity of PLC-β2 was significantly

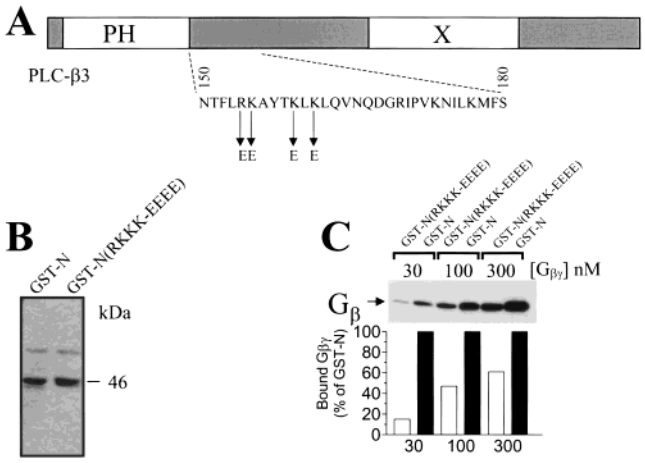

FIGURE 6: Mutation of basic residues in the GST-N fusion protein reduces $G\beta_1\gamma_2$ binding. (A) Schematic representation of the N-terminus of PLC-β3 showing the amino acid sequence of the $G\beta\gamma$ binding region (Asn^{150}−Ser^{180}) and the location of the four basic residues mutated to glutamic acid. (B) Coomassie blue-stained SDS−10% PAGE of the fusion proteins GST-N and GST-N(RKKK-EEEE). (C) Western blot for Gβ in order to compare the $G\beta\gamma$ binding ability of GST-N and GST-N(RKKK-EEEE) fusion proteins. In vitro binding assays were carried out as described under Experimental Procedures and the relative intensity of Gβ bands was quantitated by laser densitometry. Data [■, GST-N; □, GST-N(RKKK-EEEE)] are expressed as a percentage of that bound to GST-N at each concentration of $G\beta\gamma$.

higher, and $G\beta\gamma$ subunits gave an approximately 3-fold enhancement of activity. The addition of GST-N inhibited PLC-β3 activity with an IC_{50} of 0.5 μM and a maximal 76% inhibition at 3 μM. A similar potency and degree of inhibition was observed with GST-N in PLC-β2 activity assays. GST-X, GST-Y, and boiled GST-N had no effect at either 1 or 3 μM on PLC-β2 or -β3 activity. To determine whether the observed inhibition of $G\beta\gamma$-stimulated activity was due to a suppression of PLC catalytic activity rather than $G\beta\gamma$ stimulation, the effect of the GST-N fusion protein on basal Ca^{2+}-stimulated PLC activity was examined. GST-N had negligible effects on basal activity of PLC-β2 or -β3 (Figure 5). This was most clearly demonstrated in experiments with PLC-β2 where basal activity was higher (Figure 5B). Moreover, a submaximal dose of Gαq (10 nM) in the presence of AlF_4^- stimulated PLC-β3 activity 20-fold, and this response was unaffected by 3 μM GST-N (Figure 5C).

Effect of Point Mutations on Gβγ Binding and of PLC-β3 Activity. Experiments with GST fusion proteins indicated that the first 180 amino acids of PLC-β3 are involved in binding $G\beta\gamma$ and that amino acids 150−180 just distal to the PH domain are required for this interaction. This region shows some similarity with the $G\beta\gamma$ binding region of β-ARK, in which a series of basic residues have been shown by mutational analysis to be critical for $G\beta\gamma$ binding (*29*). Thus we mutated four corresponding basic residues (^{154}Arg, ^{155}Lys, ^{159}Lys, and ^{161}Lys) to glutamic acid and examined the effect on $G\beta\gamma$ binding and G protein-stimulated PLC activity.

A comparison of $G\beta\gamma$ binding to the wild-type GST-N and mutant fragment is shown in Figure 6. Binding to the mutant was reduced by between 40% and 85% over a range of $G\beta\gamma$ concentrations but was not completely abolished. In parallel with this result, the EC_{50} for $G\beta\gamma$ activation of the mutant PLC-β3(RKKK → EEEE) enzyme (EC_{50} = 36 nM)

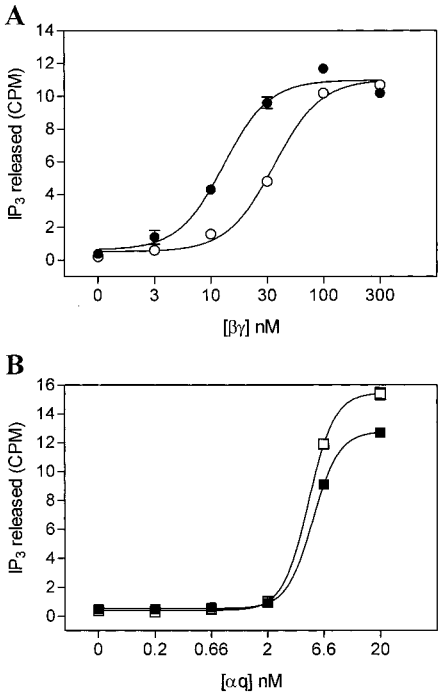

FIGURE 7: Effect of mutations in PLC-β3 on activation by either G$\beta\gamma$ or Gαq. Activation of the wild-type PLC-β3 (●) and mutant PLC-β3(RKKK-EEEE) (○) was measured over a range of concentrations of G$\beta_1\gamma_2$ for 30 min at 30 °C. (B) Same as in panel A except that activation of the wild-type PLC-β3 (■) and mutant PLC-β3(RKKK-EEEE) (□) was measured over a range of concentrations of Gαq in the presence of AlF_4^-. Each point represents the mean of duplicate determinations and the data are representative of three separate experiments (cpm $\times$ 10^{-2}).

was reduced by about 3-fold as compared with the wild-type PLC-β3 (EC_{50} = 13 nM), with no reduction in the maximal activity at saturating G$\beta\gamma$ concentrations (Figure 7A). The effect of the mutations was specific for G$\beta\gamma$-dependent activation since basal PLC activity with the wild-type and mutant enzymes were similar and Gαq-dependent activation of the wild-type and mutant enzymes was similar (Figure 7B).

DISCUSSION

Previously, we have observed that PLC-β3 activity is regulated by chemoattractant receptors and that this phospholipase may be a site for cross-regulation of receptors that activate PLC via G$\beta\gamma$ subunits (*22*−*24*). To begin to elucidate the mechanisms of PLC-β3 regulation, we sought to identify the regions of the enzyme that interact with G$\beta\gamma$. In the present study, a region at the N-terminus of PLC-β3 that is important for G$\beta\gamma$ binding and for activation of PLC-β3 by G$\beta\gamma$ subunits has been defined. An N-terminal fragment of PLC-β3 (GST-N), encompassing the PH domain and putative EF-hand motifs, bound G$\beta_1\gamma_2$ in vitro. Binding to the fragment was inhibited by $G\alpha_{i1}$·GDP implying that only free G$\beta\gamma$ can interact with this sequence from PLC-β3, and this is consistent with the fact that the heterotrimer (G$\alpha\beta\gamma$) cannot activate PLC. The GST-N fragment also inhibited G$\beta\gamma$-stimulated PLC-β2 and -β3 activity in in vitro assays but had no effect on basal or Gαq-stimulated PLC activity. The IC_{50} for this inhibition (0.5 μM) is 100-fold lower than the IC_{50} value reported for the QEHA peptide derived from adenylyl cyclase 2 (*30*) or peptides derived from the Y-box of PLC-β2 (*21*).

Binding of G$\beta\gamma$ to the GST-X fragment was also observed, although to a lower extent than GST-N, and the binding region was localized to amino acids 290−404. However, binding to this fragment was not effectively inhibited by $G\alpha_{i1}$·GDP, suggesting that the heterotrimer can interact with the GST-X fragment of PLC-β3. The Gα-binding surface of G$\beta\gamma$ may not be the only region of effector interaction; recently it has been demonstrated that the heterotrimer can interact with PLC-β2 (*31*). Since GST-X did not suppress G$\beta\gamma$ activation of PLC-β, it is unlikely that this interaction is relevant for enzyme activation by G$\beta\gamma$. GST fusion proteins from the Y-box and C-terminus of PLC-β3 did not bind to G$\beta\gamma$ in vitro or have significant effects in PLC activity assays, suggesting that G$\beta\gamma$ may not contact these regions of PLC-β3.

The sequences within the N-terminus of PLC-β3, which are important for binding G$\beta\gamma$, were further delineated by using a series of truncated GST fusion proteins derived from GST-N. This approach enabled the G$\beta\gamma$-binding site to be localized within the first 180 amino acids of PLC-β3. GST-5−180 bound G$\beta\gamma$; however, truncation of a further 30 amino acids (GST-5−150) led to a marked loss of G$\beta\gamma$ binding, indicating that amino acids 150−180, just distal to the PH domain (which extends to approximately amino acid 143), are required for the interaction. These experiments were complicated by the presence of proteolytic products, which were recognized by an antibody to GST, and by larger contaminating proteins in the GST fusion preparation, which were not recognized by the same antibody. An excess of glutathione beads was used in the G$\beta\gamma$ binding assay to provide sufficient binding capacity for the GST fusion proteins. These other proteins did not appear to influence G$\beta\gamma$ binding since the crude GST-5−180 binds G$\beta\gamma$ as well as GST-N, which is at least 95% pure. The G$\beta\gamma$ binding region defined here is similar to that reported in several other studies which demonstrate that C-terminally extended PH domains from various proteins interact with G$\beta\gamma$ in vitro (*10*, *32*, *33*). The physiological significance of some of these interactions remains to be established; however, the interaction of the extended PH domain from β-adrenergic receptor kinase 1 (β-ARK-1) with G$\beta\gamma$ has been characterized in vitro and in vivo (*29*, *34*). The region of β-ARK-1 responsible for binding G$\beta\gamma$ is not identical to the PH domain but encompasses the C-terminal portion of the PH domain plus adjacent downstream sequence, and basic residues within the adjacent α-helical sequence are critical for G$\beta\gamma$ binding.

The defined N-terminal region does not contain the putative G$\beta\gamma$ binding motif, QXXER, described by Chen et al. (*30*). It does, however, show some similarity with the G$\beta\gamma$ binding regions of β-ARK and PLC-β2 with the presence of a series of basic residues (Figure 6A) (*29*). Thus we focused on ^{154}Arg, ^{155}Lys, ^{159}Lys, and ^{161}Lys as potentially key residues involved in binding G$\beta\gamma$. Binding of G$\beta\gamma$ to the mutant GST-N with this series of basic residues mutated to glutamic acid was reduced and the EC_{50} for G$\beta\gamma$-dependent activation of the enzyme was decreased 3-fold (Figure 6 and 7). This partial attenuation of G$\beta\gamma$ binding caused by the mutations contrasts with a complete loss of binding following mutation of corresponding residues in the G$\beta\gamma$ binding region of β-ARK (29). Since the mutations did not completely abolish G$\beta\gamma$ binding to this N-terminal region of PLC-β3, the basic residues may not directly mediate the

interaction in this region. Nonetheless, the 3-fold reduction in EC_{50} for the mutant enzyme is consistent with the conclusion that Gβγ interaction with this region is important for PLC-β3 activation. Since the mutations did not alter basal PLC activity or the Gαq-dependent activation of the enzyme the effect was specific for Gβγ-dependent activation. These data and the observation that Gβγ-dependent activation at saturation is not effected by the mutations suggest that the mutations did not have effects on catalysis directly and that the observed effect was not due to destabilization of the enzyme structure. Further studies will have to be performed to determine exactly which amino acids directly contribute to the binding site for Gβγ subunits.

Although there is evidence for subtle differences in residues of Gβγ involved in activation of PLC-β2 and PLC-β3 (*35*−*37*), it seems likely, considering the high homology between PLC-β1, PLC-β2, and PLC-β3, that similar regions of each isozyme are involved in binding and activation by Gβγ. In support of this hypothesis, it was recently demonstrated that Gβγ subunits bind to the extended PH domains from PLC-β1 and PLC-β2 (*38*). Moreover, the data demonstrating that both PLC-β2 and -β3 activity were inhibited by GST-N is consistent with this model, suggesting that both effectors share an interaction surface on Gβγ. Previously, a region in the Y-box of PLC-β2 was defined as a Gβγ binding site; therefore, Gβγ may interact with both the N-terminal region (*38*) and the catalytic domain of PLC-β2 (*18*−*21*). Similarly, multiple interactions may be involved in Gβγ activation of PLC-β3. The GST-Y fragment from PLC-β3 used in this study encompasses the Gβγ binding region from PLC-β2; however, binding of Gβγ to GST-Y was barely detectable and GST-Y did not have any inhibitory effects on PLC activity. It is possible that the GST portion of the fusion protein occludes the Gβγ binding site in this fragment of PLC-β3, or alternatively this site of interaction may be unique to PLC-β2.

The data presented provide evidence for a Gβγ binding region at the N-terminus of PLC-β3, and amino acids 150−180 just distal to the PH domain were found to be required for the interaction. Mutation of a series of basic residues in this region reduced the affinity of the interaction and specifically, albeit modestly, reduced the EC_{50} for Gβγ-dependent activation of the mutated enzyme. Thus, while these basic residues may not directly mediate the interaction with Gβγ, the data provide evidence for an N-terminal Gβγ binding region of PLC-β3 that is involved in activation of the enzyme. Since Gβγ regulation of the widely expressed PLC-β3 is likely to be a mechanism for pertussis toxin-sensitive receptor-mediated signaling for many receptors, such as the chemoattractant receptors (*4*, *5*, *24*), these results have immediate impact on understanding the mechanisms involved in this process. The identification of the Gβγ binding domain of PLC-β3 will be useful in further delineation of the regulation of this important enzyme in receptor signaling.

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REFERENCES

1. Exton, J. H. (1996) *Annu. Rev. Pharmacol. Toxicol. 36*, 481−509.
2. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem. 66*, 475−509.
3. Rhee, S. G., and Bae, Y. S. (1997) *J. Biol. Chem. 272* (24), 15045−8.
4. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) *Nature 360* (6405), 684−6.
5. Katz, A., Wu, D., and Simon, M. I. (1992) *Nature 360* (6405), 686−9.
6. Parker, P. J., Hemmings, B. A., and Gierschik, P. (1994) *Trends Biochem. Sci. 19* (2), 54−5.
7. Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D. (1993) *Cell 73* (4), 629−30.
8. Gibson, T. J., Hyvonen, M., Musacchio, A., Saraste, M., and Birney, E. (1994) *Trends Biochem Sci. 19* (9), 349−53.
9. Lemmon, M. A., Falasca, M., Ferguson, K. M., and Schlessinger, J. (1997) *Trends Cell Biol. 7*, 237−242.
10. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) *J. Biol. Chem. 269* (14), 10217−20.
11. Wang, D. S., Shaw, R., Winkelmann, J. C., and Shaw, G. (1994) *Biochem. Biophys. Res. Commun. 203* (1), 29−35.
12. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature 371* (6493), 168−70.
13. Rameh, L. E., Arvidsson, A., Carraway, K. L., 3rd, Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D. S., Chen, C. S., and Cantley, L. C. (1997) *J. Biol. Chem. 272* (35), 22059−66.
14. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W. B., Kawakami, Y., and Kawakami, T. (1997) *J. Biol. Chem. 272* (20), 13033−9.
15. Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995) *J. Biol. Chem. 270* (20), 11707−10.
16. Essen, L. O., Perisic, O., Cheung, R., Katan, M., and Williams, R. L. (1996) *Nature 380* (6575), 595−602.
17. Kim, C. G., Park, D., and Rhee, S. G. (1996) *J. Biol. Chem. 271* (35), 21187−92.
18. Wu, D., Katz, A., and Simon, M. I. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90* (11), 5297−301.
19. Kuang, Y., Wu, Y., Smrcka, A., Jiang, H., and Wu, D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93* (7), 2964−8.
20. Yan, K., and Gautam, N. (1997) *J. Biol. Chem. 272* (4), 2056−9.
21. Sankaran, B., Osterhout, J., Wu, D. Q., and Smrcka, A. V. (1998) *J. Biol. Chem. 273* (12), 7148−7154.
22. Ali, H., Fisher, I., Haribabu, B., Richardson, R. M., and Snyderman, R. (1997) *J. Biol. Chem. 272* (18), 11706−9.
23. Ali, H., Sozzani, S., Fisher, I., Barr, A. J., Richardson, R. M., Haribabu, B., and Snyderman, R. (1998) *J. Biol. Chem. 273* (18), 11012−6.
24. Ali, H., Richardson, R. M., Haribabu, B., and Snyderman, R. (1999) *J. Biol. Chem. 274* (10), 6027−6030.
25. Romoser, V., Ball, R., and Smrcka, A. V. (1996) *J. Biol. Chem. 271* (41), 25071−8.
26. Mumby, S. M., and Linder, M. E. (1994) *Methods Enzymol. 237*, 254−68.
27. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) *J. Biol. Chem. 268* (19), 14367−75.
28. Kozasa, T., and Gilman, A. G. (1995) *J. Biol. Chem. 270* (4), 1734−41.
29. Touhara, K., Koch, W. J., Hawes, B. E., and Lefkowitz, R. J. (1995) *J. Biol. Chem. 270* (28), 17000−5.
30. Chen, J., DeVivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J., Logothetis, D. E., Hildebrandt, J. D., and Iyenagar, R. (1995) *Science 268* (5214), 1166−9.
31. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry 37* (44), 15563−15574.
32. Mahadevan, D., Thanki, N., Singh, J., McPhie, P., Zangrilli, D., Wang, L. M., Guerrero, C., LeVine, H., 3rd, Humblet, C.,

Saldanha, J., Gutkind, J. S., and Najmabadi-Haske, T. (1995) *Biochemistry 34* (28), 9111−7.
33. Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91* (23), 11256−60.
34. Koch, W. J., Inglese, J., Stone, W. C., and Lefkowitz, R. J. (1993) *J. Biol. Chem. 268* (11), 8256−60.
35. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998) *J. Biol. Chem. 273* (43), 28298−28304.
36. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., and Hamm, H. E. (1998) *Science 280* (5367), 1271−4.
37. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) *J. Biol. Chem. 273* (26), 16265−72.
38. Wang, T. L., Pentyala, S., Rebecchi, M. J., and Scarlata, S. (1999) *Biochemistry 38* (5), 1517−1524.

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