Dynamic regulation of G-protein signaling in the phototransduction cascade ensures the high temporal resolution of vision. In a key step, activated $\alpha$-subunit of transducin ($\text{G}_\alpha$-GTP) activates the cGMP phosphodiesterase (PDE) by binding the inhibitory $\gamma$-subunit (PDE$\gamma$). Significant progress in understanding the interaction between $\text{G}_\alpha$ and PDE$\gamma$ was achieved by solving the crystal structure of the PDE$\gamma$ C-terminal peptide bound to $\text{G}_\alpha$ in the transition state for GTP hydrolysis (Slep, K. C., Kercher, M.A., He, W., Cowan, C.W., Wensel, T.G., and Sigler, P.B., 2001, Nature 409, 1071). However, some of the structural elements of each molecule were absent in the crystal structure. We have probed the binding surface between the PDE$\gamma$ C-terminus and activated $\text{G}_\alpha$ bound to guanosine 5'-O-[3-thio]-triphosphate (GTP$\gamma$S) using a series of full-length PDE$\gamma$ photoprobe species. For each of seven PDE$\gamma$ photoprobe species, expressed protein ligation allowed one benzoyl-L-phenylalanine substitution at selected hydrophobic C-terminal positions, and the addition of a biotin affinity tag at the extreme C-terminus. We have detected photocrosslinking from several PDE$\gamma$ C-terminal positions to the $\text{G}_\alpha$-GTP$\gamma$S N-terminus, particularly from $\text{G}_\alpha$ residue 73. The overall percentage of crosslinking to the $\text{G}_\alpha$-GTP$\gamma$S N-terminus was analyzed using a far-Western method for examining $\text{G}_\alpha$-GTP$\gamma$S proteolytic digestion patterns. Furthermore, mass spectrometric analysis of crosslinks to $\text{G}_\alpha$ from a benzoyl-phenylalanine replacement at PDE$\gamma$ position 86 localized the region of photoinsertion to $\text{G}_\alpha$ N-terminal residues $\text{G}_\alpha$(22-26). This novel $\text{G}_\alpha$/PDE$\gamma$ interaction suggests that the transducin N-terminus plays an active role in signal transduction.

To date, X-ray crystallography (1-4) has described many key features of G-protein/effecter interactions. However, the majority of X-ray structures of G-protein $\alpha$-subunits, including the $\alpha$-subunit of transducin ($\text{G}_\alpha$), lack information concerning the organization of their N-terminals. For example, X-ray analysis of the complex formed between the PDE$\gamma$ C-terminus, $\text{G}_\alpha$, and the catalytic domain of the ninth member of the Regulator of G-Protein Signal family (RGS9), required prior removal of the $\text{G}_\alpha$ N-terminus (1). Intriguingly, the N-terminus was present in the X-ray structure of the transducin $\text{G}_\alpha_\beta_\gamma$ heterotrimer (4) where it adopts an extended $\alpha$-helix participating in extensive contacts with transducin $\beta$-subunit. This interaction, called the N-terminal interface, contributes
roughly one-third of the total binding energy of \( \Gamma \alpha - \Gamma \text{GDP} \) towards the \( \beta \)-subunit (4). The photocrosslinking study reported here extends the role of the \( \Gamma \alpha \) N-terminus to interactions with \( \Gamma \text{DE} \gamma \), suggesting a possible regulatory role for the G-protein N-terminus in interactions with effectors.

The extreme \( \Gamma \alpha \) N-terminus is myristoylated (5, 6). This myristoyl group is required for formation of the resting state \( \Gamma \alpha \beta \gamma \) heterotrimer (4, 7), but is not primarily responsible for membrane attachment (8, 9, 10, 11). Compared to other G-protein \( \alpha \)-subunits that are also palmitoylated, \( \Gamma \alpha \) is uniquely soluble in low-salt buffers or cell cytosol-like buffers, and translocates to the lipid membrane only when in the transition state or its \( \text{AlF}_4^- \) analog (8, 9, 12, 13). Myristoylation of the \( \Gamma \alpha \) N-terminus may modulate transducin activity (8, 14). Although the precise mechanism is not fully understood, intramolecular binding sites for the \( \Gamma \alpha \) N-terminus and its myristoyl group have been proposed (15, 16). The peptidyl portion of the \( \Gamma \alpha \) N-terminus also makes contact with peptidyl sites within \( \Gamma \beta \gamma \). In this work, we demonstrate that the \( \Gamma \alpha \) N-terminus also makes contact with the C-terminus of \( \Gamma \text{DE} \gamma \).

\( \Gamma \text{DE} \gamma \) maintains nanomolar binding affinity to activated \( \Gamma \alpha \) through two discrete regions. The central portion (residues 24-46) and the C-terminal region (residues 46-87) each contribute roughly half of the binding energy towards transducin (17). The \( \Gamma \text{DE} \gamma \) C-terminal region also modulates GTPase-activating protein (GAP) affinity towards transducin, primarily through the action of seven hydrophobic residues (18). The \( \Gamma \text{DE} \gamma \) C-terminus interacts primarily with the \( \alpha \) helix and conserved switch regions (1, 19). It is also thought that key conformational changes in \( \Gamma \text{DE} \gamma \) residue L76 and surrounding residues, initiated by \( \Gamma \alpha \) binding, may displace the \( \Gamma \text{DE} \gamma \) C-terminal domain from the \( \Gamma \alpha \beta \) catalytic site (20). In the X-ray crystal structure of the RGS9-bound model of \( \Gamma \text{DE} \gamma /\Gamma \alpha - \text{AlF}_4^- \) interactions, a peptide \( \Gamma \text{DE} \gamma (50-87) \) interacts primarily with the \( \alpha \) helix of \( \Gamma \alpha - \text{AlF}_4^- \), a binding surface also detected by a peptide truncation study (21) and a photocrosslinking experiment utilizing full-length \( \Gamma \text{DE} \gamma \) (22). In this crystal structure information concerning the \( \Gamma \alpha \) N-terminus or the myristoyl modification was not available.

Fluorescence anisotropy and electron spin resonance (ESR) measurements of the inhibitory G-protein \( \alpha \)-subunit, \( \Gamma \alpha \), revealed that the conformation of the N-terminus is dynamic (15, 16), where activation (i.e. dissociation from the \( \Gamma \beta \gamma \) subunits) results in the ‘melting’ of helical structure within the \( \Gamma \alpha \) N-terminus. Considering the high homology and structural similarity of \( \Gamma \alpha \) to \( \Gamma \beta \) (23) it is plausible to speculate that the local structure of the \( \Gamma \alpha \) N-terminus is also modulated by activation. Perhaps a ‘melted’ \( \Gamma \alpha \) N-terminus may interact with \( \Gamma \text{DE} \gamma \).

We have employed a library of intein-derived, full-length \( \Gamma \text{DE} \gamma \) benzophenone photoprobes, generated by Expressed Protein Ligation (EPL: 24, 25), to screen for photoinsertion into the N-terminus of \( \Gamma \alpha, \Gamma \text{GTP} \gamma \). The limited proteolysis strategy for selective proteolysis of the \( \Gamma \alpha - \Gamma \text{GTP} \gamma \) N-terminus (26, 27) was used to detect photoinsertion into the \( \Gamma \alpha \) N-terminus by two independent methods. Biotin/streptavidin far-Western blot analysis revealed that the W70Z, F73Z, and L81Z and I86Z photoprobes demonstrated significant levels of photoinsertion into the \( \Gamma \alpha - \Gamma \text{GTP} \gamma \) N-terminus. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometric (MALDI-TOF MS) analysis of crosslinked peptide fragments localized crosslinking from \( \Gamma \text{DE} \gamma \) I86Z to \( \Gamma \alpha \) N-terminal residues \( \Gamma \alpha (22-26) \).

**EXPERIMENTAL PROCEDURES**

*Materials-* The IMPACT EPL kit and restriction enzymes were from New England Biolabs. Nickel-nitritotriacetic acid (Ni-NTA) resin and plasmid purification kits were acquired from Qiagen. Endoproteinase Lys-C (endo Lys-C), endoproteinase Glu-C (endo Glu-C), GTP\( \gamma \)S, GDP, 4-(N-maleimido)-
benzophenone (MBP), trifluoro-acetic acid (TFA), N-ε-p-tosyl-L-lysine chloromethyl ketone (TLCK) and acetonitrile (ACN) were purchased from Sigma. POROS R2 resin was purchased from Applied Biosystems. All remaining chemicals were purchased from Fisher Scientific.

Mutagenesis- The polymerase chain reaction (PCR) was used to amplify the PDEγ(1-61) cDNA from a bovine retinal rod PDEγ cDNA (28), using forward (5'-CAT ATG CAT ATG AAC CTC GAG C -3') and reverse (5'-TGC TCT TCC GCA GAT AAC GGT GAT GTC G-3') primers compatible with intein ligation. The amplified DNA was then subcloned into the unique SmaI site of pBlueBac. The fragment was excised with NdeI and SapI, then ligated into the intein pTXBI vector (New England Biolabs).

Escherchia Coli (E. Coli) XL1 Blue cells were transformed with the PDEγ(1-61)/intein pTXBI vector. Plasmid DNA was purified from individual colonies and screened by digestion with XbaI and BamHI. Positive clones were further verified by DNA sequencing. A hexylhistidine (6HIS) affinity motif was added to the PDEγ N-terminus using forward (CAT ATG CAC CAT CAT CAT CAT ATG) and reverse (GTA TAC GTG GTA GTG GTA GTG GTA TAC) primers. Positive clones were further verified by DNA sequencing. The vector containing hexylhistidine-tagged PDEγ(1-61) intein fusion protein cDNA was then transformed into BL21(DE3) cells.

Synthesis of C-Terminal PDEγ Peptides-The PDEγ C-terminal peptides and the rhodopsin (Rh) 3rd intracellular loop peptide VKEAAAQQQESATTQKAEEKVTR, residues 230-252, were synthesized (25 µmol scale) by standard Fmoc chemistry (29) on an Applied Biosystems Synergy 432A peptide synthesizer at the University of Wisconsin Peptide Synthesis Center. The seven PDEγ(62-87ZXG) C-terminal synthetic peptides were created based on the PDEγ C-terminal sequence CDITVIAPEAFNHLEHALAQYGIIIXG where the appropriate benzoyl-L-phenylalanine (Z) and biotinyl-L-lysine/glycine (XG) substitutions were incorporated using the Fmoc derivatives of benzoyl-L-phenylalanine (Bachem) and biotin-L-lysine (Anaspec). The N-terminal residue of each peptide was cysteine to accommodate the intein ligation chemistry, and an additional C68A replacement was made to minimize disulfide formation. The C-terminal peptides were deprotected with 0.3 M dithiothreitol (DTT) in 90% TFA, precipitated three times in tert-butyl-methyl-ether, resuspended in H2O and lyophilized. The sequence and molecular mass of each PDEγ C-terminal peptide is reported as follows: CDITZIAPWEAFNHLEHALAQYGIIIXG (V66Z; [M+H]+ 3557.4), CDITVIAPEAFNHLEHALAQYGIIIXG (W70Z; [M+H]+ 3470.2), CDITVIAPEAFNHLEHALAQYGIIIXG (F73Z; [M+H]+ 3510.1), CDITVIAPEAFNHLEHALAQYGIIIXG (L78Z; [M+H]+ 3545.5), CDITVIAPEAFNHLEHALAQYGIIIXG (L81; [M+H]+ 3543.7), CDITVIAPEAFNHLEHALAQYGIIIXG (I86Z; [M+H]+ 3543.0).

Intein-Mediated Synthesis and Purification of PDEγ Photoprobes- The PDEγ(1-61)/intein fusion protein was overexpressed in E. coli by standard methods, and purified by the method of Evans (24). After cell lysis by sonication, the PDEγ(1-61)/intein construct was incubated overnight with chitin beads (10 mL per L cell extract) at 4 °C, in the presence of lysis buffer (50 mM NaH2PO4, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF, pH 7.0). Bead-bound PDEγ(1-61) was washed with 10 volumes of high-salt buffer (50 mM NaH2PO4, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF, pH 7.0), followed by 10-mL of low-salt buffer (50 mM NaH2PO4, 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, pH 7.0). Bead-bound PDEγ(1-61) was transferred to a 50 mL Falcon tube and then eluted with an equal volume of 0.5 M β-mercaptoethanesulfonic acid (MESNA) in low-salt buffer. The activated intermediate PDEγ(1-61)/MESNA was aliquoted, and frozen at -80 °C until use. Preparations were stable for up to one month. To initiate intein ligation, 1 mg of C-terminal
PDEγ peptide was solubilized in 50-uL DMSO, then added to a 2-mL aliquot of PDEγ(1-61)/MESNA eluate. The EPL mixture was then incubated for 24 hours at room temperature (22-24 °C). The resultant full-length PDEγ photoprobes were purified over Ni-NTA resin (Qiagen) in the presence of a urea-containing column wash buffer (20 mM NaH2PO4, 8 M urea, 20 mM β-mercaptoethanol, pH 7.0). After washing with 10 column volumes of wash buffer, photoprobes were eluted in batch using low-pH imidazole buffer (100 mM NaOAc 250 mM imidazole, 8 M urea, 20 mM β-mercaptoethanol, pH 4.0). Full-length photoprobes were purified from excess PDEγ(1-61) by HPLC on a self-packed POROS R2 column, utilizing an acetonitrile gradient from 10-50% over 40 minutes, in the presence of 0.1% TFA. The PDEγ concentration was determined by the Bradford/Lowry method (30) and also by the level of Coomassie staining when analyzed using SDS-polyacrylamide gel electrophoresis (PAGE) gels. The full-length PDEγ photoprobe was lyophilized on a speed-vacuum centrifuge (Savant) and stored at -80 °C.

Mass spectrometric measurement by MALDI-TOF MS identified molecular masses consistent with those of appropriately modified full-length photoprobes, as determined using the Protein Prospector software (31). These data, reported as [M+H]+ are as follows: V66Z: 11130.5 (calc. 11132), W70Z: 11179.5 (calc. 11180), F73Z: 1129.4 (Na+ salt, calc. 1128), L76Z: 11140.8 (calc. 11140) L78Z: 11137.4 (calc. 11140) L81Z: 11138.2 (calc. 11140), I86Z: 11137.5 (calc. 11140).

Preparation of Maleimido-Benzophenone PDEγ photoprobes- The F73C and I87C PDEγ polypeptides with a C68A mutation were prepared as described previously (32). The 4-(N-maleimido)benzophenone (MBP) derivative was prepared as described for PDEγ peptides (33) with minor modifications. The appropriate PDEγ polypeptide was treated with a 15 fold excess of 4-(N-maleimido)benzophenone, in the presence of 40 mM NaH2PO4 (pH 6.7, 10 mM EDTA, and 40% ACN). The polypeptide photoprobe was purified over a self-packed POROS R2 column, which eluted at ~37% percent acetonitrile when chromatographed at 0.7% per minute, with 0.1% TFA.

Purification of Gαγ-GTPγS and Gαγ(26-350)-GTPγS- ROS membranes were purified from dark-adapted frozen retinas (W.L. Lawson Co). Both the Gαγ-GTPγS and Gβγ subunits were released from the purified ROS membranes then purified over a Blue Sepharose CL-6B column by standard methods (34). Purity of Gαγ-GTPγS was assessed by SDS-PAGE. To generate Gαγ(26-350)-GTPγS, Gαγ-GTPγS was first treated with endo Lys-C with 1:100 (w/w enzyme:protein) for 18 hours, and Gαγ(26-350)-GTPγS was isolated over Blue Sepharose as described for intact Gαγ-GTPγS.

Recombinant RGS9/Gβ5 for use in GTPase turnover measurements was obtained by published procedure (35).

GTPase Assays- The ability of each PDEγ photoprobe species to stimulate the RGS9-catalyzed GTPase activity of Gαγ was measured using the single turnover approach (36, 37). The assays were conducted at room temperature (22-24 °C) in a buffer containing 25 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 8 mM MgCl2. Urea-treated ROS membranes, lacking endogenous RGS9 activity, were used as a source for the photoexcited rhodopsin required for transducin activation. The reactions were initiated by the addition of 10-µL of 0.6 µM [32P] GTP (~10^5 disintegrations/min/sample) to 20 µL of urea-treated ROS membranes (20 µM final rhodopsin concentration) reconstituted with transducin heterotrimer (1 µM) and recombinant RGS9-Gβ5 complex (0.5 µM). The reactions were performed either in the absence or presence of PDEγ derivatives (1 µM). The reaction was stopped by the addition of 100 µL of 6% perchloric acid, then 32P, formation was measured with activated charcoal as described (37).
Photocrosslinking of PDEγ Benzophenone Photoprobes to Gaγ-GTPγS - To initiate photocrosslinking experiments 20 μg of PDEγ photoprobe was incubated with 100 μg Gaγ-GTPγS for 20 minutes at room temperature. The total reaction volume was 200-μL, in crosslinking buffer comprised of 20 mM Tris (pH 7.4), 100 mM NaCl, and 8 mM MgCl2. Samples were irradiated with 350 nm UV light in 10 min. increments for a total of 30 min. in a Rayonet UV irradiator (Southern New England Ultraviolet Company, CT), at 4 °C. The percentage of PDEγ photoinsertion into Gaγ-GTPγS, relative to the total quantity of Gaγ-GTPγS present in each crosslinking reaction, was determined from Coomassie-stained gels by measuring the intensity of both the crosslinked and unreacted Gaγ-GTPγS. Because PDEγ stains poorly on the 15% SDS-PAGE gels relative to Gaγ-GTPγS, no adjustment was necessary to account for PDEγ content in the crosslinked bands.

Endo Lys-C Digestion of PDEγ/Gaγ-GTPγS Crosslinks and Far-Western Analysis - Limited digestion experiments were performed as described previously with minor modifications (26, 27). Endo Lys-C digestion of crosslinks, which removes the transducin N-terminus selectively and the N-terminal half of PDEγ, was performed for 18 hours at room temperature (1:100 w/w enzyme:total protein). Aliquots (2- or 5-μL) were electrophoresed on duplicate 15% SDS-PAGE minigels (Hoefer). Protein bands were either stained with Coomassie Blue or transferred to nitrocellulose membranes as described in the subsection on analytical methods. The extent of PDEγ photoprobe crosslinking to the Gaγ-GTPγS protein core (‘Internal XL’) was determined by first measuring the relative level of biotin-positive signal representing intact, crosslinked PDEγ/Gaγ-GTPγS species (50 kDa) prior to limited digestion, termed here ‘Total XL’. Limited endo Lys-C removes crosslinks from PDEγ to the Gaγ N-terminus, leaving only unreacted Gaγ at 36 kDa and/or PDEγ-crosslinked Gaγ(26-350)-GTPγS at 41 kDa. The crosslinked band at 41 kDa reflects proteolytic removal of the PDEγ N-terminal half as well as the Gaγ N-terminus. The relative level of biotin-positive signal remaining associated with the 41 kDa band, representing crosslinks to Gaγ(26-350)-GTPγS, is denoted as ‘Internal XL’. The ratio of ‘Internal’ crosslinking to ‘Total’ crosslinking was defined as percent of ‘Internal’ crosslinking. The percent photoinsertion into the Gaγ-GTPγS N-terminus (N-Terminal XL) was inversely proportional to the degree of ‘Internal’ photoinsertion.

Endo Glu-C Digestion of PDEγ/Gaγ-GTPγS Crosslinks and HPLC Purification of Fragments - After crosslinking was performed, the unpurified reaction products were treated directly with endo Glu-C protease at a ratio of 1:20 w/w enzyme/protein. The digest mixture was allowed to incubate 18 hours at room temperature, quenched with 100 μM TLCK, and frozen at -80 °C. Protein fragments were purified by HPLC over a POROS R2 column, utilizing a steep gradient from 10 to 90% ACN over 20 minutes. Individual 1-mL fractions were collected, frozen, and dried by vacuum centrifugation.

MALDI-TOF MS Analysis of Crosslinking Reactions - Individual, lyophilized HPLC fractions subjected to the endo Glu-C digestion procedure were resuspended in 5-μL of a solution of 20% ACN. A 0.5-μL aliquot of each fraction was spotted onto an individual well on the MALDI target. To this, 0.5-μL of saturated α-cyano-4'-hydroxyl-cinnamic acid (CHCA) matrix solution (70% ACN, 0.1% TFA) was added. Samples were analyzed on a Bruker REFLEX II MALDI-TOF mass spectrometer (Billerica, MA) using a 337 nm N2 laser and both positive reflectron and positive linear modes. All spectra (at least 50 shots) were calibrated with an appropriate combination of mass standards including bradykinin (1060.6), neurtensin (1672.9), insulin (5734.6) and cytochrome C (12360).

Analytical Methods - SDS-PAGE (15%) was performed as described by Laemmli (38), and
tricine SDS-PAGE (16.5%) was performed by the method of Schagger and von Jagow (39). Protein molecular weight standards from Sigma and prestained standards from Bio-Rad were used to approximate molecular weight. The homogeneity of PDEγ photoprobe preparations was assessed using 16.5% Tricine gels, and all other experiments were performed with 15% SDS-PAGE gels. Protein bands were transferred to 0.2 µm nitrocellulose (Pierce Biotechnology) and subjected to far-Western analysis according to the manufacturer’s instructions. Enhanced chemiluminescence (ECL) detection of biotin was performed using streptavidin-conjugated horseradish peroxidase (HRP) and ECL reagents obtained from Pierce Biotechnology. Densitometric measurements of Coomassie-stained gel bands and of far-Western blots were acquired with a HP ScanJet laser scanner and quantified with NIH Image.

RESULTS

Characterization of PDEγ Photoprobes- All seven full-length PDEγ photoprobes (Fig. 1A) were purified to homogeneity. The progress of the two-step purification by Ni-NTA chromatography, followed by HPLC, is illustrated for the I86Z photoprobe (Fig. 1B and 1C). Purified photoprobe preparations were homogeneous as judged by SDS-PAGE analysis (Fig 1D, lane 1). Analysis of two PDEγ-based standards (Fig. 1D, lane 2), demonstrated that the apparent molecular weight of the full-length PDEγ photoprobes on the SDS-PAGE gel was greater than both wt PDEγ (MW ≈ 11kDa) and the PDEγ(1-61) fragment (MW ≈ 7 kDa). The anomalous apparent migration at approx. 14 kDa was presumably due to the 6HIS and biotin/glycine additions. MALDI-TOF mass spectrometry (see ‘Experimental Procedures’) indicated, however, that these photoprobes attained the correct molecular mass.

The maximal percentage of photoinsertion of the seven PDEγ C-terminal photoprobes into Gαγ-GTPγS was evaluated at saturating concentrations of Gαγ-GTPγS (Fig. 2A and 2B). The F73Z photoprobe demonstrated the highest level of crosslinking to Gαγ-GTPγS. In contrast, a low level (approximately 10%) of the V66Z photoprobe to Gαγ-GTPγS was detected on Coomassie-stained SDS-PAGE gels, even at saturating concentrations of PDEγ. The W70Z, L76Z, L78Z L81Z and I86Z photoprobes showed greater than 30% crosslinking.

The functional activity of each PDEγ photoprobe was determined by its ability to stimulate the RGS9-catalyzed GTPase activity of Gαt in reconstituted membrane preparations (Fig. 3A). The role of PDEγ in this reaction is to enhance the affinity between activated Gαt and RGS9 (40), and it has been demonstrated that the 6HIS addition to PDEγ N-terminus has no effect on functional activity towards the Gαt-GTPase or the cGMP phosphodiesterase catalytic core (37). All PDEγ photoprobes except W70Z were functionally active and retained between 50% and 80% of the wild type PDEγ activity. The activity of W70Z PDEγ photoprobe was severely impaired and amounted to no more than 5% of the wild type PDEγ activity, consistent with a previous report that alanine replacement at this position abrogated the PDEγ GTPase activating protein (GAP) activity (18). A species of the F73Z photoprobe lacking the biotin-lysine/glycine modification retained wild-type activity levels (data not shown). Although the F73Z photoprobe containing the biotin modification retained only 70% activity in the turnover assay, percent crosslinking for both the F73Z with biotin and the F73Z photoprobe lacking biotin were identical (data not shown).

The specificity of crosslinking of PDEγ photoprobes to Gαγ-GTPγS was addressed in protection experiments (Fig. 3B). Protection of PDEγ/Gαγ-GTPγS crosslinking by the prior addition of wt PDEγ was complete, demonstrating the specific nature of the light-dependent photoinsertion. The dark control confirmed that crosslinks failed to form in the absence of UV irradiation. Irradiation with 350 nm UV light as described under ‘Experimental Procedures’ resulted in the
formation of the 50 kDa band representing PDEγ/Gαγ-GTPγS crosslinked species. Furthermore, PDEγ photoinsertion into Gαγ-GTPγS was blocked by the addition of 20 µM wild-type PDEγ. In contrast, addition of a large excess (350 µM) of a peptide representing the 3rd intracellular loop of rhodopsin had minimal effect on the extent of Gαγ-GTPγS crosslinking.

To determine the extent of photoinsertion of the F73Z and I86Z PDEγ photoprobes into the Gαα(GDP)βγ heterotrimer, crosslinking experiments were performed with 10 µM of either PDEγ photoprobe in the presence of 12 µM Gαα(GDP)βγ. Photoinsertion of the F73Z and I86Z photoprobes into the heterotrimer was minimal (Fig. 3C). The F73Z photoprobe crosslinked to Gαα to less than 5%, and the I86Z photoprobe did not exhibit a measurable level of photoinsertion into Gαα(GDP). Crosslinking to the β-subunit was not observed in either case. These experiments confirmed that the PDEγ photoprobes crosslinked primarily to activated Gαγ-GTPγS.

Analysis of PDEγ/Gαγ-GTPγS Crosslinks by Limited Proteolysis and Far-Western Blot

After photoactivation, crosslinking of intact PDEγ photoprobes to intact Gαγ yielded a 50 kDa crosslinked band, which contained the biotin-labeled crosslinks. The biotin-positive PDEγ/Gαγ crosslinked complexes were detected by far-Western analysis (Fig. 4A), utilizing streptavidin-conjugated HRP to visualize the biotin incorporated into the PDEγ C-terminus. Limited proteolysis of the PDEγ/Gαγ crosslinked species with endo Lys-C selectively removed the Gαα N-terminus. If PDEγ were crosslinked only to the Gαα N-terminus, all PDEγ crosslinks would have been removed concomitantly by limited digestion. In this case the trimmed Gαα(26-350) molecule lacking the biotin-labeled crosslink would form a 36 kDa gel band that would remain silent in far-Western analyses with streptavidin.

In contrast, post-crosslinking proteolytic removal of the Gαα N-terminus with endo Lys-C would not remove PDEγ crosslinks that inserted into Gαα(26-350), termed here ‘Internal’ crosslinks. The PDEγ portion of the crosslink is split into a 5 kDa C-terminal fragment (residues 46-87XG), as well as several smaller PDEγ N-terminal fragments. Because all the benzoyl-L-phenylalanine replacements reported in this work occur beyond position V66, the biotinylated PDEγ C-terminus does not become separated from PDEγ/Gαα ‘Internal’ crosslinks. In this case, the species representing PDEγ(46-87XG) crosslinked to Gαα(26-350) migrated as a 41 kDa (36 kDa + 5 kDa) band on SDS-PAGE gels, which was visible in streptavidin-based far-Western experiments.

The degree of insertion of the PDEγ benzophenone photoprobes into the N-terminus of Gαγ-GTPγS was assessed by first measuring the percentage of biotin-labeled ‘Internal’ crosslinks remaining on the Gαα(26-350) after limited endo Lys-C proteolysis. These represent crosslinks to the Gαα ‘core’. Six of the seven photoprobes were screened for photoinsertion into the Gαα-GTPγS N-terminus, with the exception of the V66Z photoprobe, which did not appreciably form crosslinks.

The photoinsertion level of the W70Z photoprobe (Fig. 4A) was moderate, and roughly equally proportioned between N-Terminal and ‘Internal’ crosslinking. Although this replacement had a deleterious effect on GTP turnover (Fig. 2A), the unsaturated aryl rings of the benzophenone made functionally non-productive contacts with Gαα-GTPγS. The primary site of photoinsertion for the F73Z photoprobe was the Gαα-GTPγS N-terminus (Fig. 4A), since limited proteolysis of the Gαα N-terminus removed the majority (>90%) of the biotin-labeled PDEγ crosslinks from Gαα. The L76Z and L78Z photoprobes crosslinked primarily into ‘Internal’ Gαα regions, as indicated by the higher percentage of biotin label remaining covalently bound to Gαα(26-350) after limited
proteolysis. In contrast, the L81Z and I86Z photoprobe crosslinked substantially to both the Goa_s-GTPγS N-terminus as well as to 'Internal' regions. These data demonstrate position-dependent crosslinking from several positions within the PDEγ C-terminus to the N-terminus of Goa_s-GTPγS.

Potential deleterious conformational effects of the biotinyl-lysine/glycine addition to the C-terminus of the intein-derived photoprobe were evaluated in an independent experiment using two PDEγ photoprobe species incorporating a maleimido-benzophenone moiety at either position 73 (F73CysMBP) or 87 (I87CysMBP). In this experiment, the full-length PDEγ photoprobe contained a maleimido-benzophenone addition to a cysteine engineered into the appropriate position but retained the intact, unmodified extreme PDEγ C-terminus (i.e. no biotin addition). The F73CysMBP photoprobe (Fig. 4B, lane 1) crosslinked to Goa at a level similar to the F73Z intein-based photoprobe. Furthermore, limited endo Lys-C digestion completely reduced the PDEγ/Goa crosslinked molecule (50 kDa) to the 36 kDa Goa_s(26-350) species (Fig. 4B, lane 2), demonstrating photoinsertion predominantly into the Goa N-terminus. Although the PDEγ I87CysMBP photoprobe crosslinked to Goa at levels higher than that of PDEγ I86Z intein-derived photoprobe (approx. 50%; Fig. 4B, lane 3), limited endo Lys-C digestion resulted in formation of both the 36 kDa Goa_s(26-350) species and the 40-41 kDa trimmed, crosslinked PDEγ/Goa_s(26-350) species (Fig. 4B, lane 4). Therefore, the I87CysMBP PDEγ photoprobe crosslinked to both Goa_s(26-350) and the Goa N-terminus. Since position-dependent photoinsertion into the Goa N-terminus by the F73CysMBP and I87CysMBP PDEγ photoprobe confirmed the results obtained with the intein-derived PDEγ photoprob, we concluded that the presence of the biotinyl-lysine/glycine addition to the PDEγ C-terminus did not significantly affect the conformation of the PDEγ C-terminus when bound to Goa_s-GTPγS.

To confirm the importance of the Goa N-terminus as a photoinsertion site, crosslinking experiments were carried out directly on Goa_s(26-350)-GTPγS, termed ΔNT, where the N-terminus was removed prior to crosslinking (Fig. 4C). In contrast to the robust crosslinking to intact, wild-type Goa_s-GTPγS (lane 1), the F73Z photoprobe did not crosslink appreciably to Goa_s(26-350)-GTPγS (lane 2), emphasizing the role of the Goa N-terminus as the predominant photoinsertion site. Compared to intact Goa_s-GTPγS (lane 3), the I86Z photoprobe retained a substantial degree of crosslinking into Goa_s(26-350)-GTPγS (lane 4) despite prior removal of the Goa N-terminus. This indicated that crosslinking to the Goa protein core was significant for this photoprobe. These data support the results of the far-Western/proteolysis experiments identifying the Goa N-terminus as the primary target of the F73Z photoprobe, while the PDEγ I86Z photoprobe crosslinked to both the Goa N-terminus and the protease-resistant Goa protein 'core'.

**Direct Detection of PDEγ/Goa_s-GTPγS Crosslinked Peptides by MALDI-TOF MS**

The PDEγ I86Z photoprobe was selected for further analysis of the photoinsertion site within the Goa N-terminus because of the small size of its PDEγ contribution to endo Glu-C digested crosslinks. After crosslinking of the I86Z photoprobe to Goa_s-GTPγS, the reaction mixture was subjected to limited endo Glu-C digestion. Then, the crude digest was chromatographed over a POROS R2 HPLC column. MALDI-TOF MS analysis of individual HPLC fractions was performed to identify PDEγ peptide fragments that were crosslinked to the Goa N-terminus.

A unique, crosslinked peptide was identified at m/z 1900 (Fig. 5A), reflecting the presence of contributions from both PDEγ and Goa_s. The PDEγ contribution of 1325 Da reflects complete endo Glu-C digestion of the PDEγ C-terminus to yield PDEγ(81-87XG), termed Fragment I. The mass of the Goa peptide (574...
Da) identified this peptide as DAEKD (Table I). This signal in the mass spectrum could not be attributed to unreacted $\text{G}_\alpha$-GTP$\gamma$S fragments, excess unreacted PDE$\gamma$ fragments or intramolecularly-crosslinked PDE$\gamma$ fragments. A second peptide signal at m/z 1585 was assigned to either $\text{G}_\alpha$ (16-17) or $\text{G}_\alpha$ (25-26), but could not be assigned to one unique photoinsertion site within $\text{G}_\alpha$. To further validate the light-activated crosslinking approach, control experiments were performed in which identical crosslinking mixtures were treated similarly, but were not photoactivated (Fig. 5B). No analyte signals were detected at m/z 1585 and 1900, which confirmed the uniqueness of the crosslinked peptides to genuine photolabeling experiments.

In the same experiment, a third crosslinked peptide was identified at m/z 1944 (data not shown) from the ninth fraction after chromatography over POROS resin. This peptide eluted at 70% ACN, and reflected crosslinking of an incompletely digested PDE$\gamma$ C-terminal fragment to $\text{G}_\alpha$ (23-24). In this case, one missed endo Glu-C cleavage event resulted in a slightly larger contribution from the PDE$\gamma$ peptide fragment PDE$\gamma$(78-87XG), termed PDE$\gamma$ Fragment II (Fig. 5C) that contributed 1705 Da to the crosslinked species. A background signal due to the presence of incompletely digested, non-crosslinked PDE$\gamma$ Fragment II was present in the mass spectrum of the dark control that was not subjected to UV irradiation (data not shown).

The digestion experiment was repeated several times in duplicate, and each time the crosslinked peptides were purified over the POROS column. This approach revealed variation in the position of proteolytic cleavage, as well as heterogeneous photoinsertion into the $\text{G}_\alpha$ N-terminus. Overlapping crosslinked peptide fragments indicated the clustering of photoinsertion sites into the distal portions of the $\text{G}_\alpha$-GTP$\gamma$S N-terminal region, residues 22-26 (Table I). Four unique crosslinked peptides were identified. The smallest sequence identified in these experiments was AE(23-24) at 217 Da. The peptide AEKD was also identified in these experiments. Overall, the overlapping nature of peaks identified in this manner confirmed crosslinking of PDE$\gamma$ photoprobes to the $\text{G}_\alpha$-GTP$\gamma$S N-terminus, and localized them primarily to the region $\text{G}_\alpha$(22-26).

DISCUSSION

The $\text{G}_\alpha$ interactions with PDE$\gamma$ are critical for both effector activation and rapid inactivation of $\text{G}_\alpha$ by RGS9. Multiple studies based on X-ray crystallography (1), alanine scanning mutagenesis (18), sequential truncation of PDE$\gamma$ C-terminal residues in a C-terminal peptide study (21) and a crosslinking study (22) revealed that the effector activating region of PDE$\gamma$ is located within the residues 46-87 and that it interacts with the switch II/3 region of $\text{G}_\alpha$. However, some aspects of the molecular mechanism of effector activation remain unknown, particularly regarding the role of the $\text{G}_\alpha$ N-terminus, which is missing in the $\text{G}_\alpha$/PDE$\gamma$/RGS9 crystal structure.

The N-terminus of $\text{G}_\alpha$ plays an important role in $\text{G}_\alpha$ function. First, it serves as the site of $\text{G}_\alpha$ myristoylation. Second, it is one of the interacting sites between $\text{G}_\alpha$ and $\text{G}_\beta$. Third, and most intriguingly, it was suggested to adopt different conformations in the GDP- and GTP-bound forms of $\text{G}_\alpha$ thus serving as an additional, ‘fourth’ switch region in the molecule (15, 16). The latter is evident from electron paramagnetic resonance studies indicating that in the activated $\text{G}_\alpha$-GTP$\gamma$S species devoid of myristate, the $\text{G}_\alpha$ N-terminus adopts a random coil (15). Furthermore, these authors predicted that the N-terminus of an activated $\text{G}_\alpha$ molecule binds intramolecularly to a hydrophobic binding site within $\text{G}_\alpha$ ‘Internal’ regions. It is therefore reasonable to speculate that the PDE$\gamma$ C-terminus, being strongly hydrophobic, would also provide a binding surface for the lipophilic N-terminus of $\text{G}_\alpha$. A second EPR investigation (16) indicated that in properly
myristoylated, activated Gαt species, myristate confers some degree of structure onto N-terminal residues, although it remains to be determined whether the N-terminus folds onto itself or onto Gαt ‘Internal’ regions. The strong homology of Gαt to Gαi suggests that myristate might also influence the conformation of the Gαt N-terminus, and may also influence interactions with PDEγ.

The present crosslinking study employing full-length PDEγ C-terminus with the flexible N-terminus of Gαt. This interaction is contingent on Gαt activation. The PDEγ F73Z photoprobe demonstrated the greatest degree of photoinsertion into the Gαt-GTPγS N-terminal region. In contrast, the L76Z and L78Z photoprobes crosslinked primarily into the Gαt-GTPγS protein core, while the V66Z photoprobe did not substantially photoinsert into Gαt-GTPγS. Photoinsertion into the region Gαt(22-26) was demonstrated for the PDEγ I86Z photoprobe when crosslinked PDEγ/Gαt molecules were proteolytically fragmented using endo Glu-C proteinase, then analyzed by MALDI-TOF MS.

Molecular modeling with energy minimization using the Sybyl v. 6.8 software package (Tripos Associates, St. Louis, MO) determined that the reactive benzophenone carbonyl (crosslinking radius 3.2 Å) could photoinsert into a carbon source 9.8 Å distant from the PDEγ polypeptide backbone. This also includes the length of the phenylalanine-based sidechain (6.6 Å). Either there is direct contact between Gαt and residues in positions 73 and 86 of PDEγ, or the N-terminus of Gαt-GTPγS and C-terminus of PDEγ come within 9.8 Å of each other upon binding without making discrete contacts. Both possibilities indicate association of the Gαt N-terminus with PDEγ upon Gαt activation. This re-arrangement may be important for providing a high affinity interface for the interaction of Gαt with PDEγ.

The position-dependent crosslinking of the PDEγ C-terminal photoprobes to Gαt-GTPγS did not conform to predictions derived from the Gαt-GDP*AlF4 transition state crystal structure of Slep, et al. (1). According to this structure, the L76Z and I86Z photoprobes are unlikely to generate crosslinks to Gαt, because L76 and I86 of PDEγ form instead intramolecular PDEγ/PDEγ contacts. However, the L76Z and I86Z crosslinking data demonstrate both N-Terminal and ‘Internal’ photoinsertion into Gαt. Surprisingly, the F73Z photoprobe crosslinked primarily to the Gαt N-terminus, in contradiction to predictions from the crystal structure (1) that the F73Z photoprobe should form crosslinks only to switch II/α3 region of Gαt(26-350). Finally, the V66Z photoprobe failed to crosslink to Gαt, while the crystal structure predicted robust crosslinking to Gαt(26-350).

The crosslinking experiments described here offer several unique advantages over X-ray crystallographic analysis of PDEγ complexes with Gαt. First, use of full-length PDEγ provides greater affinity for Gαt, much stronger than micromolar Kd’s available for C-terminal peptides (19), such as the PDEγ (46-87) peptide employed in crystallographic analysis. Indeed, this higher affinity of full-length PDEγ allowed study of its interactions with Gαt-GTPγS, while the crystal structure analysis required inclusion of a RGS9 fragment to act as a ‘molecular glue’. Furthermore, in terms of diffractable data within the X-ray structure, PDEγ and RGS9 were represented by fragments spanning no more than half their sequence, which could introduce some artifacts during crystallization.

Although it is possible that incorporation of benzophenone and biotinyl-lysine components may perturb the interaction of PDEγ with Gαt, we have tried to mitigate this potential by replacing only hydrophobic amino acids with benzoyl-L-phenylalanine, and incorporating the biotinyl-lysine/glycine affinity motif at the extreme PDEγ C-terminus. Furthermore, position-dependent photo-insertion into the Gαt-GTPγS N-terminus was confirmed using
the F73ZCysMBP and I87ZCysMBP PDE_γ photoprobe, which did not require modification of the extreme PDE_γ C-terminus.

Overall, the crosslinking data suggest new interactions between the PDE_γ C-terminus and Ga_α in the ‘Signaling State’, which involve the first 25 N-terminal amino acids of Ga_α and particularly residues 22-26. This finding advances our knowledge about the nature of interaction between transducin and its effector, cGMP PDE. It appears that the C-terminus of PDE_γ interacts simultaneously with the previously identified the switch II/α3 region of Ga_α and a novel switch region, the N-terminus of Ga_α. We speculate that this additional interaction may increase the affinity of Ga_α for PDE_γ with a consequent increase in the efficiency of effector activation by transducin.

Although it is too early to speculate on potential regulatory effects of interactions between the Ga_α N-terminus and PDE_γ, a physical interaction has been detected in this study. Furthermore, the overall pattern of the crosslinking from the seven full-length PDE_γ photoprobe to Ga_α-GTPγS suggests that the pre-catalysis GTPγS-modeled ‘Signaling State’ is distinct from that of the transition state for GTP hydrolysis in the presence of RGS9.

**FOOTNOTES**

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6 The abbreviations used are: Ga_α, transducin α-subunit; PDE, cGMP phosphodesterase; PDE_γ, PDE6 inhibitory γ-subunit; GTPγS, guanosine 5’-O-[3-thio]-triphosphate; RGS9, regulator of G-protein signaling #9; Gaβγ, transducin heterotrimer; GDP, guanosine diphosphate; AlF_4-, aluminum tetrafluoride; GAP, GTPase activating protein; EPL, expressed protein ligation; Ni-NTA, nickel-nitriloacetic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MBP, 4-(N-maleimido)-benzophenone; TFA, trifluoroacetic acid; TLCK, N-ε-p-tosyl-L-lysine chloromethyl ketone hydrochloride; ACN, acetonitrile; PCR, polymerase chain reaction; 6HIS, hexylhistidine; Z, benzoyl-L-phenylalanine; XG, biotin-lysine/glycine affinity motif; DTT, dithiothreitol; MESNA, β-mercapto-ethanesulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; FPLC, fast performance liquid chromatography; ROS, rod outer segments; CHCA, α-cyano-4-hydroxycinnamic acid; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase.

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FIGURE LEGENDS

**Fig. 1.** Synthesis, purification and characterization of full-length PDEγ photoprobes. A. Benzoyl-L-phenylalanine (Z) replacements were made throughout the PDEγ C-terminus (V66, W70, F73, L76, L78, L81 and I86), at hydrophobic positions known to influence PDEγ GAP (GTPase activating protein) activity towards Ga-t-GTPγS. Each photoprobe contained both hexylhistidine (6HIS) and biotin-lysine/glycine (XG) motifs. B. Full-length PDEγ photoprobes were purified from excess synthetic peptide by chromatography over Ni-NTA resin as described in ‘Experimental Procedures’. Lane 1: urea-solubilized synthetic mixture. Lane 2: Elution of PDEγ photoprobe, excess PDEγ(1-61) and gyrA intein fragment from Ni-NTA resin. C. Full-length PDEγ photoprobes (e.g. PDEγ I86Z) were readily purified from excess PDEγ(1-61) and the intein fragment by reverse-phase HPLC as described under ‘Experimental Procedures’, and analyzed by spectrophotometric analysis of the HPLC trace at 215 nm. D. A Coomassie-stained SDS-PAGE gel revealed that the purified PDEγ I86Z photoprobe (lane 1) was >95% pure and migrated at a higher molecular weight compared to either wild-type PDEγ and the PDEγ(1-61) precursor (lane 2).

**Fig. 2.** Percent photoinsertion of each full-length PDEγ photoprobe into Ga-t-GTPγS was assessed by SDS-PAGE analysis. The percentage of PDEγ/Ga crosslink formation was determined as described under ‘Experimental Procedures’. Crosslinking reactions were performed at saturating concentrations of PDEγ, at a molar ratio of at least 1.5:1. Individual aliquots were electrophoresed on 15% SDS-PAGE gels. A. Coomassie-stained gel demonstrating crosslinking yield for each of the seven photoprobes, at saturating concentrations of photoprobe. B. Percent photoinsertion was determined by quantifying the Coomassie staining of both the crosslinked and non-crosslinked transducin bands. Data represent the average of triplicate measurements.

**Fig. 3.** Characterization of PDEγ C-terminal photoprobe functional activity and crosslinking potential. A. The ability of each PDEγ photoprobe to promote GTP hydrolysis by transducin was tested in single turnover GTPase assays as described under ‘Experimental Procedures’. The experimentally determined rate constants of GTP hydrolysis were plotted as percent of that measured for wild type PDEγ after subtracting the rate constant value obtained in the absence of PDEγ. All of the PDEγ photoprobes demonstrated GAP activity similar to that of unmodified PDEγ except the W70Z photoprobe, which was severely compromised. B. Crosslinking from PDEγ to Ga-t-GTPγS is specific, as demonstrated for the F73Z photoprobe. Crosslinking reactions were performed as described under ‘Experimental Procedures’, and analyzed by Coomassie-stained gel: dark control (lane 1), photoinsertion as a result of irradiation at 350 nm (lane 2), and protection by the addition of wt PDEγ at a molar ratio of 2:1 (lane 3). In contrast, the presence of an unrelated peptide from the Rhodopsin (Rh) 3rd loop did not interfere with crosslinking of PDEγ photoprobes to Ga-t-GTPγS (lane 4). C. The ability of the F73Z and I86Z PDEγ photoprobes to photoinsert into the Gα,βγ heterotrimer was examined. The control lane containing irradiated heterotrimer (but no PDEγ photoprobe) demonstrated some faint Coomassie-stained protein bands in the 49-50 kDa range (lane 1). The F73Z photoprobe crosslinked the heterotrimer α-subunit to an extent of less than 5% (lane 2) while the I86Z photoprobe did not demonstrate crosslinking to the transducin heterotrimer (lane 3).
**Fig. 4.** Crosslinking of PDEγ C-terminal photoprobes to the Gαt-GTPγS N-terminus.

A. Screening for biotin by far-Western analysis of crosslinking reactions before (Total XL, “-” lanes) and after (“+” lanes) removal of the Gαt-GTPγS N-terminus by limited endo Lys-C proteolysis revealed the extent of N-Terminal crosslinking for the W70Z, F73Z, L76Z, L78Z, L81Z and I86Z PDEγ C-terminal photoprobes. The percentage of PDEγ crosslinks remaining covalently crosslinked to the Gαt(26-350) core (Internal XL) was compared to the total extent of crosslinking before digestion (Total XL). Percent photo-incorporation into the Gαt-GTPγS N-terminus was measured as described in ‘Experimental Procedures’ and estimated from at least two experiments, where the percent of N-Terminal crosslinking (NTERM) was calculated as (Total-Internal XL).

B. Intact crosslinks between Gαt-GTPγS and the F73CysMBP (lane 1) and I87CysMBP (lane 3) PDEγ photoprobes were treated to limited Endo-Lys C proteolysis as described in Experimental Procedure (lanes 2 and 4, respectively). The presence of crosslinks to either Gαt at 50 kDa or Gαt(26-350) at 41 kDa was evaluated on a 15% SDS-PAGE gel stained with Coomassie Blue.

C. Crosslinking to Gαt(26-350)-GTPγS. Photoinsertion of the F73Z and I86Z intein-based PDEγ photoprobes to Gαt(26-350) was evaluated. The Gαt-GTPγS N-terminus was removed by limited endo Lys-C proteolysis to generate Gαt(26-350)-GTPγS (∆NT), and this truncated species was purified by gel filtration as described in ‘Experimental Procedures’. Photoinsertion of the F73Z and I86Z PDEγ photoprobes into both intact Gαt-GTPγS (lanes 1 and 3) and Gαt(26-350)-GTPγS (lanes 2 and 4) was assessed by SDS-PAGE. Photoinsertion into the Gαt-GTPγS N-terminus is reported as a percentage, as described in ‘Experimental Procedures’.

**Fig. 5.** MALDI-TOF MS identification of PDEγ C-terminal photoprobe insertion into the Gαt-GTPγS N-terminus.

PDEγ/Gαt-GTPγS crosslinks were subjected to limited endo Glu-C proteolysis, and peptide fragments were fractionated by HPLC as described in ‘Experimental Procedures’. MALDI-TOF MS analysis of the chromatographed fractions from photocrosslinking experiments identified several crosslinked peptides. A. **Photocrosslinking experiment.** As an example, the mass spectrum from the seventh HPLC fraction identified two crosslinked peptides at m/z 1585 and 1900. Subtraction of the mass of the completely digested PDEγ C-terminal contribution [PDEγ (81-87XG)] from the mass of XL\_\text{ANT} identified two putative photoinsertion sites, into either Gαt(15-16) or Gαt(25-26). The crosslinked peptide at m/z 1900 (XL\_\text{NTERM}) identified crosslinking between Gαt(22-26) and PDEγ(81-87XG). B. **Dark control.** These peptide signals were completely absent from the mass spectrum of dark control fractions treated similarly. C. **PDEγ contributions to crosslinks.** Complete digestion of the PDEγ C-terminus resulted in the formation of PDEγ(81-87XG), the smallest possible PDEγ contribution to PDEγ/Gαt crosslinks, termed PDEγ Fragment I. In the case of incomplete endo Glu-C digestion of the PDEγ C-terminus, a larger PDEγ contribution to PDEγ/Gαt crosslinks was present. This fragment spanned PDEγ residues PDEγ(78-87XG), and is identified as PDEγ Fragment II.
Table I. Identification of crosslinks from PDEγ 186Z to the Gαt N-terminus \(^a\)

\[
\begin{array}{cccccc}
\text{Observed Mass (m/z)} & \text{Mass of PDEγ Contribution (Da)} & \text{Mass of Gαt Fragment (Da)} & \text{Predicted Mass (Da)} & \text{Gαt Sequence} \\
1585 & 1325 & 260 & 260 & 25-26 or 15-16 \\
1808 \(^e\) & 1325 & 483 + 22 & 483 & 23-26 + Na\(^+\) \\
1900 & 1325 & 575 & 576 & 22-26 \\
1944 \(^e\) & 1705 & 217 + 22 & 217 & 23-24 + Na\(^+\) \\
\end{array}
\]

\(^a\) Tabulation of crosslinking results, showing the masses of crosslinked peptides observed in the MALDI-TOF mass spectrum, the PDEγ contribution to crosslinks and the Gαt contribution to crosslinks.

\(^b\) Sequence of the Gαt N-terminal peptides participating in crosslinks.

\(^c\) The mass of the crosslinked peptides was determined as the [M+H]\(^+\) monoisotopic mass.

\(^d\) The mass of the transducin peptides was determined as the [M+H]\(^+\) monoisotopic mass.

\(^e\) Assigned as the [M+Na]\(^+\) ion.
Figure 1

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I. 

J. 

K. 

L. 

M. 

N. 

O. 

P. 

Q. 

R. 

S. 

T. 

U. 

V. 

W. 

X. 

Y. 

Z.
Figure 2

A.

B.

Position of Benzophenone Replacement

% Photoinsertion into \( \alpha_t \)-GTP/S
Figure 4

A. PDEγ Photoprobe

| Total XL | Internal XL | Endo Lys-C | % N-terminal Photoinsertion |
|----------|-------------|------------|-----------------------------|
| W70Z     | F73Z        | L76Z       | L78Z | L81Z | L86Z |

- + + + + + +

---

50 kDa | 41 kDa

B. F73 CysMBP | I87 CysMBP

| Total XL | Gα1 | Endo Lys-C |
|----------|-----|------------|
| 1        | 2   | 3          | 4         |

- + - +

---

50 kDa | 41 kDa | 36 kDa

C. % Crosslinking to Gα1-GTPγS

|     | 1 | 2 | 3 | 4 |
|-----|---|---|---|---|
| wt  | 50% | 10% | 30% | 20% |
| ΔNT | 5%  | 5%  | 5%  | 5%  |

% Reduction in Crosslinking

- F73Z | I86Z

- + - + + +

90% 49%
Figure 5

A. Photocrosslinking Experiment, Fraction 7

B. Dark Control, Fraction 7

C. PDEγ C-Terminal endo Glu-C fragments

PDEγ(62-87) → PDEγ Fragment I
m/z 1325

C D I T V I A P W E A F N H L E L H E L A Q Y G Z I X G

PDEγ Fragment II
m/z 1705
The N-terminus of GTPγS-activated transducin α-subunit interacts with the C-terminus of the cGMP phosphodiesterase γ-subunit
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