THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 6, Issue of February 8, pp. 4294–4300, 2002

Printed in U.S.A.

Mutations in the Carboxyl-terminal Domain of Phospholipase C-β1 Delineate the Dimer Interface and a Potential $G\alpha_q$ Interaction Site*

Received for publication, October 4, 2001, and in revised form, November 15, 2001
Published, JBC Papers in Press, November 29, 2001, DOI 10.1074/jbc.M109612200

Olga Ilkaeva, Lisa N. Kinch, Ruth H. Paulssen‡, and Elliott M. Ross§

From the Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041

The carboxyl-terminal domain of phospholipase C-β is required for its stimulation by $G\alpha_q$ and for its $G\alpha_q$-specific GTPase-activating protein (GAP) activity. We subjected this domain to a combination of deletion and alanine/glycine scanning mutagenesis to detect mutations that would inhibit either responsiveness to G_q or G_q GAP activity. Most mutations that altered either response or GAP activity diminished both in parallel. Many of these mutations map at the interface at which the carboxyl-terminal domain was recently shown to form a dimer (Singer, A. U., *et al.* (2001) *Nat. Struct. Biol.*, 9, 32–36). Most others clustered in an area that is a plausible $G\alpha_q$ binding site. In addition, one mutation that differentially inhibited GAP activity relative to responsiveness to $G\alpha_q$ mapped in this region at a location modeled to be in close contact with the switch II region of $G\alpha_q$. This is the site at which RGS proteins are thought to exert their GAP activity. Last, a deletion mutation differentially inhibited the response of phospholipase C-β1 to $G\alpha_q$ without blocking GAP activity. Its location in the molecule suggests that moving the attachment point of the catalytic domain can disrupt its ability to be activated by $G\alpha_q$.

Phospholipase C (PLC)[1]-βs are metazoan PIP_2-selective PLCs whose activity is stimulated by heterotrimeric G proteins. They are structurally distinguished from the G protein-unresponsive PLCs γ, δ, and ε by a distinctive sequence amino-terminal to the catalytic domain and by a ~40-kDa carboxyl-terminal extension (1–3). PLC-β enzymes are stimulated both by GTP-bound Gα subunits of the G_q family ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14/15}$, and $G\alpha_{16}$) and by Gβγ subunits, although relative sensitivity to $G\alpha_q$ and Gβγ varies among the four mammalian members of the PLC-β family (2, 3). Stimulation of phospholipase activity by G proteins can exceed 100-fold *in vitro*, although both activity and relative stimulation are strongly influenced by the lipid interface at which the PIP_2 substrate is found (2).

In addition to their role as G_q-regulated effector enzymes, PLC-βs are also GTPase-activating proteins (GAPs) specific for members of the $G\alpha_q$ family (4). PLC-β1 can increase the rate of hydrolysis of GTP bound to G_q over 1000-fold (5). The GAP activity of PLC-βs is thought to provide enhanced temporal responsiveness to signaling processes mediated by G_q and PLC-β and to minimize spontaneous activation (noise) in these pathways (6, 7).

The carboxyl-terminal domain of PLC-β, excluding the last ~20 amino acid residues, is required for the enzyme to respond to activated $G\alpha_q$ (8, 9). Fragments of this domain, some as small as 15 kDa, display G_q GAP activity independent of the PLC-β catalytic and amino-terminal domains (10), and several mutations in this region inhibit response to $G\alpha_q$ (11). It is thus likely that the carboxyl-terminal region of PLC-β constitutes all or part of the $G\alpha_q$ binding site. This idea is supported by the observation that the carboxyl-terminal domain can inhibit stimulation of PLC-β by G_q either using purified proteins in solution (10) or in cells (12), presumably by binding and sequestering $G\alpha_q$. In addition to displaying intrinsic GAP activity, the tails can potentiate and inhibit the GAP activity of PLC-β1 in a biphasic process and display biphasic stimulation/inhibition by themselves (10). The complex and biphasic regulatory behavior of the tails, both alone and in combination with full-length PLC-β1, led us to suggest that the tails can oligomerize both with themselves and with full-length PLC-β1. The idea that PLC-β may function as a dimer also agrees with the concentration-dependent activity described for PLC-β2 (13) and with the observation of fluorescence resonance energy transfer between differently fluorophore-labeled PLC-β2 molecules.[2]

The structure of the carboxyl-terminal tail of turkey PLC-β2 was recently solved at the atomic level by Singer *et al.* (14). The tail is a long, three-stranded coiled coil. Two tails bind to form an antiparallel dimer with its interface along the long axis of the coiled coils. The validity of the dimeric structure of the tails found in the protein crystal is supported by the functional data cited above.

Prior to the solution of the tertiary structure of the PLC-β tail, we attempted to locate sites in the carboxyl-terminal region of PLC-β1 that, when mutated, would block its G_q GAP activity. We report here the location of one mutation that selectively inhibits GAP activity relative to responsiveness to stimulation by G_q and another that inhibits responsiveness with minimal loss of GAP activity. Most inactivating mutations, however, diminish both effects in parallel. Many of these mutations lie at the dimer interface. Others, along with the mutation that disproportionately inhibits GAP activity, point to a likely site of interaction with $G\alpha_q$.

* Supported by National Institutes of Health Grant GM30355 and R. A. Welch Foundation Grant I-0982 (to E. M. R.) and by a grant from the Norwegian Research Council for Science and the Humanities (to R. H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Institute of Clinical Medicine, University of Tromsø, Tromsø, N-9037 Norway.

§ To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041. Tel.: 214-648-8717; Fax: 214-648-2994; E-mail: ross@utsw.swmed.edu.

[1] The abbreviations used are: PLC, phospholipase C; GTPγS, guanosine 5′-*O*-(thiotriphosphate); GAP, GTPase-activating protein; PIP_2, phosphatidylinositol 4,5-bisphosphate.

[2] L. Runnels and S. Scarlata, personal communication.

EXPERIMENTAL PROCEDURES

Mutagenesis of the cDNA that encodes the carboxyl-terminal domain of rat PLC-β1 was performed on a 1130-bp *Ava*I cDNA fragment in pUC19. Most mutagenesis was performed using PCR, usually by the QuikChange protocol (Stratagene). After the sequences of the mutants were verified, the *Ava*I fragment was returned to the full-length cDNA in bacterial expression vector pQE60 (Qiagen), which was modified to encode a His_6 tag and include a *Not*I site in the multiple cloning site. For expression in Sf9 cells, the cDNA was transferred as a *Not*I-*Bam*HI fragment to pVL1392 (15), and recombinant baculoviruses were prepared as described previously (16). The deletion mutant Δ1123–38 was prepared in the Tail32 construct (10), and that fragment was inserted into the full-length cDNA in pVL1392.

Some PLC-β1 constructs were expressed in Sf9 cells and purified as described previously (17). $G\alpha_q$, $G\beta_1\gamma_2$, and m1 muscarinic acetylcholine receptor were expressed in Sf9 cells and purified as described previously (17). Phospholipid vesicles that contain G_q and muscarinic 1 acetylcholine receptor were reconstituted as described previously (17).

PLC-β1 was routinely expressed in *Escherichia coli* SG13009/pREP4 as described previously (18). For evaluation of the activities of mutant PLC-β1s, the protein was partially purified by one-step affinity chromatography on Ni^{2+}-NTA-agarose (Qiagen). The high-salt membrane extract from 1 liter of cells (18) was mixed with 0.5 ml of Ni^{2+}-NTA-agarose (Qiagen) for 1 h at 4 °C. The slurry was poured into a column and washed with 5 ml of buffer Y (20 mM Tris-Cl (pH 7.5), 1 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 10% glycerol, and 10 mM 2-mercaptoethanol), 5 ml of 0.3 M NaCl in buffer Y, and 10 ml of buffer Y plus 0.3 M NaCl and 10 mM imidazole. PLC-β1 was eluted in 1 ml of 0.1 M NaCl plus 150 mM imidazole in buffer Y.

PLC-β1 that has been partially purified from *E. coli* is only 2–4% pure. To normalize multiple preparations to a fixed concentration of active enzyme, we assayed PLC activity in the presence of 5 μM Ca^{2+}, as described below. The specific activity of rat PLC-β1 in this assay is identical for highly purified preparations of enzyme from Sf9 cells or *E. coli* (18), and total activity in an assay is proportional to the amount of enzyme added over the range of concentrations used here (data not shown). To determine the concentration of active PLC-β1, we divided its activity by the specific activity of the purified enzyme. This concentration was consistent with the total amount of immunoreactive PLC-β1 determined by Western blotting.

PLC activity was determined by the method of Blank *et al.* (19), which measures hydrolysis of $[^3H]PIP_2$ added as mixed phospholipid vesicles. Reactions were initiated by the addition of enzyme, and the reaction was allowed to proceed at 30 °C for 10 min. Ca^{2+}-stimulated activity was determined in the presence of 5 μM Ca^{2+}, which was maintained with an EGTA buffer. $G\alpha_q$-stimulated activity was measured in the presence of both 0.2 μM Ca^{2+} and $G\alpha_q$ that had been previously been activated by incubation with GTPγS (17). Routine $G\alpha_q$-stimulated assays used for screening mutants contained 2 nM $G\alpha_q$, which is approximately equal to the EC_{50} (median effective concentration) (20).

G_q GAP activity was determined according to the increase in receptor-stimulated steady-state GTPase activity in phospholipid vesicles that contained trimeric $G\alpha_q\beta_1\gamma_2$ and muscarinic 1 acetylcholine receptor. Assays were performed as described previously (4, 21).

The structural model of the carboxyl-terminal domain of rat PLC-β1 was based on the atomic coordinates of the amino-terminal domain of turkey PLC-β2 (14). This construct contains a designed deletion between residues 960 and 1001 (PLC-β1 residue numbers). The corresponding amino acid residues display 33% sequence identity and 52% similarity, with a 2-residue deletion and a 1-residue insertion (both in loops). The insertion, residues 947–950 in PLC-β1, is in a region that is poorly conserved among the PLC-βs. The deletion, 2 residues between PLC-β1 residues 1084 and 1085, is characteristic of PLC-β1; the sequence in this region is distinctive for each of the four PLC-β isoforms. Residues in the following groups were counted as similar: (A, S, T, C), (Y, F, I, L, V, M), (K, R), (E, D, Q, N), and (H, Y, F). To create the model, the turkey PLC-β2 side chains were replaced with the corresponding rat side chains using the Biopolymer module of the Insight II graphics package. Overlapping side chains were adjusted by manual rotamer, and energy was minimized using the Discover module of the same graphics package. Structural images of the resulting model were produced with the program Bobscript (22).

TABLE I

Deletion and replacement mutations of PLC-β1

Mutations are identified by the first and last residues mutated. A and N denote alanine/glycine substitutions, and R denotes deletions. Each mutant protein was purified and assayed for its response to GTPγS-activated $G\alpha_q$ and its G_q GAP activity. Regulatory behaviors are tabulated as +, wild type; c, response to activated $G\alpha_q$ decreased only at low concentrations of the mutant PLC-β1; m, minor decrease; −, significant decrease.

Mutation	Begin	End	$G\alpha_q$ response	G_q GAP activity	Group
A1	1018	1028	+	+	I
A2	1043	1052	−	−	III
A3	1057	1064	−	−	III
A4	1081	1082	+	+	I
A5	1092	1097	+	+	I
A6	1101	1109	−	−	III
A7	904	916	m	+	II
A8	917	927	−	−	III
A9	928	943	−	−	III
A10	944	957	m	+	II
A11	965	975	+	+	I
A12	976	990	+	+	I
A13	998	1007	+	+	I
R14	1007	1012	c	+	II
R15	1062	1070	−	−	III
R16	993	998	c	+	II
N17	1076	1078	+	+	I
N18	1085	1091	+	+	I
R19	958	964	c	+	II

RESULTS AND DISCUSSION

Mutations in the Carboxyl-terminal GAP Domain—To delineate sites in PLC-β1 that are involved in regulatory interaction with $G\alpha_q$, we initially mutated stretches of 6–15 amino acid residues in the carboxyl-terminal domain. Mutations were confined to residues 903–1143, which corresponds to the fragment previously referred to as Tail 32 (10). This region is required for stimulation by G_q (8). Relatively small fragments of this domain, 15–33 kDa in size, also display intrinsic G_q GAP activity and inhibit stimulation of intact PLC-β1 by $G\alpha_q$ (10). The region tested here excludes carboxyl-terminal residues 1145–1216, which are not required for interaction with $G\alpha_q$ (8) (confirmed in this study). Bovine, rat, and human PLC-β1s differ at only 7 amino acid residues in this region, and all differences but one are highly conservative. Each mutant PLC-β1 protein was purified and assayed both for sensitivity of its phospholipase activity to stimulation by G_q and for G_q GAP activity. All mutant proteins displayed about the same specific activity of Ca^{2+}-stimulated phospholipase activity according to the ratio of measured activity to the amount of PLC-β1 protein determined by Western blotting and were similar to wild type PLC-β1 according to total expression and relative purification.

The initial multisite mutations produced three general phenotypes (Table I). Group I, mutations A1, A4, A5, A11, A12, A13, N17, and N18, displayed no obvious loss of either GAP activity or sensitivity to stimulation by GTPγS-activated $G\alpha_q$. Group II, deletion mutations R14, R16, and R19, also displayed normal G_q GAP activity. Their sensitivity to stimulation by activated $G\alpha_q$ was also normal, but only when the concentration of the mutant PLC-β1 was >3 nM in the assay medium. At lower concentrations, their sensitivities to stimulation by $G\alpha_q$ were reduced up to 60%. Because the effect of these mutations is relatively minor and is detected only at low PLC-β1 concentrations, it is unlikely that they alter interaction with $G\alpha_q$ directly. We consider them to be without phenotype in this sense. Their subtle phenotype may reflect a decrease in stability of the PLC-β1 dimer (see below), but we have not characterized them further. Mutants A7 and A10 behaved much like others in Group II, but displayed a somewhat greater loss of responsiveness to $G\alpha_q$ (~80%). We did not determine whether

TABLE II
Carboxyl-terminal uncoupling mutations in PLC-β1

Residues in regions of the group III mutants (Table I) were mutagenized in smaller groups and evaluated as described in Table I. Those residues whose mutations impaired coupling are listed in the second column. This column includes three lysine residues that were implicated in responsiveness to Gα$_q$ by the studies of Kim *et al.* (11). For grouped amino acid residues, the number given is that of the first residue.

Region	Altered coupling	Not tested
A2	ECQ1048	T 1043 VAE 1045
A3/R15	K1058 K1063 K1069	I 1060 L1068
A6	SY 1101	I 1103 VV 1106
A8/A9	K921 Q923 KHY 925	SFV 918 L 922 MK 930 LVKR 933 KKTT 939

response to Gα$_q$ was greater at high concentrations of the mutant proteins.

Group III mutants displayed varied but significant loss of both responsiveness to Gα$_q$ and G$_q$ GAP activity. The group III mutants were mutations A2, A3, A6, A8, A9, and R15 (Table I). To further delimit the amino acid residues that contribute to interaction with Gα$_q$, we then mutated subsets of these regions. We concentrated on residues that are conserved among the four mammalian PLC-β isoforms because all four are sensitive to stimulation by Gα$_q$, and all are G$_q$ GAPs (3, 23–25).[3] The second round of mutants helped localize regions important for regulatory interactions between PLC-β1 and Gα$_q$, as shown in Table II. This initial screen also indicated that at least 61% (147 of 241) of the amino acid residues in the Tail 3 region of PLC-β1 may be either deleted or mutated to alanine or glycine without significant loss of regulatory interactions with Gα$_q$.

Uncoupling Mutations in PLC-β1—The significantly uncoupled mutants displayed defects in both their responses to stimulation by activated Gα$_q$ and their G$_q$ GAP activities, but specific patterns of behavior varied (Table III). As shown in Fig. 1*A,* all of the class III mutants displayed decreased responsiveness to activated Gα$_q$ in terms of apparent affinity. The EC$_{50}$ for Gα$_q$ was increased by about 10-fold for each mutant. For several mutants (A3 and A1101 for example), the increased EC$_{50}$ was the only significant cause of decreased response. Others also displayed a decreased maximal response to Gα$_q$, and the amounts of protein used in the assays shown in Fig. 1*A* were varied over a 12.5-fold range to yield comparable maximal activities. The loss of response to G$_q$ was most pronounced for mutant A923 (residues Gln^{923}, His^{926}, Tyr^{927}) in which the decrease in maximally stimulated activity approached 90%. Mutant A1048 also displayed a significant loss of maximal response. All mutants in class III that displayed a decreased maximally stimulated phospholipase activity displayed the same loss in apparent affinity for activated Gα$_q$.

When the mutant PLC-β1 proteins were assayed for G$_q$ GAP activity in the steady-state GTPase assay, all the class III alanine/glycine replacement mutants displayed decreased maximal G$_q$ GAP activity. The decrease ranged from about 50%, for mutant A923 to 85% for mutant A3. However, concentration dependence for GAP activity was essentially unchanged for all these mutants (Fig. 1*B*; Table III). This pattern contrasts

[3] S. Mukhopadhyay, G. H. Biddlecome, and E. M. Ross, unpublished data.

TABLE III
Regulatory properties of PLC-β1 mutants

Mutant PLC-β1s were assayed for phospholipase activity in the presence either of 5 μM Ca^{2+} or 10 nM GTPγS-activated Gα$_q$. Stimulation by Gα$_q$ is expressed as the ratio of that activity to activity measured in the presence of Ca^{2+}. The Ca^{2+}-stimulated activities varied from 0.11 pmol/min/ng to 0.14 pmol/min/ng. G$_q$ GAP activity was measured in the steady-state, receptor-coupled assay over a range of PLC concentrations. Maximal activity and EC$_{50}$ are both shown. Data shown are taken from at least three experiments with duplicate determinations in each assay. The error parameter is S.D.

	Response to Gα$_q$-GTPγS	GAP EC$_{50}$	GAP V_{max}
	(fold vs. Ca^{2+})	*(nM)*	*(pmol/min)*
Wild type	2.96 ± 0.45	1.3 ± 0.2	3.1 ± 0.3
A923	0.12 ± 0.2	2.4 ± 0.5	1.5 ± 0.2
A3	0.68 ± 0.09	2.0 ± 0.4	0.49 ± 0.08
A9	0.80 ± 0.14	2.4 ± 0.5	1.05 ± 0.16
A1101	1.48 ± 0.32	1.4 ± 0.3	1.4 ± 0.25
A1048	1.27 ± 0.19	2.3 ± 0.5	0.92 ± 0.13

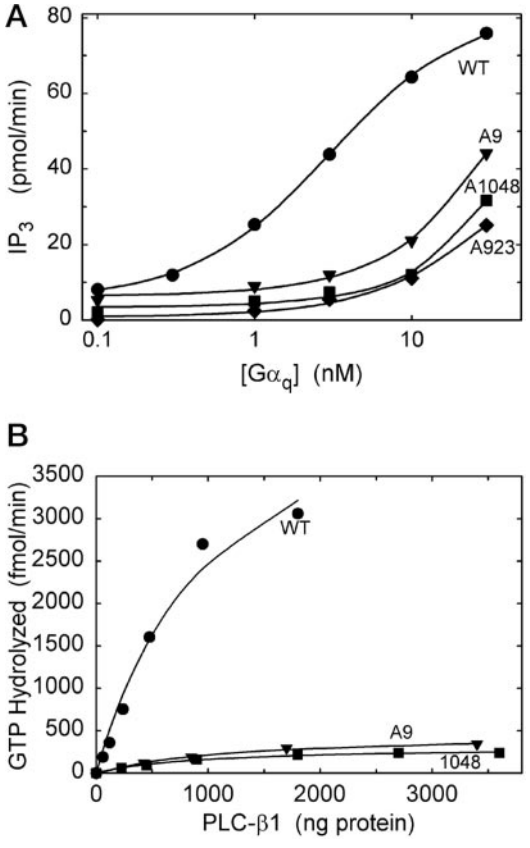

FIG. 1. **Interaction of PLC-β1 mutants with Gα$_q$.** Mutants of PLC-β1 described in Table I were expressed in *E. coli* and partially purified as described under "Experimental Procedures." *A*, phospholipase activity was measured under standard in the presence of increasing concentrations of GTPγS-activated Gα$_q$. Amounts of the different PLC-β1 mutants were chosen to yield easily measurable activities: wild type, 120 ng (●); A923, 1500 ng (♦); A9, 200 ng (▼); and A1048, 450 ng (■). *B,* G$_q$ steady-state GAP activities were measured in the presence of increasing concentrations of wild type PLC-β1 (●) and mutants A9 (▼) and 1048 (■).

with that observed for responses to Gα$_q$, where the concentration of activated Gα$_q$ needed to increase phospholipase activity was increased, but often without significant loss of the maximum response. The behavior of the mutants in the two assays thus seems to differentiate between interactions needed to exert GAP activity and those needed to respond to activated Gα$_q$. However, GAP activity in the steady-state GTPase assay depends on a relatively stable interaction among receptor, G$_q$,

and GAP over the time course of multiple GTPase cycles (5, 7, 17). Consequently, a decrease in maximal GAP activity in this assay may actually represent a decrease in affinity of the mutant PLC-β1 for Gα$_q$. The simplest interpretation of these results is therefore that the alanine/glycine substitutions in the PLC-β1 carboxyl-terminal region decrease affinity for Gα$_q$ without altering the GAP activity of the PLC-β1 protein when it is bound to its Gα$_q$ target.

Uncoupling Mutations Map to the Homodimer Interface or to a Candidate Gα$_q$ Binding Site—Singer *et al.* (14) recently determined the tertiary structure of a fragment of the carboxyl-terminal domain of turkey PLC-β2 that corresponds to residues 904–1174 of mammalian PLC-β1. To crystallize this fragment, they also deleted a region that is not well conserved among PLC-β isoforms, residues 961–1000 in the rat enzyme. Their tail construct contained 231 amino acid residues that are 33% identical and 60% similar to the corresponding portion of mammalian PLC-β1. The PLC-β tail is essentially a three-stranded coiled coil. Two tails bind to form a dimer with the dimer interface along the long axis of each monomer (14). A dimeric structure for PLC-β mediated by the carboxyl-terminal domain is consistent with the regulatory behavior of tail constructs similar to the one used by Singer *et al.* (14).

Because of the strong sequence similarity between the rat PLC-β1 and turkey PLC-β2, we were able to model the carboxyl-terminal domain of PLC-β1 according to that of PLC-β2 (Fig. 2). Those residues that could be mutated to alanine or glycine with minimal effect are *cyan*, and those whose mutation (individually or as part of a small group) caused significant uncoupling from Gα$_q$ are *red*; those generally unconserved residues that were not tested are *light gray-green*. Note that the number of residues grouped as being phenotypically sensitive to mutation is an overestimate because we did not mutate each residue in this group separately. Some residues shown in *red* may therefore be phenotypically neutral.

The residues in the GAP domain of PLC-β1 whose mutations cause uncoupling are distributed in two distinct groups (Fig. 2). One group lies along the dimer interface, and the other lies on one surface of the dimer. The relevant mutations on the dimer interface include residues Lys^{1058}, Lys^{1065}, Lys^{1069}, and Tyr^{1102} (Fig. 2*B, vermilion*). These conserved residues all contain large side chains that extend outward from the helices and make contact with residues in the opposite monomer. The side chains may contribute to the stability of the dimer, the correct alignment or orientation of the coils, or the regulatory interaction surface outlined by the second group of mutated residues. We have not attempted to assign the precise interfacial contacts in which these residues participate because the structure shown in Fig. 2 remains a model and because several nonidentical residues are found in turkey PLC-β2. In addition, the residues in the monomer that lie across the dimer interface from these four side chains do not yield severe phenotypes when mutated individually or in groups (Fig. 2*C, cyan*). Support of this idea is provided by the phenotypically neutral A1 mutation (residues 1018 through 1028). It includes several residues (Gln^{1021}, Tyr^{1025}, and Lys^{1028}) that are apparently poised to interact with these functionally important side chains. Such data suggest that the stability or specificity of the dimer interface may not provide the only contribution to the loss of activity displayed by the Lys^{1058}, Lys^{1065}, Lys^{1069}, and Tyr^{1102} mutations. The surface representation of these mutations diagrammed in Fig. 2*C* illustrates the accessibility of these side chains to the protein exterior and their position relative to the remaining group of essential residues. Lys^{1069} is particularly exposed.

The second group of functionally important residues maps to one surface of the dimeric carboxyl-terminal domain and may thus outline a site of interaction with Gα$_q$. These residues include Lys^{921}, Lys^{925}, Glu^{1048}, and Lys^{1063} (Fig. 2*A, fuchsia*). They lie on two of the three helices of each monomer and are directed outward from a slightly concave surface near the center of the long axis of the dimer. The residues in each monomer are oriented essentially symmetrically with respect to the center of the long axis of the dimer. The region delimited by these mutations describes the surface onto which Singer and Sondek (14) were able to dock two molecules of Gα. The phenotypes of these eight mutations are consistent with their direct interaction with Gα$_q$ and support the proposed binding of Gα$_q$ in this region. These residues are certainly involved in the coupling process that allows Gα$_q$ to activate the phospholipase activity of PLC-β. The other possible function for this region would be regulatory interaction of the carboxyl-terminal domain with the catalytic core of PLC-β. Distinguishing these possibilities or assigning specific residues to one function or the other awaits solution of the structure of the intact PLC-β molecule and its complex with activated Gα$_q$.

Several of the 13 residues whose mutation altered PLC-β-Gα$_q$ coupling could not readily be assigned either to the dimer interface or to potential interaction with Gα$_q$. His^{926}, Gln^{1050}, and Gln^{1050} lie within the coiled coil of each monomer. They may participate in helix-helix interactions, but their importance has not been tested in single mutations. We speculate that Ser^{1101}, Cys^{1049}, and Tyr^{927} are actually phenotypically neutral because they were only mutated in combination with other residues that contribute to the dimer interface.

Loss of Response to G$_q$ without Loss of GAP Activity in a Deletion Mutant—In addition to the uncoupled mutations discussed above, we also characterized one mutation in which residues 1123–1138 of rat PLC-β1were deleted (Δ1123–38). This mutant displayed a G$_q$ GAP phenotype qualitatively different from those of the mutants discussed above, and we therefore expressed it in Sf9 cells and purified it completely (17) to study its behavior more carefully. The Δ1123–38 PLC-β1 was stimulated only slightly by activated Gα$_q$, to a maximal activity about 2% that of wild type PLC-β1 (Fig. 3*A*). In addition, stimulation occurred only at relatively high Gα$_q$ concentrations, with an EC$_{50}$ at least 50-fold above that of wild type (Fig. 3*A*). In contrast, the maximal G$_q$ GAP activity of Δ1123–38 PLC-β1 was unaltered (Fig. 3*B*). Its EC$_{50}$ as a GAP was increased about 15-fold in the steady-state, receptor-coupled assay (20-fold in Fig. 3*B*). The increase in EC$_{50}$ in both assays probably indicates decreased affinity for activated Gα$_q$, but such a decrease cannot account significantly for the loss of responsiveness to Gα$_q$.

Although it was less sensitive than wild type to stimulation by Gα$_q$, Δ1123–38 PLC-β1 displayed an ~4-fold increased basal phospholipase activity in the absence of Gα$_q$. No other mutant displayed this effect. Because maximal activation by Gα$_q$ is decreased and basal activity is increased, Gα$_q$ stimulates the activity of Δ1123–38 PLC-β1 only about 0.6% as much as that of wild type (see the Fig. 3 legend). The elevated basal phospholipase activity of Δ1123–38 PLC-β1 does not result from increased sensitivity to Ca^{2+}. Although both wild type and Δ1123–38 PLC-β1 have very low activity at low nanomolar concentrations of Ca^{2+}, there was no obvious increase in the potency of Ca^{2+} at concentrations above 10 nM. We have not determined whether the elevated basal activity of Δ1123–38 PLC-β1 results from an increased V_{max}, decreased K_m, or both.

The behavior of the Δ1123–38 mutant shows minimally that the deleted region is unnecessary for Gα$_q$ binding and GAP activity, although the deletion does decrease affinity for Gα$_q$ somewhat. The loss of responsiveness to Gα$_q$ and the increase in basal activity suggest that this region may be involved in

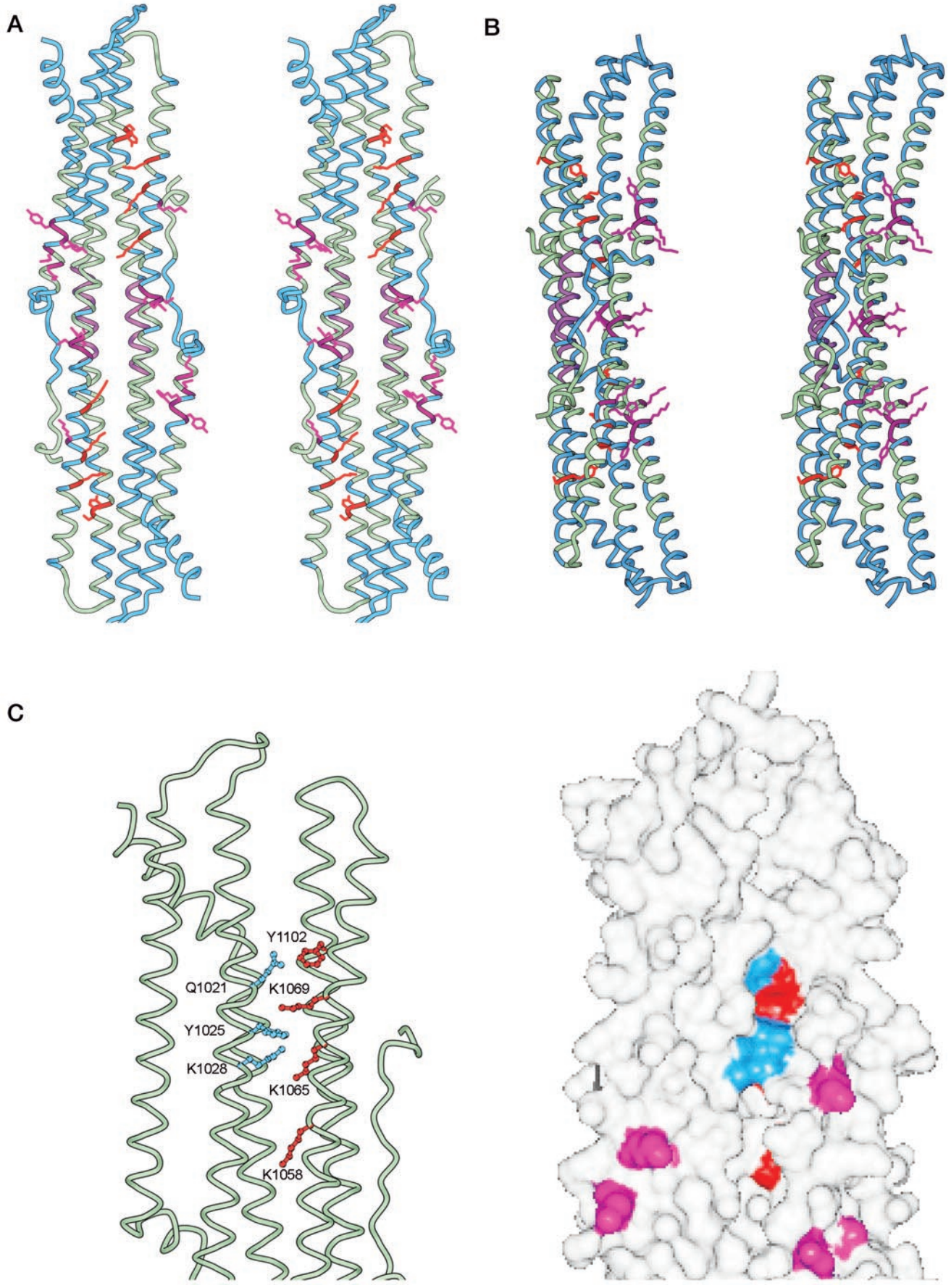

FIG. 2. **Locations of mutations in the carboxyl-terminal Gα$_q$-interaction domain of PLC-β1.** The structure of the carboxyl-terminal region of PLC-β1, amino acid residues 903–1174, was modeled on the structure of the homologous domain of turkey PLC-β2, determined by Singer and Sondek (14), as described under "Experimental Procedures." Residues shown in *cyan* are those whose mutation caused little or no change in

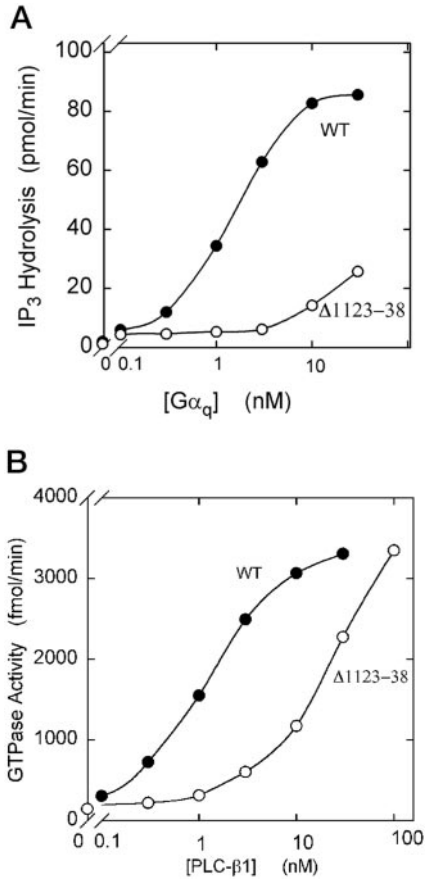

FIG. 3. **Regulatory interactions between Δ1123–38 PLC-β1 and Gα$_q$.** *A,* the phospholipase activities of wild type (●) or Δ1123–38 (□) PLC-β1, both that expressed in Sf9 cells and that purified as described previously (17), was measured in the presence of increasing concentrations of GTPγS-activated Gα$_q$ ([Ca^{2+}]$_{free}$ = 0.2 μM). The concentration of wild type PLC-β1 was 0.1 nM, and the concentration of Δ1123–38 PLC-β1 was 1.0 nM to compensate for their different Gα$_q$-stimulated specific activities. Data are averages of duplicate determinations. In this and two similar experiments, the activity of wild type PLC-β1 in the presence of 10 nM activated Gα$_q$ was 20.0 ± 2.4 μmol PIP_2 hydrolyzed/min/mg and that of Δ1123–38 PLC-β1 was 0.5 ± 0.1 μmol PIP_2 hydrolyzed/min/mg (error parameter is S.D.). Activity in the presence of 5 μM Ca^{2+} was 6.3 ± 0.9 μmol PIP_2 hydrolyzed/min/mg for wild type and 26.4 ± 6.3 μmol PIP_2 hydrolyzed/min/mg for Δ1123–38. *B,* G$_q$ GAP activity was measured according to the increase in agonist-stimulated steady-state GTPase activity in the presence of increasing concentrations of wild type (●) or Δ1123–38 (○) PLC-β1. In the same matched reference experiments, the EC_{50} for GAP activity of Δ1123–38 was 13-fold higher than that of wild type.

regulatory coupling between the carboxyl-terminal region of the PLC-β molecule and its central catalytic domain. The deletion of residues 1123–1138 falls in the middle of the third of the three helices in the PLC-β carboxyl-terminal domain (Fig. 3, *A* and *B*). It would therefore be predicted to either grossly change the packing of the basic coiled-coil structure of this domain or shift the registry of the helix-helix interaction. The latter seems more likely because GAP activity is retained. This deletion apparently leaves the basic structure of the carboxyl-terminal domain intact, either by terminating helix-helix interactions at the initiation of the deletion or by replacing these interactions with helical elements carboxyl-terminal to the deletion.

Conclusions—Unbiased mutagenic scanning of the carboxyl-terminal region of PLC-β1 indicates that a significant majority of the amino acid residues can be replaced by alanine and/or glycine with little, if any, loss to its regulatory functions. This structural stability is consistent with the extended coiled-coil structure of this domain (14). The naive mutagenesis approach used here, initial replacement of contiguous segments with alanine and a few glycine residues, may be generally useful when applied to protein domains of unknown structure, particularly if they are predicted to contain large runs of α-helix. This is true for the PLC-β carboxyl-terminal domain.

More importantly, those mutations that did cause a significant loss of regulatory interaction with G$_q$ clustered in two regions. The occurrence of several key residues at the homodimer interface both supports the validity of this unusual quaternary structure and demonstrates the role of PLC-β homodimerization in its regulation. If a mutated carboxyl-terminal domain that cannot dimerize can still fold in cells, it will provide a platform for experiments to isolate separate steps in the G$_q$-PLC-β signaling pathway in cells. Mutations at the dimer interface may also help us dissect the energetics of interaction with two Gα$_q$ molecules.

The cluster of key amino acids on one slightly concave face of the homodimer is consistent with the possibility that this region contains at least some of the binding interface for Gα$_q$ and suggests possibly important loci for regulatory coupling between these two molecules. The surface of this region is positively charged, and several uncoupling mutations in this region were of Lys residues, consistent with the binding of a negatively charged face of Gα$_q$. The site shows no sequence similarity or similarity of tertiary structure with the Gα binding sites of adenylyl cyclase (26) or the γ subunit of cyclic GMP phosphodiesterase (27), other G protein-regulated effectors whose structures are known. The mutations in this region will be powerful aids in assessing intermolecular interactions when a structure for the Gα$_q$-PLC-β complex becomes available.

Acknowledgments—We sincerely thank Alex Singer and John Sondek (University of North Carolina) for discussing their results and ours and for sharing the coordinates of the PLC-β2 carboxyl-terminal domain well before submission of their manuscript. We thank Suzanne Scarlata for discussing her unpublished work and Suchetana Mukhopadhyay for help and advice during the initiation of this project.

REFERENCES

1. Rhee, S. G., and Choi, K. D. (1992) in *Advances in Second Messenger and Phosphoprotein Research* (Putney, J. W., Jr., ed), pp. 35–61, Raven Press Ltd., New York
2. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem.* **66,** 475–509
3. Rebecchi, M., and Pentyala, S. (2000) *Physiol. Rev.* **80,** 1291–1335
4. Berstein, G., Blank, J. L., Jhon, D.-Y., Exton, J. H., Rhee, S. G., and Ross, E. M. (1992) *Cell* **70,** 411–418
5. Mukhopadhyay, S., and Ross, E. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9539–9544
6. Ross, E. M. (1995) *Recent Prog. Horm. Res.* **50,** 207–221
7. Ross, E. M., and Wilkie, T. M. (2000) *Annu. Rev. Biochem.* **69,** 795–827
8. Wu, D., Jiang, H., Katz, A., and Simon, M. I. (1993) *J. Biol. Chem.* **268,** 3704–3709
9. Park, D., Jhon, D.-Y., Lee, C.-W., Ryu, S. H., and Rhee, S. G. (1993) *J. Biol. Chem.* **268,** 3710–3714
10. Paulssen, R. H., Woodson, J., Liu, Z., and Ross, E. M. (1996) *J. Biol. Chem.* **271,** 26622–26629
11. Kim, C. G., Park, D., and Rhee, S. G. (1996) *J. Biol. Chem.* **271,** 21187–21192
12. Beekman, A., Helfrich, B., Bunn, P. A., Jr., and Heasley, L. E. (1998) *Cancer*

interaction with Gα$_q$ (see the text for criteria). Those shown in *shades of red* were sensitive to mutation: those shown in *vermilion* in or near the dimer interface, and those shown in *fuchsia* in a potential G$_q$ binding surface. Those shown in *light gray-green* were not tested (see "Results" and Tables I and II). Residues in *purple* are those removed in the Δ1123–38 mutant. *A,* stereo view facing the dimer interface. *B,* stereo view of the surface on which remaining sensitive residues are clustered and which may therefore contribute to Gα$_q$ binding. *C,* detailed version of *A*; the key mutated residues are labeled.

Res. **58,** 910–913
13. Paterson, A., Boyer, J. L., Watts, V. J., Morris, A. J., Price, E. M., and Harden, T. K. (1995) *Cell. Signaling* **7,** 709–720
14. Singer, A. U., Waldo, G. L., Harden, T. K., and Sondek, J. (2002) *Nature Struct. Biol.* **9,** 32–36
15. Jenkinson, S., Nahorski, S. R., and Challiss, R. A. J. (1994) *Mol. Pharmacol.* **46,** 1138–1148
16. Parker, E. M., Kameyama, K., Higashijima, T., and Ross, E. M. (1991) *J. Biol. Chem.* **266,** 519–527
17. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J. Biol. Chem.* **271,** 7999–8007
18. Meij, J. T. A., and Ross, E. M. (1996) *Biochem. Biophys. Res. Commun.* **225,** 705–711
19. Blank, J. L., Ross, A. H., and Exton, J. H. (1991) *J. Biol. Chem.* **266,** 18206–18216
20. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., and Ross, E. M. (1992) *J. Biol. Chem.* **267,** 8081–8088
21. Wang, J., Tu, Y., Mukhopadhyay, S., Chidiac, P., Biddlecome, G. H., and Ross, E. M. (1999) in *G Proteins: Techniques of Analysis* (Manning, D. R., ed), pp. 123–151, CRC Press, Boca Raton, FL
22. Esnouf, R. M. (1997) *J. Mol. Graph. Modl.* **15,** 112–113, 132–134
23. Smrcka, A. V., and Sternweis, P. C. (1993) *J. Biol. Chem.* **268,** 9667–9674
24. Biddlecome, G. H. (1997) *Regulation of Phospholipase C-β1 by G_q and m1 Muscarinic Cholinergic Receptor*. Doctoral dissertation, University of Texas Southwestern Graduate School of Biomedical Sciences, Dallas, TX
25. Lee, C.-W., Lee, K.-H., Lee, S. B., Park, D., and Rhee, S. G. (1994) *J. Biol. Chem.* **269,** 25335–25338
26. Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) *Science* **278,** 1943–1947
27. Slep, K. C., Kercher, M. A., He, W., Cowan, C. W., Wensel, T. G., and Sigler, P. B. (2001) *Nature* **409,** 1071–1077