Role of the G Protein γ Subunit in βγ Complex Modulation of Phospholipase Cβ Function*

The G protein βγ complex regulates a wide range of effectors, including the phospholipase C isozymes (PLCs). Different domains on the β subunit are known to contact phospholipase Cβ and affect its regulation. In contrast, the role of the γ subunit in Gβγ modulation of PLCβ function is not known. Results here show that the γ subunit C-terminal domain is involved in mediating Gβγ interactions with phospholipase Cβ. Mutations were introduced to alter the position of the post-translational prenyl modification at the C terminus of the γ subunit with reference to the β subunit. These mutants were appropriately post-translationally modified with the geranyleranyl moiety. A deletion that shortened the C-terminal domain, insertions that extended this domain, and a point mutation, F59A, that disrupted the interaction of this domain with the β subunit were all affected in their ability to activate PLCβ to varying degrees. All mutants, however, interacted equally effectively with the Gα subunit. The results indicate that the G protein γ subunit plays a direct role in the modulation of effector function by the βγ complex.

The G protein βγ complex modulates the function of a number of effectors (1). The precise molecular mechanisms underlying the activation or inhibition of an effector by the G protein βγ complex is not clear. Regions on the β subunit and effectors important for interaction between Gβγ and effector molecules have been identified. A domain on the G protein β subunit was initially shown to contact domains on several effector molecules (2). Later studies have identified several regions on the β subunit that interact with effectors divided into regions that are involved in stabilizing interaction and others involved in modulating effector activity (3–8). Although there is increasing evidence that the G protein γ subunits specify contact with receptors (9, 10), their role in effector regulation has been less clear. Early evidence indicated that the prenyl modification at the C terminus of the γ subunit is a requirement for the βγ complex action on effectors (11). More recently, it has been shown that if the type of prenylation on γ subunits, farnesyl (C15) or geranyleranyl (C20), is altered through mutational alteration of the protein, the ability of the mutant βγ complex to act on effectors is altered (12). These results confirmed the role for the prenyl moiety in effector activation. The reasons for the requirement of the prenyl modification were, however, not clear. It was unclear whether the prenyl group was required because it assisted the βγ complex association with membranes, and as a consequence, brought the βγ complex in close proximity to membrane-bound effector molecules or whether the prenyl group actually interacted with effectors and stabilized this critical protein-protein interaction event. Recent evidence supports the latter mechanism, indicating that the prenyl moiety physically contacts at least one effector, PLCβ, and facilitates βγ interaction with PLCβ (13).

To further define the role of this prenyl modification in effector interaction, we have designed a set of mutant forms of the γ subunit. These mutant γ subunits are predicted to yield γ subunit prenyl modifications that will be in an altered position on the three-dimensional structure of the βγ complex as compared with the wild type. Two of these mutants extend the C-terminal tail of the γ subunit by inserting Gly residues immediately upstream of the Cys that is geranyleranylated. Gly residues will also increase the conformational flexibility of the attached prenyl moiety. A third mutant has 10 residues upstream of the Cys residue deleted, thus shortening the C-terminal tail domain. Ten residues were removed because this domain has previously been shown to be the minimum sequence critical for receptor interaction of the G protein heterotrimer (9, 10, 14). A fourth mutant contains an Ala substituted for Phe at position 59. This Phe residue interacts with six residues on the β subunit (15). In the absence of this interaction, the C-terminal domain of the mutant γ subunit is expected to occupy a distinctly different position with reference to the β subunit when compared with the wild type. The mutant βγ complexes were purified, and high performance liquid chromatography (HPLC) combined with mass spectrometry was used to determine whether these C-terminal domain mutants were still appropriately prenylated with geranyleranyl. All mutants were prenylated. The mutants were compared with the wild type for their relative abilities to activate PLCβ. All the mutants activated PLCβ2 less effectively in comparison with the wild type with the deletion being completely ineffective. The mutations did not have an impact, however, on the ability of the βγ complexes to interact with the α subunit. Subtle alterations in the position of the prenyl moiety on the βγ complex thus have a strong impact on the ability of the βγ complex to interact effectively with an effector, PLCβ. This result indicates that the γ subunit prenyl moiety directly interacts with an effector and not with membranes. Second, it supports the notion that the particular position of the prenyl

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1 The abbreviations used are: PLCβ, phospholipase Cβ isozyme; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; PIP2, phosphatidylinositol 4,5-bisphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GTPγS, guanosine 5‘-3-O-(thio)triphosphate; wt, wild type.
moiety with reference to the β subunit domains that interact with the effector is critical for effective regulatory contact between the G protein βγ complex and PLCβ.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylethanolamine were purchased from Avanti Polar Lipids. [3H]IP₃ (specific activity: 6.5 Ci/mmol) was from PerkinElmer Life Sciences, and [3H]NAD (specific activity: 22 Ci/mmol) was from ICN Biomedicals. All other compounds were purchased from Sigma unless otherwise indicated.

Expression and Purification of Recombinant G Protein Subunits—
The mutant γ subunits were created by using the QuikChange mutagenesis kit (Stratagene) on pFast-BAC vector (Invitrogen) carrying the γ subunit with a His tag at the N-terminal region. GATEWAY system (Invitrogen) was used to generate recombinant γ5 and γ6 type baculoviruses. Sf9 insect cells were co-infected with β{γ₅}-wt and γ{γ₅}-wt or γ{γ₆}-mutant baculoviruses according to the manufacturer’s protocols. The expressed βγ{γ₅}-wt or βγ{γ₆}-mutant proteins were expressed, solubilized from the membrane fractions, and purified using nickel-nitritolriacetic acid Superflow resin (Qiagen) as described (13, 16, 17) with slight modifications. Before eluting the βγ complex, endogenous α subunits that might be bound to the βγ complex were removed with the addition of aluminum fluoride in the final washing step. The proteins were frozen in liquid nitrogen and stored at −80 °C. The proteins were separated in 15% SDS gels with bovine serum albumin standards, and the concentrations of the proteins were determined after scanning and analyzing them with IMAGEJ software (rsb.info.nih.gov/ij/). The purity of the purified protein was always more than 95%, and the yield was 0.5 mg/liter of Sf9 cell culture.

Wild type α, subunit was purified from bacteria co-expressing N-methyl transferase as described before (18). The protein purity was determined by SDS gel electrophoresis with appropriate standards of known concentration. The active α, protein concentration was estimated based on GTPγS binding.

HPLC-Mass Spectrometry Analysis—The prenylation of the γ subunits was confirmed with HPLC (Beckman System GOLD). The βγ subunits were run on a C₁₈, 300-A column (diameter, 4.6 × 250 mm; particle size, 5 μm; Jupiter, Phenomenex) with a linear acetonitrile gradient (containing 0.1% trifluoroacetic acid) from 30 to 80% in 50 min. The flow rate was 1 ml/min. The absorbance was recorded at 205 nm. The purified βγ complexes or some of the peak fractions from the HPLC eluate were collected and analyzed with MALDI-mass spectrometry.

Tryptic Digestion—Tryptic digestion protocol of the βγ complex was modified from a previous method (19). Briefly, about 1.2 μg of βγ complex and 50 ng of trypsin were mixed in trypsin buffer (50 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol, 50 μM GDP). At the final step, 200-μl fractions were collected, and the peak fraction was determined using Bradford Reagent (Bio-Rad). The proteins were washed with 14 ml of 6% trichloroacetic acid. The filters were homogenized in scintillation liquid overnight and counted with a scintillation counter.

RESULTS AND DISCUSSION

Synthesis and Characterization of Mutant Forms of the G Protein γ Subunit—Four different mutant forms of the γ subunit were synthesized. Baculovirus vectors containing the β₁ subunit and various γ subunit forms were co-expressed in Sf9 insect cells, and the expressed proteins were purified. The alterations introduced at the C terminus of the γ subunit were expected to reposition the prenyl moiety with reference to the β subunit domains known to contact the effector, PLCβ. These changes in the various mutant forms in comparison with the wild type are represented diagrammatically on a model of the crystal structure of the G protein βγ complex in Fig. 1. In the wild type, the γ subunit is bound to one side of the FIG. 1. Diagrammatic representation of the G protein βγ₅ mutant C-terminal domains. Sequences corresponding to the C-terminal 19 residues of the wild type γ₅ are shown. The potential orientation of the C-terminal protein sequence and the prenyl moiety in the free βγ complex are diagrammatically represented to indicate putative differences in this region of the βγ complex between the various mutants. In the case of the 3G and 6G insertions (underlined), the domain extending from the point of insertion (wavy line) is also shown in gray in a different orientation to emphasize the potential for conformational flexibility. The Ala substitution at position 59 is underlined. The CAX motif is boxed. The approximate position of the Phe-59 residue on the γ₅ subunit and the β₁ subunit site with which this residue interacts are also shown. The model of the three-dimensional structure of the β₁γ₅ complex (30) has been generated using MolMol (www.mol.biol.ethz.ch/wuthrich/software/molmol/).

Phospholipase C Assay—Phospholipase C assays were conducted as described previously (16) except that cholate was omitted in the βγ complex dilution buffer. In each reaction tube, phospholipid vesicles (50 μm PIP₂, 200 μm phosphatidylethanolamine, and [3H]IP₃ giving 10,000 cpm) and 2 ng of PLCβ2 (a kind gift from Dr. A. Smrcka) were mixed in varying concentrations of βγ complex as shown in the figures. The final incubation was performed for 7 min at 30 °C.

Statistical Analysis—Student’s t tests were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

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Fig. 2. Interaction of the Phe-64 residue of the γ subunit with the β subunit. The model depicts residues on the β subunit that interact with the Phe-64 residue on the γ subunit. An enlarged view of the surfaces between blades 7 and 1 in the folded β subunit structure is shown. The model shown is based on the crystal structure of β1γ1 (30) where the Phe-64 residue contacts six different residues, anchoring the C-terminal γ subunit domain to the β subunit. The model was generated as discussed in the legend for Fig. 1.

β subunit torus-like structure. The γ subunit C-terminal tail domain is positioned in apposition to the β sheets traditionally referred to as “blades 6, 7, and 1.” G protein βγ complexes crystallized initially did not contain the prenyl moiety (15, 21). Furthermore, the C-terminal residues of the γ subunit did not resolve in these structures. The precise orientation of the prenylated tail could not therefore be inferred. The crystal structure of Gβγ containing the prenylated γ subunit was later obtained as a complex of β1γ1 with phosducin (22). In this crystal structure, the C-terminal region of the γ subunit was again disordered. It is not possible, therefore, to predict the precise orientation of the C-terminal γ subunit domain in the wild type Gβγ complex. However, it is possible to visualize overall changes in the mutants designed for these experiments, as shown in Fig. 1. The removal of the last 10 residues upstream of the prenylated Cys residue will result in a shortened γ subunit with the prenyl moiety repositioned in apposition to the β sheet blades in the β subunit. The addition of 3G or 6G residues will (i) extend the C-terminal domain upstream of the prenyl moiety and (ii) increase the mobility of the prenyl moiety with reference to the γ subunit as compared with the wild type due to the addition of Gly residues that are conformationally flexible. Together this should result in the prenyl moiety occupying positions in three-dimensional space that are distinct from the wild type. In the γ5-F59A mutant, the Phe residue at position 59 was substituted with Ala to disrupt the binding of the C-terminal domain to the β subunit. As shown in Fig. 2, the corresponding Phe residue (Phe-64) in the γ1 subunit interacts with six different residues in the cleft between blades 7 and 1 of the β1 subunit. This Phe residue is conserved in all mammalian γ subunits (9). Mutating the hydrophobic Phe residue to Ala is expected to result in the C-terminal domain of the γ subunit loosing its anchor on the β subunit and repositioning itself (Fig. 1).

The wild type γ5 and the four C-terminal mutants were purified as described and analyzed on an HPLC hydrophobic column using an acetonitrile gradient (described under “Experimental Procedures”). Under the chromatography conditions used here, the β subunit separates from the γ subunit. Fig. 3 shows the chromatograms of the γ subunit forms examined. Chromatograms of the wild type and the point mutant γ5-F59A showed a single peak, whereas the others showed two. Proteins corresponding to each of these peaks were isolated, and the masses of the corresponding proteins were determined using mass spectrometry. The results from this analysis are shown in Table I. In the chromatogram for each γ subunit (Fig. 3), protein corresponding to the peak that appeared earlier was prenylated, proteolyzed, and carboxyl-methylated. Protein corresponding to the peak that appeared later was prenylated but not proteolyzed. Similar to other proteins that are prenylated, the G protein γ subunits undergo three distinct post-translational processing steps (23). First, they are prenylated, and then the last three residues are proteolytically removed. Finally, the protein is carboxyl-methylated. Consistent with earlier reports that the only determinant of prenylation is the CAAX box sequence, all the mutants are appropriately prenylated with the geranylgeranyl moiety. However, the proteases do not seem to recognize the mutant sequences effectively, and a fraction of the γ5−3G, γ5−6G, and γ5−Δ mutants retain the last three residues (Ser-Phe-Leu). Previous evidence indicates that the retention of the last three residues does not significantly alter the properties of the γ subunit (24). The relative positions of the chromatographic peaks corresponding to the various γ subunit forms are consistent with their expected hydrophobic properties (Fig. 3). γ5−6G appears earliest followed by γ5−3G. The additional Gly residues would be expected to reduce the overall hydrophobicity of these two proteins in that order. The point mutant contains an Ala residue in place of the hydrophobic Phe residue at position 59 and runs faster than...
The alteration of the amino acid sequence at the C terminus (with the exception of the activity of the wild type at any of the concentrations tested traditionally used to measure the interaction between the phobic Phe and Leu residues. which would increase the overall hydrophobicity of the prenylated protein. Together these results indicated that the mutant wild type. The deletion appears last, consistent with the removal of 10 residues from the relatively small γ subunit, which would increase the overall hydrophobicity of the prenylated protein. The geranylgeranylated forms that retain the last three residues appear after the geranylgeranylated and proteolyzed forms consistent with the retention of the hydrophobic Phe and Leu residues.

**Tryptic Digest of Wild Type and Mutant βγ Complexes**—First, we examined these βγ complexes using an assay that can detect gross changes in the conformation of the β subunit. The wild type βγ complex, when digested with trypsin, yields two fragments of ~24 and 13 kDa as demonstrated before (31). Significant changes in the three-dimensional structure of the β subunit would be expected to generate other fragments on trypsin digestion due to the exposure of masked Lys and Arg residues. All the mutants yielded the same fragments as the wild type. The deletion appears last, consistent with the removal of 10 residues at the C terminus of the γ subunit after HPLC separation of the βγ complex (Fig. 3). Together these results indicated that the mutant βγ complexes were not significantly altered in their structure.

**Interaction of the α Subunit with Mutant and Wild Type βγ Complexes Measured Using the ADP-ribosylation Assay**—Next we examined the βγ complexes containing the wild type and the mutant γ subunit forms for their ability to interact with the α subunit using the ADP-ribosylation assay. This test was performed to determine whether more subtle alterations in the structure of the βγ complex forms had occurred as a result of the mutations. Pertussis toxin catalyzes the transfer of the ADP-ribosyl group from NAD to the C-terminal Cys residue in several α subunit types. This transfer is considerably enhanced by the G protein βγ complex. The assay has therefore been traditionally used to measure the interaction between the α subunit and the βγ complex. Fig. 5 shows the results of ADP-ribosylation assays performed with the different γ subunit mutants. The dependence of α, ADP-ribosylation by pertussis toxin on increasing concentrations of βγ complex was examined. None of the mutants showed significant differences from the activity of the wild type at any of the concentrations tested (with the exception of γα-3G at 50 nM concentration, p < 0.05). The alteration of the amino acid sequence at the C terminus and the repositioning of the prenyl moiety with reference to the β subunit therefore had no effect on the interaction of the mutant βγ complexes with the α subunit. These results also indicate that the γα-3G, γα-6G, and γα-Δ forms that retain the last three residues have no significant impact on interaction with the α subunit.

**Phospholipase Cβ2 Activation by the Wild Type and Mutant βγ Complexes**—The phospholipase Cβ isozymes break PIP₂ to inositol trisphosphate and diacylglycerol. These isozymes are activated by the G protein subunits, α and the βγ complex (25). As shown in Fig. 6, we examined the ability of the various mutants as well as the wild type βγ complex to activate the phospholipase Cβ2 isozyme. The recombinant form of PLCβ2 purified after expression using the baculovirus and SF9 insect cell system was used (16, 26). βγ concentration-dependent activation of PLCβ was measured in vitro. The wild type βγ complex stimulated the basal PLCβ activity about 20-fold. The βγ-3G, βγ-6G, and βγ-Δ F59A mutants were significantly less active in stimulating the enzyme activity at concentrations of 50, 100, 200, and 400 nM βγ complex (Fig. 5). The deletion was not active at all concentrations tested. It is highly unlikely that the inability of γα-Δ to activate PLCβ is due to the fraction of the processed form that retains the C-terminal three residues because the geranylgeranylated, proteolyzed form is about 40% of the geranylgeranylated form (Fig. 3), but even at a concentration of 400 nM βγ-Δ complex, no detectable activity is elicited from PLCβ2.

Two different models can account for the unexpectedly dramatic effect of these mutants on their ability to regulate PLCβ activity. The attenuation of βγ function is especially striking in the case of the deletion and also in the case of the F59A mutant since this alters only one residue at the C terminus of the γ
subunit. One model is based on the proposal that the prenyl moiety is buried in a cavity between blades 6 and 7 of the β subunit when it interacts with effectors, thus altering the conformation of the β subunit and exposing the appropriate residues for interaction with the effector molecule (7). With regard to this model, it should be noted that the crystallographic basis for the presence of the prenyl moiety is not conclusive since the electron density in the cavity was surmised to be the prenyl moiety and the last several residues of the γ subunit were unresolved (22). Extensive analysis of β subunit mutants that were altered at residues potentially in contact with the prenyl moiety or residues that showed conformational changes between free βγ complex and phosducin-bound βγ demonstrated that they were significantly affected in their ability to activate PLCβ (7). This result is consistent with the proposal that the prenyl moiety resides in the cavity between blades 6/7 when interacting with an effector, thus bringing about a conformational change in residues in the β subunit required for binding the effector. However, the crystal structure of an unmodified βγ complex with phosducin deduced by another group (27) shows that the cavity between β subunit blades 6 and 7 still occurs even in the absence of the prenyl moiety. This result is not entirely consistent with the notion that the positioning of the prenyl moiety in the β subunit cavity is important for inducing the β subunit conformation required for interaction with an effector. Despite this concern, it is to be noted that some of the results obtained here from the analysis of the mutant βγ complexes are consistent with the above model. The γΔ would be expected to shorten the C-terminal tail of the γ subunit significantly, thus preventing the prenyl moiety from reaching the pocket between blades 6 and 7 of the β subunit. The γF59A mutant may not similarly have the prenyl moiety located appropriately in the β subunit pocket because an important anchoring site for the γ subunit-terminal domain on the β subunit has been removed. The increasingly stronger effects of the 3G and 6G mutants on their ability to activate PLCβ is less clear within the context of this model. The addition of the Gly residues would be expected to extend the C-terminal tail but also to increase the conformational flexibility of this region. These mutants can also affect the positioning of the prenyl moiety in the β subunit pocket.

An alternative model invokes the direct interaction of the prenyl moiety with PLCβ. Recent results indicate that PLCβ has a site that binds the prenyl moiety (13). A prenylated peptide potently inhibits Gβγ stimulation of PLCβ. Fluorescence-based assays indicate direct interaction between the prenylated peptide and PLCβ. These results are consistent with the location of the prenyl moiety in a pocket present in PLCβ during βγ complex interaction with the effector enzyme. This role for the γ subunit prenyl moiety is also supported by the evidence from the crystal structure of the of Cdc42/RhoGDI complex (28). The prenyl moiety at the C terminus of Cdc42 is buried in a hydrophobic pocket present in the protein partner RhoGDI. In this model for the interaction of the Gβγ complex with PLCβ, interaction is facilitated by the γ subunit prenyl moiety as well as several domains on the β subunit. Two-hybrid interaction, peptide studies, and mutational analyses have implicated several such domains on the β subunit in interaction with PLCβ and other effectors (2–8, 29). The prenyl moiety is predicted to stabilize these interactions of PLCβ with β subunit domains by directly anchoring itself in a hydrophobic pocket in PLCβ. The interaction between the βγ complex and PLCβ can be disrupted due to mutations that alter the β subunit binding sites, removal of the prenyl moiety, or most relevant to the results here, altering the position of the prenyl moiety with reference to the other binding sites on the β subunits. All mutants designed here are expected to alter the relative position of the prenyl moiety with reference to the β subunit surfaces postulated to contact the PLCβ and result in impaired interaction between the βγ complex and effector. The decreased ability of Gβγ containing the γ subunit mutants to activate PLCβ (Fig. 5) is consistent with this expectation. Finally, it cannot be discounted that the two different mechanisms outlined above are both involved in Gβγ regulation of PLCβ. The prenyl moiety may reside in the β subunit cavity transiently and then locate itself in a pocket in the PLCβ enzyme, stabilizing the interaction between the two proteins at different points of time during PLC activation.

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