Characterization of a Phospholipase C β2-Binding Site Near the Amino-terminal Coiled-coil of G Protein βγ Subunits*

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In previous work (Sankaran, B., Osterhout, J., Wu, D., and Smrcka, A. V. (1998) J. Biol. Chem. 273, 7148–7154), we showed that overlapping peptides, N20K (Asn564–Lys583) and E20K (Glu574–Lys593), from the catalytic domain of phospholipase C (PLC) β2 block Gβγ-dependent activation of PLC β2. The peptides could also be directly cross-linked to βγ subunits with a heterobifunctional cross-linker succinimidyl 4-[N-maleimidomethyl]-cyclo-hexane-1-carboxylate. Cross-linking of peptides to Gβ1 was inhibited by PLC β2 but not by αi1(GDP), indicating that the peptide-binding site on βi1 represents a binding site for PLC β2 that does not overlap with the αi1-binding site. Here we identify the site of peptide cross-linking and thereby define a site for PLC β2 interaction with β subunits. Each of the 14 cysteine residues in βi1 were altered to alanine. The ability of the PLC β2-derived peptide to cross-link to each βγ mutant was then analyzed to identify the reactive sulfhydryl moiety on the β subunit required for the cross-linking reaction. We find that C25A was the only mutation that significantly affected peptide cross-linking. This indicates that the peptide is specifically binding to a region near cysteine 25 of βi1 which is located in the amino-terminal coiled-coil region of βi1 and identifies a PLC-binding site distinct from the α subunit interaction site.

Guanine nucleotide-binding proteins (G proteins) are a large group of structurally similar proteins consisting of three subunits (α, β, and γ) that are central molecules coupling seven-transmembrane domain-spanning receptors to downstream effector molecules. Activation of G proteins begins with a ligand-induced conformational change of the receptor which catalyzes the release of GDP from the α subunit in exchange for GTP (1, 2). In the GDP-bound heterotrimeric state, αGDP+βγ, neither αGDP nor βγ can regulate effector activity. Upon receptor-catalyzed G protein activation, the heterotrimer dissociates into free α(GTP) and free βγ subunits. It is well understood that both α(GTP) and βγ subunits can interact with a variety of downstream effector molecules including enzymes and ion channels. GTP is hydrolyzed to GDP, and reassociation of α(GDP) with βγ results in deactivation of βγ-dependent signaling. Despite detailed knowledge of α- and βγ subunit functions, the mechanism for how βγ subunits activate its variety of effectors is not entirely understood.

Effector-binding sites on the surface of βγ are beginning to be mapped. The putative competition between α(GDP) and effectors for βγ forms the premise for recent studies to map effector-binding sites at the α subunit-binding interface on β. The three-dimensional structure of the G protein heterotrimer reveals that the β subunit is a β-propeller with seven “blades” and an amino-terminal α-helix (3, 4). The α subunit binds to a portion of the top of the β-propeller and along side one of the blades of the propeller. Two groups have shown that alanine substitution of α-contacting residues on the top surface of the β-propeller differentially affected the ability of βγ to regulate various target molecules (5, 6). One of these groups tested whether the sides of the β-propeller may be important for effector interactions. Residues along the outer strand of each of the 7 blades of the β-propeller were altered, and each βγ mutant was tested for its ability to stimulate phospholipase C (PLC) β2 and regulate adenyl cyclases (7). Mutations in three of the blades eliminated the ability of βγ to activate PLC β2, whereas these same mutations did not affect regulation of adenyl cyclase isoforms 1 and 2. Consistent with these studies, synthetic peptides from discrete blade regions of β were used in competition experiments to define a region located near the interface between α and βγ as being important for activation of PLC β2 (8).

A screen for dominant-negative yeast Gγ mutants that could interfere with mating factor signaling identified different regions involved in β subunit-effector interactions (9). In particular, one set of mutations maps to an amino-terminal region involved in a coiled-coil interaction with γ subunits on the opposite side of the β subunit from the α subunit-binding site. Taken together, these studies show that the top surface of β, where many α-contact points are located, is a region critical for effector binding and regulation. However, effector-binding sites are being mapped to a variety of regions on the surface of βγ. Multiple interaction regions are reported even for the same effector. It has been suggested that distinct sets of contacts may define the specificity of the interaction of βγ with various effectors (i.e. each effector will have a characteristic footprint along the sides of the molecule).

In previous work (10) we showed that overlapping peptides, N20K (Asn564–Lys583) and E20K (Glu574–Lys593) from the catalytic domain of phospholipase C (PLC) β2, block PLC activation by βγ and could be directly cross-linked to β subunits with a heterobifunctional cross-linker (SMCC). In the study presented here we have identified the site of cross-linking of N20K to the β subunit thereby defining a binding site for PLC β2. This approach identified a site distinct from those mapped by site-directed mutagenesis but consistent with the region identi-

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The abbreviations used are: G protein, GTP-binding protein; PLC, phospholipase C; SMCC, succinimidyl 4-[N-maleimidomethyl]-cyclo-hexane-1-carboxylate; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase.
tified in yeast β subunits as being important for β subunit-effector interactions (9).

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were purchased from Biosynthesis (Lewisville, TX) and had a purity of greater than 90% based on high pressure liquid chromatography analysis, and identities were confirmed by mass spectrometry. Biotin-labeled peptide (B-N20K) was purchased from Alpha Diagnostic International (San Antonio, TX). Succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (SMCC) and horseradish peroxidase-conjugated NeutrAvidin were from Pierce. Trypsin and 2-aminooethanol (monoethanolamine) were from Sigma. PVDF membrane was from PerkinElmer Life Sciences. Nitrocellulose membrane was from Schleicher & Schuell.

β2-Cysteine mutants in pALTER (11) were kindly provided by Dr. Eva J. Neer.

**Construction of Recombinant Baculoviruses and S9 Culture**—Each β-cysteine mutant was subcloned into the baculovirus transfer vector pFASTBAC (Life Technologies, Inc.). Recombinant baculoviruses were generated using standard manufacturer’s procedures. S9 cells were grown at 27 °C in SF900 medium (Life Technologies, Inc.).

**Purification of Phospholipase C β2**—Baculovirus constructs directing expression of recombinant His6-PLC β2 were used to infect 800 ml of S9 cells at a density of 2.5 × 10^6 cells/ml. PLC β2 was purified according to published procedures (12).

**Purification of Wild Type β1γ2, Cysteine-mutated β1γ2 and S9 βγ Subunits**—For purification of wild type β1γ2, baculovirus constructs encoding β1-, γ2-, and Hisa-tagged αs1 were obtained from Alfred Gilman’s laboratory. 800 ml of S9 cells at 2.5 × 10^6 cells/ml were simultaneously infected with the three constructs, and the βγ subunits were purified according to published procedures (13) and modified as in Romoser et al. (12). The protein was concentrated on a 0.3-ml macro-prep ceramic hydroxyapatite (Bio-Rad) column and eluted into 50 mM Tris, pH 8.0, 2 mM dithiothreitol, 2 mM N-acetyl-L-cysteine, 80% glycerol, 1% SDS, 350 mM potassium phosphate, pH 8.0.

For purification of βγ-cysteine (C) mutants, 200 ml of S9 cells at 2.5 × 10^6 cells/ml were simultaneously infected with baculovirus constructs encoding each βγ-cysteine mutant, γc2, and Hisa-tagged αs1. Each βγ-cysteine mutant was purified according to the above procedures but modified to batch style with 0.5 ml of nickel-nitrilotriacetic-agarose. Peak fractions were pooled and concentrated on a 0.3-ml hydroxyapatite column, and protein was eluted into βγ vehicle.

For purification of endogenous S9 β, 1 liter of S9 cells at 2.5 × 10^6 cells/ml was simultaneously infected with baculovirus constructs encoding Hisa-tagged α and γc1. S9 βγ subunits were purified according to the procedures for the purification of wild type βγ as described above.

**Purification of a Subunits—Recombinant myristoylated αs1 and αg were purified from Escherichia coli coexpressing αs1 or αg with N-myristoyltransferase according to published procedures (14) with some modifications. αs1 or αg were expressed in 1 liter of E. coli containing the N-myristoyltransferase plasmid for 16 h. After preparation of the lysate, the protein was applied to a 50-ml column of Q Sepharose fast flow (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM Tris, pH 8.0, 2 mM dithiothreitol, 2 mM MgCl2. The column was washed with 150 ml of equilibration buffer followed by elution of the protein with a 400-ml linear gradient to 250 mM NaCl in equilibration buffer. Fractions containing α subunits subunits were further purified by fast protein liquid chromatography phenyl-Superose chromatography resulting in two peaks of α subunits eluting from the column. The second peak corresponding to myristoylated α subunit was pooled and concentrated; 50 μM GDP was added, frozen in aliquots in liquid N2, and stored at −80 °C. Functionality of the α subunits was confirmed by the ability to be ADP-ribosylated by pertussis toxin and the ability to inhibit βγ-mediated activation of PLC β2.

**Chemical Cross-linking**—We used two different cross-linking protocols in this study. Protocol 1, used for the experiments described in Figs. 1 and 7, has been described previously (10). Here, βγ subunits at the indicated concentrations were mixed with N20K (NRSYVISS-FTEKAYDLLSK) peptide followed by addition of SMCC to 200 μM from a 2-ml stock solution. The reaction was allowed to proceed for 5 min. For protocol 2, N20K was reacted with SMCC for 10 min followed by addition of 500 mM ethanolamine, pH 7.3, to inactivate the unreacted succinimide moiety. βγ subunits were then added, and the reaction was allowed to proceed at room temperature for 1.5 min. For both protocols reactions were quenched with SDS sample buffer (32 mM Tris, pH 6.8, 5% glycerol, 1% SDS, 350 mM 2-mercaptoethanol, bromphenol blue (final concentrations), resolved on SDS-PAGE, and transferred over-night onto a PVDF membrane (PerkinElmer Life Sciences). For some experiments (as indicated) cross-linked species were detected with horseradish peroxidase (HRP)-conjugated NeutrAvidin (Pierce). In other experiments cross-linked β subunits were detected with the carbonyl-terminal anti-β subunit antibody B600 and/or amino-terminal specific β subunit antibody following secondary antibody linked to HRP and development with Amersham Pharmacia Biotech chemiluminescence reagents.

**Cross-linking to Trypsin Digestion of βγ Subunits**—Wild type or cysteine mutant βγ was digested with trypsin at a βγ:trypsin ratio of 75:1, for 30 min at 30 °C. The digestion was stopped by addition of 62.5 μM phenylmethylsulfonyl fluoride. Digested βγ (80 nl), 100 μM N20K, and cross-linker were mixed as in cross-linking protocol 1 and analyzed by immunoblotting with an amino-terminal specific β subunit antibody. Phospholipase C Assay—PLC assays were performed as described previously (12). Briefly, purified PLC β2 was mixed with sonicated phospholipid vesicles containing 50 μM phosphatidylinositol 4,5-bisphosphate, 200 μM phosphatidylethanolamine, and [3H]phosphatidylcholine (6000–8000 cpm/assay), with or without 100 nM purified β1γ2 or mutants and/or peptides. Reactions were allowed to proceed from 3 to 5 min at 30 °C. Intact lipids and proteins were precipitated with bovine serum albumin and 10% trichloroacetic acid and removed by centrifugation. Supernatant containing soluble [3H]inositol 1,4,5-trisphosphate was analyzed by liquid scintillation counting.

**RESULTS**

**PLC β2 Inhibits Peptide Cross-linking to βγ Subunits but a Subunits Do Not**—We have previously shown that two peptides derived from PLC β2 (N20K and E20K) are specifically and chemically cross-linked to both G protein β and γ subunits using a heterobifunctional cross-linking reagent (SMCC) (10). SMCC contains maleimide- and succinimide-reactive groups, which react with SH— and NH— moieties, respectively. Since the peptides have no cysteine residues, cross-linking must be through a primary amine on the peptide and a sulfhydryl group in the β subunit. The cross-linking reaction is equally effective if the amino terminus of the peptide is acetylated indicating that one of the two lysine residues within the N20K peptide sequence is involved in the reaction.

Previous data suggest that cross-linking of N20K to βγ subunits could be blocked by PLC β2 but not αG (10). To analyze this in greater detail, the concentrations of α subunits and PLC β2 required to inhibit N20K cross-linking to the β subunit were examined (Fig. 1). Cross-linking of β subunit to the peptide is indicated by the increase in apparent molecular weight of the immunoreactive β subunit after incubation with peptide and SMCC. The uppermost band is β cross-linked to γ (γi1), and bands with γ subunits and γ-subunit antibodies show two bands in between are β cross-linked to the peptide. There is one prominent peptide cross-linked species and a second minor peptide cross-linked species. PLC β2 (90 nm) significantly inhibited cross-linking of 10 μM peptide (N20K) to 30 nm βγ and completely inhibited the cross-linking at 150 nm PLC β2. This concentration dependence is
consistent with the EC_{50} of \(-50–100\) nM for PLC activation by \(\beta\gamma\) subunits (15). On the other hand \(\alpha\gamma\) (GDP) did not inhibit cross-linking of the peptide up to 250 nM. This same preparation of \(\alpha\) subunit was able to block \(\beta\gamma\)-mediated activation of PLC \(\beta2\) by 95\% at a 2:1 ratio of \(\alpha\) to \(\beta\gamma\) (not shown). These results indicate that the peptide and \(\alpha\) subunit-binding sites on \(\beta\gamma\) are distinct and that the peptide-binding site is within the binding site for PLC \(\beta2\) on \(\beta\gamma\).

**Recombinant Myristoylated \(\alpha\) Subunits Do Not Prevent PLC from Inhibiting Cross-linking of the Peptide—**As discussed, \(\alpha\) subunits have been suggested to inhibit \(\beta\gamma\) subunit-mediated PLC \(\beta2\) activation by competing for binding of the PLC \(\beta2\) to the \(\beta\gamma\) subunits. To test whether \(\alpha\gamma\) (GDP) inhibits the binding of PLC \(\beta2\) to \(\beta\gamma\) subunits, we tested whether excess \(\alpha\gamma\) (GDP) (250 nM) altered the ability of PLC \(\beta2\) to block peptide cross-linking to \(\beta\gamma\) (Fig. 2). For this experiment the cross-linking protocol was modified to eliminate intermolecular \(\beta\) cross-linking to \(\gamma\) and reduce the number of cross-linked species (see “Experimental Procedures” cross-linking protocol 2 and the figure legend). Although PLC \(\beta2\) did not completely inhibit peptide cross-linking to \(\beta\) subunits in the absence of \(\alpha\) in this experiment (compared with Fig. 1), cross-linking was still significantly inhibited when PLC \(\beta2\) was added. In the presence of the \(\alpha\) subunit, PLC \(\beta2\) also inhibited cross-linking, but a higher concentration of PLC was required to observe the inhibition. Similar results were observed with \(\alpha\gamma\) (GDP). This suggests that PLC \(\beta2\) can bind to \(\beta\) subunits at the peptide interaction site even in the presence of \(\alpha\) subunits, but \(\alpha\) subunits may alter the affinity of PLC for \(\beta\gamma\).

**Purification of Cysteine-mutated \(\beta\) Subunits—**To identify the location of peptide cross-linking and thus map a region for PLC \(\beta2\) binding that does not coincide with \(\alpha\) subunit binding, we used a sulfhydryl mutagenesis strategy similar to that designed by Garcia-Higuera et al. (11). Since cross-linking can only occur between a cysteine residue from \(\beta\) and lysine from the peptide, we characterized cross-linking of the peptide to a series of \(\beta\) subunit mutants where each cysteine residue had been individually mutated to alanine.

We obtained the \(\beta1\)-cysteine mutant cDNAs from Dr. Eva J. Neer and created baculovirus constructs for expression and purification of each cysteine mutant from Sf9 insect cells. Fig. 3A displays a Coomassie-stained panel of 11 of the 14 purified \(\beta1\)-\(\gamma2\)-cysteine mutants. The numbers indicate the positions of the cysteine residue in the \(\beta1\) primary sequence. The Cys\textsuperscript{166}, Cys\textsuperscript{140}, and Cys\textsuperscript{217} proteins are not shown but are of similar purity. Cys\textsuperscript{103}, Cys\textsuperscript{114}, Cys\textsuperscript{211}, Cys\textsuperscript{318}, and Cys\textsuperscript{271} are doublets in which the top band of each doublet is Sf9 \(\beta\) that copurifies. It will be shown in later experiments that Sf9 \(\beta\) does not cross-link to N20K, and therefore, any cross-linking that is observed must be to the expressed mutant \(\beta\) subunit. The concentration of each \(\beta1\)-cysteine mutant was estimated by immunoblot analysis because the preparations contained Sf9 \(\beta\), and we wanted to base the analysis on equal amounts of the expressed \(\beta\) mutants. The concentration of each \(\beta1\)-cysteine mutant (lower band in the case of doublets) was estimated by comparing the band intensities to a range of intensities produced by known amounts of wild type \(\beta1\) protein (an example of such an estimation for Cys\textsuperscript{114}) is shown in Fig. 3B).

Each of the mutants was able to bind to His-tagged \(\alpha\) and elute with addition of a G protein activator (AlF\textsubscript{4}\textsuperscript{-}), suggesting that they were properly folded and active. To be sure that the mutants were able to activate phospholipase C, 13 of the 14 mutants were tested for their ability to activate phospholipase C \(\beta2\) in an in vitro phospholipase C assay (Fig. 4). All of the mutants that were tested were able to activate PLC \(\beta2\) between 3- and 5-fold compared with 7-fold for wild type \(\beta1\).\(\gamma2\). Thus, while their efficacy is slightly diminished (by \(-\frac{1}{2}\) relative to wild type \(\beta1\)\(\gamma2\) in general these mutants are properly folded and capable of interacting with \(\alpha\) subunits and PLC \(\beta2\). It is not clear that these mutants have a reduced ability to activate PLC \(\beta2\), but we suspect that it is not a result of the mutations themselves but rather is a consequence of the difficulties in quantitating the mutants as well as repeated freezing and thawing of the samples. Regardless, this cannot be responsible for any differences observed in the cross-linking experiments since there is no correlation between the activities and the degree of cross-linking.

**Cross-linking of Biotin-labeled Peptide to Wild Type \(\beta1\)\(\gamma2\) Dimer—**For the survey of the ability of the peptide to cross-link to the mutants, we modified cross-linking protocol 2 (see “Experimental Procedures”) to use an amino-terminal biotin-modified form of the N20K peptide (B-N20K) which we could detect with horseradish peroxidase-conjugated avidin (HRP-NeutrAvidin from Pierce). With this modified peptide our procedure for monitoring cross-linking to \(\beta\gamma\) subunits allows only the peptide cross-linked species of \(\beta\) to be detected.

To confirm that the biotin modification did not affect the activity of the peptide, we tested whether the B-N20K would block PLC activation by \(\beta\gamma\). Consistent with our previous report (10), B-N20K inhibited \(\beta\gamma\)-stimulated PLC activity with an IC\textsubscript{50} of \(-50\) \(\mu\)M with 88\% inhibition of activity occurring at
We purified Sf9 to determine whether the peptide cross-links to this mutant linking observed with any of the cysteine mutants. Properties of the peptide and that the site of peptide cross-cleavage to isolate the amino-terminal fragment of the peptide was Cys25. As discussed, Cys103, Cys114, Cys121, Cys148, resulted in a clearly significant reduction in cross-linking of the repeated experiments the only mutation that consistently resulted in a minor effect on cross-linking, it is difficult to make conclusions as to their significance with this method since it is not strictly quantitative. Clearly, however, in multiply repeated determinations. The cross-linking reaction resulted in the presence of 0.15% octyl glucoside. The βγ peptide preincubation mixture was then diluted 1.5-fold into a PLC assay such that the final concentration of βγ subunits was 100 nM, the peptide concentrations as indicated, and octyl glucoside was 0.1%. CaCl2 was 2.8 mM in the presence of 3 mM EGTA at pH 7.2, and reactions were for 5 min at 30 °C. Each point represents duplicate determinations, and the experiment was repeated twice.

100 μM (Fig. 5A). B-N20K was cross-linked to wild type β1γ2 and immunoblotted with β subunit antibodies to confirm that this peptide behaved like unmodified N20K in the cross-linking reaction (Fig. 5B). A shift to higher molecular weight was observed as has been seen for unmodified N20K. One major cross-linked species was observed, and one very minor species appeared with longer reaction times, confirming that the procedure could be used to monitor specific reaction of the peptide with the β subunit at a single site.

We went on to determine whether we could detect the cross-linked B-N20K cross-linked to β using HRP-NeutrAvidin. This method detected only one cross-linked species at a molecular weight corresponding to the cross-linked species detected with β subunit-specific antibody (Fig. 5C, indicated by the arrow on the right). Consistent with our previous report (10), PLC β2 inhibits cross-linking of B-N20K peptide to β1, further demonstrating that the biotin modification does not affect the binding properties of the peptide and that the site of peptide cross-linking represents a binding site for PLC β2 on βγ subunits.

Panel of Biotin-labeled Peptide Cross-linked to Wild Type and Cysteine Mutant β1γ2 Dimers—Fig. 6A displays the results of a comprehensive cross-linking screen of B-N20K to wild type β1γ2 and all 14 cysteine mutants. Only the cysteine 25 alteration eliminated peptide cross-linking, whereas the remaining 13 cysteine mutations did not significantly affect peptide cross-linking to β1, indicating that the major peptide cross-linking site is cysteine 25 of β1. Whereas the other mutations may have had a minor effect on cross-linking, it is difficult to make conclusions as to their significance with this method since it is not strictly quantitative. Clearly, however, in multiply repeated experiments the only mutation that consistently resulted in a clearly significant reduction in cross-linking of the peptide was Cys25. As discussed, Cys103, Cys114, Cys121, Cys148, and Cys271 show double bands with the upper band corresponding to Sf9 β that copurifies with the mutant β subunit (Fig. 3). We purified Sf9 β (see under “Experimental Procedures”) to determine whether the peptide cross-links to this β subunit. We were unable to detect cross-linking of B-N20K to Sf9 β subunit (Fig. 6B). This indicates that it is not Sf9 β contamination of the preparation that is responsible for the cross-linking observed with any of the cysteine mutants.

Cross-linking of Peptide to Trypsin-digested Wild Type and C Mutant β1γ2 Dimers—To confirm further that peptide cross-linking to the Cys25 mutant is eliminated, we performed trypsin cleavage to isolate the amino-terminal fragment of β1, where cysteine 25 is located and assayed for a shift in the molecular weight of this fragment in the presence or absence of N20K (not biotinylated) and cross-linker. In the native state only one of the 32 potential trypsin cleavage sites in β1 (arginine at position 129) is accessible to trypsin. Thus trypsin cleavage of the native βγ complex produces two fragments of 14- and 24-kDa (amino and carboxyl termini, respectively) (16). Fig. 7 shows immunoblots from a cross-linking experiment of trypsin-digested wild type and cysteine mutant β1γ2 complexes in the presence and absence of N20K, detected with an amino-terminal specific anti-β1 antibody. The cross-linking reaction resulted in appearance of an immunoreactive species at ~17 kDa (top arrow), only in the presence of peptide, for wild type β1γ2, Cys103, and Cys114. The apparent molecular weight of the cross-linked band corresponds to what would be expected if one peptide of ~2.5 kDa is covalently attached to the amino-terminal fragment of 14 kDa. No cross-linking to the amino-terminal fragment was observed for Cys25 confirming that Cys25 is a site of N20K cross-linking.
Mapping of a PLC-binding Site on Gβγ

Studied to determine effector-binding sites on Gβγ suggest that effectors bind to various regions along the Gβγ surface and that each effector will have its own characteristic set of contact points or footprints along the sides of the Gβγ complex. These contact points may serve to define the specificity of interactions between Gβγ and its effectors. All of the mapping studies to define these footprints are based on measurement of the distance between cysteine residues in protein mutants. Although we used site-directed mutagenesis, the site but rather directly assesses the location of a specific cross-linking site. Our mutations were designed to be important for effector binding (5–7). Here, we take an alternative approach that does not require prediction of the binding region, blade 2, is in yellow. The blades in the β-propeller are numbered according to the nomenclature of Sprang and co-workers (4). βCys25 and Leu20 are in yellow. The blades in the β-propeller are numbered according to the nomenclature of Sprang and co-workers (4). βCys25 and Leu20 are shown in a space-filling representation, and the distance between the Cys25 sulfur atom and an Leu20 methyl carbon is indicated. The amino-terminal helix of β involved in a coiled-coil interaction with γ is in red.

DSCUSSION

This study clearly defines the Cys25 as a site of peptide cross-linking. Cysteine 25 is located in the βγ amino-terminal coiled-coil region (Fig. 8). The cross-linker SMOC can link the sulfhydryl groups on cysteine residues to ε-amino groups on lysine that are separated by a maximum of 11.6 Å (Pierce (17)). Cross-linking of the peptide to cysteine 25 is not consistent with its binding to three previously identified PLC-binding sites, blades 2, 6, and 7. The closest of these three blades to Cys25 is blade 6. The most proximate amino acid to Cys25 on propeller blade 6 is 25 Å away. Given a lysine side chain length of 5 Å and the 11.6-Å cross-linker length, the peptide could not be binding to this region and cross-linking to cysteine 25. The closest amino acid on the surface of another putative PLC-binding region, blade 2, is 40 Å away if measured in a direct line from cysteine 25 through the protein. The distance along the surface of the protein is of course even greater. Thus the peptide cannot be binding to blade 2 and cross-link to cysteine 25. A similar argument can be made for blade 7, which is 50 Å away from Cys25. Given the length of the cross-linker the most reasonable sites for binding are the amino-terminal coiled-coil region and the sides of blade 5 or 4. We do not conclude that the results from our study and the results from site-directed mutagenesis are mutually exclusive. Given the size of PLC β2, it is possible that the enzyme can interact with multiple regions of the β subunit. Our study simply identifies an interaction site that was not implicated in other studies of mammalian β subunits.

Supporting these ideas is the observation that none of the other cysteine mutations significantly affect cross-linking. In
the cross-linking study by Garcia-Higuera et al. (11) that utilized this same approach to identify a subunit interaction sites, Cys204 and Cys271 were both found to be responsible for cross-linking of α to β by bismaleimido-hexane (16-Å cross-linker). The mutation at cysteine 25 had no effect on α subunit cross-linking but did prevent cross-linking to γ2 in their study. Thus two highly accessible cysteine residues are present at the α subunit interaction interface, but apparently they are not the major site of cross-linking of the N20K peptide. In particular Cys271 is in the loop region connecting blades 5 and 6. If the peptide were binding to blade 6 one might expect this cysteine to be a cross-linking site. This also suggests that if the peptide were binding in the α subunit interaction region it would be detected. Within or near blade 2 of the β-propeller are cysteines 114, 121, 148, 149, and 166, none of which appear to be critical for cross-linking of the peptide. However, these amino acids may not be accessible to the cross-linker since they are mostly buried and not clearly accessible to solvent. The critical data supporting a binding site other than blades 2 and 6 is that a cross-linking site at Cys25 has been identified that is not within range of these regions. The lack of a major effect of mutation of cysteine residues that are within range of these regions supports this result and speaks to the specificity of the cross-linking of the peptide.

This general location is in contrast with what has been found in some mutagenic studies but correlates well with an effector interaction domain identified in a screen for dominant negative yeast Gβγ mutants (9). Recent studies show that mutation of some of the residues in the amino terminus of yeast β subunits responsible for the dominant negative phenotype eliminates binding to Ste20p, the yeast homologue of mammalian PAK (18). Other mutants in the coiled-coil region of yeast β subunits (Leu65 and Leu49) are unable to bind to the scaffolding protein Ste6p (19). These data strongly suggest that the amino-terminal coiled-coil region is important for effector binding in yeast. Evidence for involvement of this region in interactions of mammalian β subunits with effectors is lacking. One study (20) of this region with a series of single point mutations found that some of the mutations had dramatic effects on assembly with γ subunits, whereas those mutants that did assemble had little effect on the ability of βγ subunits to activate JNK in a COS cell transfection assay. A possible explanation for this is that mammalian effectors may have contacts with other regions of the β subunit, and as such single point mutations in this region are not sufficient to disrupt effector interactions in overexpression assays. Our data support the idea that this region is important for binding of effectors to mammalian β subunits and that this interaction is important for regulation since binding of a peptide to this region blocks the ability of βγ to activate effectors.

The amino-terminal coiled-coil region of the β subunit is far from the α-interacting region located at the top of the β subunit complex making contact with blades 2 and 7 of the propeller. This general location for peptide cross-linking is consistent with our previous finding that α subunit does not block cross-linking of peptide to βγ (10), and our data show that α(GDP) subunits do not completely prevent PLC from binding to β and thereby preventing cross-linking of peptide to β. This latter finding suggests that perhaps PLC could bind near the amino-terminal region of β even in the presence of the α subunit. This idea is supported by the results of another group (21) who propose α(GDP) can bind to βγ-PLC β2 complex to form α(GDP)βγ-PLC β2. Thus, the site we have mapped may represent a region where PLC can remain bound to βγ even in the presence of α. We can envision a model in which effector and G protein heterotrimeric are precomplexed and poised for immediate activation. The top surface of the βγ complex may be critical for effector activation and may become available to the effector upon activated receptor-induced activation of α subunits without a requirement for a potentially rate-limiting dissociation of α subunits or association of PLC β2. Deactivation could involve occlusion of the effector-binding site on β by α(GDP) subunits with the βγ-PLC β2 complex without dissociation of PLC β2 from βγ. Identification of a binding site for PLC that does not overlap with α subunits supports this hypothesis and provides a starting point for understanding molecular interactions that could allow PLC to remain associated with βγ subunits in the presence of bound α subunits.

REFERENCES
1. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
2. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
3. Lambright, D. G., Sondek, J., Bohen, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311–319
4. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
5. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reveney, E., Seshiar, L. R., Rosal, R., Weng, G., Yang, C.-S., Iyengar, R., Miller, R., Jan, L. Y., Lefkowitz, R. J., and Hamm, H. E. (1998) Science 280, 1271–1274
6. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kosaza, T. (1998) J. Biol. Chem. 273, 16265–16272
7. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kosaza, T., and Neer, E. J. (1998) J. Biol. Chem. 273, 28296–28304
8. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) Science 283, 1332–1335
9. Leberer, E., Dignard, D., Hougan, L., Thomas, D. Y., and Whiteway, M. (1992) EMBO J. 11, 4805–4813
10. Sankaran, B., Osterhout, J., Wu, D., and Smrcka, A. V. (1998) J. Biol. Chem. 273, 7146–7154
11. Garcia-Higuera, L., Thomas, T. C., Yi, F., and Neer, E. J. (1996) J. Biol. Chem. 271, 528–535
12. Romoser, V., Ball, R., and Smrcka, A. V. (1996) J. Biol. Chem. 271, 25071–25076
13. Kosaza, T., and Gilman, A. G. (1995) J. Biol. Chem. 270, 1734–1741
14. Mumby, S. M., and Linder, M. E. (1994) Methods Enzymol. 237, 254–268
15. Smrcka, A. V., and Sternweis, P. C. (1993) J. Biol. Chem. 268, 9671–9674
16. Thomas, T. C., Sladek, T., Yi, F., Smith, T., and Neer, E. J. (1995) Biochemistry 32, 8628–8635
17. Means, G. E., and Feeney, R. E. (1995) Bioconjugate Chem. 1, 2–12
18. Lamb, T., Wu, C., Schrag, J. D., Whiteway, M., Thomas, D. Y., and Leberer, E. (1998) Nature 391, 191–195
19. Dowell, S. J., Bishop, A. L., Dyos, S. L., Brown, A. J., and Whiteway, M. S. (1998) Genetics 150, 1407–1417
20. Pellegrino, S., Zhang, S., Garristen, A., and Simones, W. F. (1998) J. Biol. Chem. 272, 25360–25366
21. Runnels, L. W., and Scarlata, S. F. (1998) Biochemistry 37, 15563–15574
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