Complementary Interactions of the Rod PDE6 Inhibitory Subunit with the Catalytic Subunits and Transducin

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Activation of the cyclic GMP phosphodiesterase (PDE6) by transducin is the central event of visual signal transduction. How the PDE6 inhibitory γ-subunit (Pγ) interacts with the catalytic subunits (Pαβ) and the transducin α-subunit (αt) in this process is not entirely clear. Here we have investigated this issue, taking advantage of site-specific label transfer from throughout the full-length Pγ molecule to both αt and Pαβ. The interaction profiling and pull-down experiments revealed that the Pγ C-terminal domain accounted for the major interaction with αt bound with guanosine 5’-3-O-(thio)triphosphate (αtGTPγS) in comparison with the central region, whereas an opposite pattern was observed for the Pγ-Pαβ interaction. This complementary feature was further exhibited when both αtGTPγS and Pαβ were present and competing for Pγ interaction, with the Pγ C-terminal domain favoring αt, whereas the central region demonstrated a preference for Pαβ. Furthermore, αtGTPγS co-immunoprecipitated with PDE6 and vice versa in a Pγ-dependent manner. Either Pαβ or αtGTPγS could be pulled down by the Btn-Pγ molecules on streptavidin beads that were saturated by the other partner, indicating simultaneous binding of these two partners to Pγ. These data together indicate that complementary Pγ interactions with its two targets facilitate the αtPDE6 “transducisome” formation. Thus, our study provides new insights into the molecular mechanisms of PDE6 activation.

The intricate visual transduction in rod photoreceptor cells provides a paradigm for G protein–coupled signaling. The outstanding visual sensitivity of the rod is largely due to the great signal amplification achieved by the cGMP phosphodiesterase PDE6 (rod photoreceptor cGMP phosphodiesterase), the central effector enzyme (1). Upon absorption of a single photon, light-excited rhodopsin stimulates an exchange of GTP for GDP bound in the transducin α subunit (αt) (2), which in turn relieves PDE6 from the inhibitory constraint exerted by its γ-subunit (Pγ). PDE6 activation causes rapid cGMP breakdown, which closes the cGMP-coupled ion channels, thus relaying visual signals to the brain in a form of electrical pulses (3). PDE6 in the rod is uniquely composed of a large catalytic heterodimer (Pαβ, ~100 kDa each subunit) to which bind two small identical Pγ subunits (~10 kDa) keeping the enzyme inactive in the dark (1, 4). The PDE6 structure is less well understood compared with the other key players in phototransduction. This is primarily due to the fact that solving the atomic structure of PDE6 has been hindered by the lack of an expression system to produce active Pαβ heterodimers in large amounts (5). A low resolution electron microscopy image of Pαβ has revealed a linear alignment of three distinct domains of each subunit: the tandem GAFa and GAFb domains on the N-terminal side that host non-catalytic cGMP binding and the C-terminal catalytic domain that performs cGMP hydrolysis (6). Direct allosteric communication between GAF domains and the catalytic domain has been recently reported (7).

The inhibitory Pγ subunit is an intrinsically disordered protein, yet structural elements important for its function are encoded in the free Pγ molecule (8). The Pγ sequence of 87 amino acids features a polycationic central domain (Gly19–Gly49) and a negatively charged C-terminal half that contains a linker region (Phe50–Gly61) and a hydrophobic C-terminal domain (Thr62–Ile87) (1, 9). The last C-terminal dozen or so residues (herein termed the inhibitory region) are involved in the interaction with the Pαβ catalytic domain (8, 10, 11). The very recently reported crystal structure of the chimeric PDE5/6 catalytic domain complexed with the Pγ(70–87) inhibitory peptide (5) has confirmed the previous suggestion that the highly hydrophobic C terminus (Y84–Glu87) directly blocks the cGMP entry into the catalytic pocket (12, 13). The other important Pαβ-interacting site on Pγ is the central domain, which has been shown to provide most of the binding strength for Pαβ (14). The central domain of Pγ binds to the Pαβ GAF domain (15, 16) and couples non-catalytic cGMP binding in a positively cooperative manner, thus regulating the PDE-inhibiting function of Pγ (14). Remarkably, the C-terminal domain and the central domain also constitute αt-interacting sites (17–19).

An overlap of the Pγ C-terminal αt-binding region (Thr62–Ile87) and the inhibitory region (Asn74–Ile87) forms the structural basis for transducin-mediated PDE6 activation (5, 8, 20). Various lines of evidence suggest that GTP-bound αt activates PDE6 by physically displacing the inhibitory region of Pγ from...
the αβ catalytic pocket, thus initiating the signaling state of phototransduction (5, 8, 10–12, 20). In the ensuing transition state, αGTP is converted back to the GDP-bound inactive structure, which has lower affinity with Pγ, thus releasing it to reinitiate PDE6 and terminate signaling (3). Fast visual recovery is ensured by great acceleration of the α GTase activity, which is achieved by the GTase-activating protein (GAP) complex composed of αG, Pγ, RGS9-1 (the ninth member of the regulators of G-protein signaling in photoreceptors), and its constitutive partner Gβ5 as well as the membrane anchoring protein R9AP (3, 21). Much of the molecular details of the Pγ-αG interaction in the signaling state have been learned from the crystal structure of the partial transition state complex, which includes the GDP-AlF4⁻-bound αt/i1 chimera, the half-Pγ (Gly46–Ile87), and the catalytic core of RGS9-1 (20). As visualized by this structure, a stretch of Pγ residues around Trp70 forms a tight interaction with αt that is further reinforced by additional contacts provided by some residues in the Pγ inhibitory region. Recent NMR (8) and crystallography (5) studies indicated that when the Pγ inhibitory region was associated with the chimeric PDE6/5 catalytic domain, the critical αt-binding residues Trp70 and Leu76, however, were not involved. These studies lend further support to a model of the PDE6 activation (5, 11); i.e., an engagement of αtGTP with the Pγ residues Trp70 and Leu76 triggers a conformational change involving a hingelike rigid body movement of Pγ(78–87) away from the PDE6 catalytic pocket.

Thus, Pγ plays a pivotal role, not only for turning on but also for turning off phototransduction and keeping the signaling system inactive in the dark (9). Despite a wealth of information regarding phototransduction mechanisms, dynamic interactions of Pγ with αt and Paβ, as well as RGS9-1, are not well understood. There has been controversy as to whether Pγ completely dissociates from Paβ in the process of PDE6 activation. It is possible that whereas αt sequesters the Pγ C-terminal region from the Paβ catalytic domain, the central domain of Pγ stays bound to the Paβ GAF domain until the binding is allosterically reduced by the dissociation of cGMP from the GAF domain (1). This scenario of simultaneous Pγ interactions with both αt and Paβ is consistent with the proposition of an intermediate αt/PDE6 complex during PDE6 activation (17, 22–26). Earlier studies suggested that direct αt/Paβ contacts may be a driving force in forming the intermediate complex in the presence of disc membranes (24, 27). However, it has not been determined whether the Pγ interactions with αt and Paβ contribute important elements to the intermediate PDE6 activation complex.

As presented in this study, the label transfer approach, which has proven to be powerful for systematically detecting interactions of full-length molecules (16, 28, 29), offered us an opportunity to investigate this issue from a unique perspective. The data obtained through label transfer, immunoprecipitation, and pull-down suggest that complementary interactions, in which the Pγ C-terminal domain forms a strong interaction with αt while the central region binds tightly with Paβ, assist the transducin-PDE6 complex formation, which elicits PDE6 activation.

**EXPERIMENTAL PROCEDURES**

The chemicals and reagents used in this study were from the sources described previously (16, 28) unless otherwise stated. The C-terminal Pγ peptide (Pγ(62–87)) was custom-synthesized at the Peptide Synthesis Facility of the Biototechnology Center, University of Wisconsin (Madison, WI).

**Transducin Preparation**—Using frozen dark-adapted bovine retinas (J. A. & W. L. Lawson Co.), rod outer segment (ROS) membranes were isolated, from which holotransducin was prepared as described previously (29, 30). αGDP and Pγt were then purified from holotransducin using a blue Sepharose CL-6B column. To prepare αGTPγS, GTPγS was added to ROS membranes, and αGTPγS was thus released and purified on the blue Sepharose CL-6B column. The purity of αt was determined to be >95% by SDS-PAGE and Coomassie staining. The purified proteins were stored at −80 °C.

**Preparation of PDE6**—The samples of bovine PDE6 were kindly provided by Dr. Nikolai O. Artemyev at the University of Iowa and prepared according to established methods (4). Briefly, holo-PDE6 was extracted from bleached ROS membranes, and Paβ was then obtained by removing Pγ through mild tryptic proteolysis of holo-PDE6. More vigorous tryptic treatment generated the Paβ heterodimer with a nick at Lys146/Lys147 on Pβ. It has been reported that nicked Paβ has unaltered functional properties (12, 16). Unless otherwise stated, “Paβ” refers to nicked Paβ throughout this paper. The Paβ preparations were purified to >95% by a Mono-Q column (Amersham Biosciences), as judged from Coomassie-stained SDS gels.

**Preparation of Pγ Photoprobes**—The constructs for expressing the full-length wild-type Pγ with the single cysteine at position 68 (29), and the single cysteine mutants were generated as described previously (28). They were expressed in E. coli and purified by chitin beads, followed by reversed-phase HPLC using the POROS R2 resin (31). The truncated Pγ variants (29) with and without a His8 tag at the N terminus (His8Pγ(1–61) and Pγ(1–61), respectively) were prepared using the same protocol. Full-length Pγ (>95% pure) was used for preparation of Pγ photoprobes. The radioactive [125I]ACTP-Pγ and nonradioactive [127I]ACTP-Pγ photoprobes were prepared as described earlier (28).

The maleimido benzophenone (mBP)-Pγ photoprobes were prepared as described previously (16). Briefly, Pγ was derivatized with mBP in 10–20-fold molar excess, and mBP-Pγ was then separated from unreacted Pγ and free mBP through reversed phase HPLC. Correct molecular masses of the [127I]ACTP-Pγ and mBP-Pγ photoprobes have been confirmed by electrospray ionization mass spectrometry conducted at the Chemistry Department Mass Spectrometry Facility of the University of Wisconsin (Madison, WI).

**Functional Assay of the Pγ Photoprobes**—The transducin GTase activity assay was kindly conducted by Dr. Kirill A. Martemyanov (now at the University of Minnesota) and Dr. Vadim Y. Arshavsky (now at Duke University), using a single turnover technique as described previously (32). The assay was 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.
The urea-treated ROS membranes, lacking endogenous activity of RGS9-1, 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 (10^8 dpm/sample) to 20 μl of urea-treated ROS membranes (20 μM final rhodopsin concentration) reconstituted with transducin heterotrimer (1 μM) and recombinant RGS9-1-Gβ5 complex (0.5 μM). The reactions were performed in either the absence or presence of Py derivatives (1 μM). The reaction was stopped by the addition of 100 μl of 6% peracetic acid. The 32P formation was measured with activated charcoal. All assays were conducted in the absence of reducing agent due to the presence of the disulfide linkage between the photoreactive group and Py.

**Photocross-linking/Label Transfer Using Py Photoprobes**—A scheme is presented in supplemental Fig. S1A to explain the label transfer strategy. Unless otherwise described, photocross-linking reactions were performed in the HEPES buffer (10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl2). Samples were contained in ultraclear polypropylene microcentrifuge tubes (Axygen). The reactions using [125I]ACTP-Py photoprobes were exposed to the UV light generated by an AH-6 water-jacketed 1000-watt high pressure mercury lamp for 5 s at a distance of 10 cm (28). The reactions with mBP-Py were photolyzed at 5–10°C for 2 × 15 min with a 5-min dark interval on ice in an RPR-100 Rayonet photochemical reactor equipped with a jacketed 1000-watt high pressure mercury lamp for 5 s at a distance of 10 cm (35). The reactions were washed in 32P-Py containing 10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl2). Samples were first equilibrated with the HEPES buffer (10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl2). Samples were subjected to Coomassie Blue staining and autoradiography. Immunoprecipitation Assay of the αiGTPγS-PDE6 Interaction—Co-immunoprecipitation of αiGTPγS with holo-PDE6 was carried out using nProtein A Sepharose Fast-Flow beads (Amersham Biosciences) and the antibody against bovine rod Pα (Affinity Bioreagents). For each reaction, 0.5 μl of Protein A beads were first equilibrated with the HEPES buffer (10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl2) and then incubated with 0.5 μg of the anti-Pα antibody by rotating for 1 h at 4°C. One μg/ml soybean trypsin inhibitor was included to block possible nonspecific protein binding sites on Protein A beads. The beads were washed three times with 300 μl of HEPES buffer prior to the immunoprecipitation reaction. Meanwhile, 0.5 μg of holo-PDE6 was incubated for 1–2 h on ice with 0.5 μg of αiGTPγS in the HEPES buffer containing 50 μg/ml trypsin inhibitor and 1 mM DTT. The reaction was then added to the washed Protein A beads with anti-Pα bound and incubated for 1 h by rotating at 4°C. Py peptide Pγ(1–61) or Pγ(62–87) in a 200-fold molar excess over holo-PDE6 was added as a competitor to disrupt Py interactions. After washing the beads three times with 300 μl of HEPES buffer (containing trypsin inhibitor and DTT), the immunoprecipitate was eluted with the sample buffer and subjected to SDS-PAGE and then detected by Western blotting using the anti-αi antibody (K-20, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)).

Co-immunoprecipitation of Paβ with αiGTPγS was performed similarly but in a reverse manner. Briefly, 1.5 μg of anti-αi was immobilized onto 0.75 μl of Protein-A beads. In separate tubes, 0.95 μg of Paβ was incubated with 0.1 μg of Pγ on ice for 0.5 h with or without a competitor (Pγ(1–61) or Pγ(62–87) in a 200-fold molar excess over Paβ), and 0.2 μg of αiGTPγS was then added. Following incubation on ice for 1 h, the reaction was mixed with washed anti-αi/Protein A beads and rotated for 1 h at 4°C. The Paβ immunoprecipitate was...
then eluted off of the washed beads and detected by Western blotting using the anti-Pα antibody.

**Western Blot—**Western blotting was performed as described previously (16). Low cross-link 15% acrylamide gels (33) were used for SDS-PAGE. Proteins were electrotransferred from the gel to the polyvinylidene difluoride membrane for 2 h at 45 V. Antibody dilutions were as follows: anti-Pα (Affinity Bioreagents), 1 μg/ml; anti-αt (Affinity Bioreagents), 0.5 μg/ml; horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma), 100,000–300,000-fold. The Millipore Immobilon Western horseradish peroxidase substrate was used for chemiluminescence detection of the horseradish peroxidase-labeled bands.

### RESULTS

**Profiling of the αt,GTPγS Interaction with the Full-length Pγ—**A full spectrum profiling of Pγ-αt interaction has not been reported. Previous peptide mapping studies showed that C-terminal Pγ peptides had slightly lower affinity for αt,GTPγS than peptides from the central region (17, 18). The affinity of these peptides with activated αt (1 μM), however, is nearly 100-fold lower than the full-length Pγ (10–12 nM (18, 34)). This conspicuous affinity difference indicates that the full-length Pγ molecule is required to assume an optimal conformation for binding with αt. Obviously, peptide mapping is not an optimal approach to measure relative Pγ domain contributions in the full-length Pγ-αt,GTPγS interaction. Our label transfer experiments, in which the full-length Pγ could be used, however, offered a better means to address this issue.

Eleven photoprobe sets were prepared, with [125I]ACTP site-specifically attached through mixed disulfide to the single cysteines placed at various positions throughout the Pγ molecule. Functional properties of these ACTP-Pγ probes have been carefully characterized when previously used to map the Pγ-Pαβ interaction interface, and no major change due to the ACTP modification was observed for the PDE6 inhibition potency of Pγ (28). A possible impact of ACTP on the Pγ function of αt,GTPase stimulation was further assessed in this study, which showed that the functional activities of these Pγ photoprobe sets were similar to that of the unmodified native Pγ (supplemental Fig. S1B).

We therefore used these probes in the photocross-linking/label transfer experiments to profile the Pγ-αt,GTPγS interaction. Upon UV illumination, the azide group of ACTP is photoactivated into a nitrene (35), which then inserts into the nearby Pγ-interacting site(s) on αt, forming a covalent bond with the αt backbone. After DTT reversal of the S–S link between ACTP and the cysteine on Pγ, the 125I radiolabel is transferred from Pγ to αt (see the diagram in supplemental Fig. S1A), which can be detected by autoradiography (Fig. 1A). Thus, the label transfer efficiency reflects the interaction intensity between αt and a given Pγ position where [125I]ACTP is attached.

The [125I]ACTP-Pγ photoprobe sets, which were previously proven to transfer radiolabel to Pαβ specifically (28), were shown here to also specifically transfer radiolabel to αt,GTPγS. Specificity of the observed label transfer to αt was manifested not only by the absence of radiolabel on BSA (Fig. 1A), which was included as an internal control, but also by the Pγ position dependence of label transfer (Fig. 1B). The label transfer yield from each Pγ position was quantified by normalizing the intensity of radiolabel on αt, with the specific radioactivity of the corresponding [125I]ACTP-Pγ photoprobe...
(supplemental Table S1) (28). Interestingly, the resultant profile of label transfer to α, showed a pattern in which α, GTPγS was highly labeled by [125I]ACTP from the Pγ C-terminal positions around Trp70 (positions 70, 73, and 76) (Fig. 1B).

This region is known to have intimate contacts with α, (20). [125I]ACTP from the Pγ central positions Phe30-Leu60 (and the C-terminal position Ile67), however, only moderately labeled α, GTPγS. A low level of label transfer from the Pγ N-terminal positions 16 and 21 most likely represents a background level. Consistently, experiments performed under the same conditions but using α, GDP-AlF4, which shares a high similarity with α, GTPγS in their three-dimensional structures and functional properties (20, 36), showed a similar profiling pattern of label transfer (data not shown).

To further confirm the observed Pγ-α, GTPγS interaction pattern (Fig. 1B), label transfer profiling was also carried out using Pγ photoprobe containing mBP, a photoactive group different from ACTP (Fig. 2A). Under UV light (350–365 nm), the ketone in benzophenone is activated into a diradical by disproportionation and reacts with neighboring C–H bonds (35) in α, to form a C–C link. Because the maleimide group forms a C–S bond with the cysteine on Pγ, which cannot be cleaved by DTT, the cross-linked Pγ-α, complex stays covalently linked and migrates as a band higher than the α, band on the gel after SDS/DTT treatment (Fig. 2A). The mBP-Pγ photoprobe, which had been previously characterized, showed no significant functional changes in PDE6 inhibition due to mBP modifications on Pγ (16). Moreover, in a previous study from our laboratory (29), another benzophenone probe, benzoyl-l-phenylalanine, which is very similar to mBP, caused only minor changes in the Pγ stimulation of α, GTPase when incorporated into a Pγ C-terminal position at 66, 73, 76 or 86.

In a good agreement with the above results of [125I]ACTP label transfer, the cross-linking experiments using mBP-Pγ resulted in a similar profiling pattern (Fig. 2B). Thus, the fact that profiling with two different photophores (ACTP and mBP) led to similar patterns eliminates concerns regarding possible chemical selectivities of the photoprobe.

Compared with [125I]ACTP-Pγ, the mBP-Pγ probes showed a more profound preference for the Pγ C-terminal positions in cross-linking with α, GTPγS. This difference very likely stemmed from the fact that mBP is more hydrophobic (than ACTP) and thus more similar to the C-terminal hydrophobic residues that were replaced by the photoprobe. An interesting example is Ile67, the prominent hydrophobic of which is known to play an important role in the function of Pγ (12, 13). Accordingly, the hydrophobic mBP at position 87 yielded the highest Pγ-α, GTPγS cross-link efficiency (Fig. 2B), whereas [125I]ACTP, a relatively hydrophilic probe due to the presence of a carboxyl group (Fig. 1A), resulted in less cross-link efficiency at position 87 (Fig. 1B). In this regard, the mBP cross-linking profile (Fig. 2B) may better represent Pγ domain contributions to the Pγ-α, GTPγS interaction.

Notably, both [125I]ACTP and mBP yielded a substantial cross-link at Pγ position 70 (Figs. 1B and 2B), which is critical for the Pγ-α, interaction (20). This observation agrees with our previous study in which benzoyl-l-phenylalanine replacement of Trp70 also yielded a relatively high cross-link efficiency (29). The simplest explanation is that, due to a similarity to tryptophan, the photoprobe placed at position 70 could remain in close proximity to the Trp70-interactive Trp51-Lys53 and Trp54-Ile61, which is very significant. The statistical test of each position against position 87 is shown: positions 10, 21, 30, 38, 40, 50, and 68 (**); positions 70 and 76 (**); and position 73 (not significant (ns)).

**FIGURE 2. Profiling of the Pγ-α, GTPγS interaction using mBP photoprobe.** A, photocross-linked mBP-Pγ-α, GTPγS is shown as a higher band above the α, band on the Coomassie-stained SDS-gel. The photocross-linking reactions were performed as described under “Experimental Procedures.” Each reaction included 1.6 μg of α, GTPγS and an equal molar amount of mBP-P in the HEPES buffer (10 mM HEPES, pH 7.5, 120 mM NaCl, 5 mM MgCl2). DTT was added at a concentration of 2 mM to prevent possible nongenomic cross-link. B, the photocross-link efficiency of mBP-Pγ and α, GTPγS at each Pγ position was quantified as a percentage ratio of the protein amount in the cross-link band versus the sum in both the α, band and the cross-link band. Each bar represents an average ± S.D. (error bars) of six separate experiments. The mBP derivatization positions on Pγ are listed at the bottom. The difference in cross-link efficiency of α, GTPγS with two groups of Pγ positions, Phe30-Cys60 and Trp51-Ile61, is very significant. The test of each position against position 87 is shown: positions 10, 21, 30, 38, 40, 50, and 68 (**); positions 70 and 76 (**); and position 73 (not significant (ns)).
Transducin-PDE6 Interaction

**Figure 3** Differential Py domain interactions with αiGTPγS and Paββ evidenced by pull-down experiments. Pull-down experiments were performed with Btn-Pγ or Btn-Pγ(46–87) immobilized on streptavidin beads or HisPγ(1–61) immobilized on Ni²⁺-nitrilotriacetic acid beads. Conditions are described under “Experimental Procedures” unless otherwise stated. Trypsin inhibitor instead of BSA was included to block nonspecific protein-bead interactions because BSA (66 kDa) migrates too close to tβP (70 kDa) on the gel. The gels in A–D each represent at least three similar experiments. A, pull-down of αiGTPγS by Btn-Pγ. The control without Btn-Pγ is shown in lane 1. The Pγ peptide Py(62–87) (lane 3), Py(1–61) (lane 4), or both (lane 5) in a 1000-fold molar excess over Btn-Pγ or the full-length Pγ in a 20-fold excess (lane 6) was added to compete with the Btn-Pγ-αiGTPγS interaction. B, pull-down of Paββ by Btn-Pγ. Py(1–61) (lanes 2 and 4) or Py(62–87) (lanes 3 and 5), in a 1000- or 20,000-fold molar excess over Btn-Pγ, was present, competing with the Btn-Pγ-Paββ interaction. C, pull-down of Paββ or αiGTPγS using the HisPγ(1–61) peptide immobilized to nickel beads. Lanes 1 and 5, controls without HisPγ(1–61) for pulling down Paββ (lane 2) and αiGTPγS (lane 4), respectively. Both Paββ and αiGTPγS were added in the reaction of lane 3. D, pull-down of αiGTPγS (lanes 3 and 4) or Paββ (lane 6) by Btn-Pγ(46–87). Py(62–87) in a 200-fold molar excess over Btn-Pγ(46–87) was used to compete with the Btn-Pγ(46–87)-αiGTPγS interaction. Pull-down of αiGTPγS (lane 1) or Paββ (lane 2) by the full-length Btn-Pγ was also performed to compare with the Btn-Pγ(46–87) pull-down conditions.

(Figs. 1–3) is in accord with the crystal structure of the partial GAP complex (20), in which the Pγ C-terminal domain is engaged in a hydrophobic interlock between the αi1 switch II and αi helix.

It is noteworthy that the full-length Pγ in a 20-fold excess completely abolished the αiGTPγS pull-down (Fig. 3A, lane 6), and the full-length Pγ pulled down αiGTPγS much more efficiently than the C-terminal half-peptide (compare lane 1 with lane 3 in Fig. 3D). This is consistent with the previous assessment that although either the central or the C-terminal peptides could interact with αiGTPγS separately, the full-length Pγ interacted with αi with a much higher affinity (17, 18). Here we further assert that in the full-length Pγ-αi interaction, although the C-terminal domain contributes more than the central domain, both domains are required to forge a strong Pγ-αiGTPγS interaction.

Interestingly, in contrast to the major role of the Pγ-C-terminal domain in the Pγ-αi interaction, it is Pγ(1–61) (Fig. 3C, lane 2) rather than the C-terminal half (Fig. 3D, lane 6) that efficiently pulled down Paββ. The fact that Pγ(1–61) (lane 4 in B) but not Pγ(62–87) (lane 5 in B) abrogated the Pγ-Paβ interaction also indicates a dominant role of the Pγ N-terminal side for interacting with Paββ. This conclusion is consistent with the previous observations that the Pγ central domain binds Paββ much more strongly than the C-terminal domain (14). It is noteworthy that Pγ(1–61) that was 20,000-fold (lane 4 in B) but not 1000-fold (lane 2 in B) in molar excess could disrupt the Pγ-Paββ interaction. This result reflects an exceptionally tight full-length Pγ-Paββ interaction, the optimum Kd of which is in the subpicomolar range (1, 14). Therefore, the stark contrast of the binding of Paββ and αiGTPγS to the same Pγ domain raised an important question as to whether Pγ interacts with αiGTPγS and Paββ differentially.

Pγ Interacts with αiGTPγS and Paββ in a Complementary Manner—Label transfer profiling with the full-length, photoprobe-derivatized Pγ constructs allowed us to compare the pattern of Pγ-αiGTPγS interaction (Figs. 1B and 2B) with the previously observed Pγ-Paββ interaction profile (28). A “complementary” feature of the two separately determined profiles was thus revealed, in which the Pγ central region provides most of the strength for binding with Paββ, whereas the C-terminal region accounts for the major interaction with αiGTPγS.

These data prompted us to investigate the competition between αiGTPγS and Paββ for interacting with Pγ, by comparing the [125]I-ACt label transfer to αiGTPγS and to Paββ from various Pγ positions in a systematic manner. For this purpose, label transfer to αiGTPγS and Paββ from a certain Pγ position in a photocross-linking reaction with these two partners present could be directly compared in the same lane on the SDS gel (Fig. 4A). These experiments were intended to mimic the signaling state when αiGTP interacts with and displaces Pγ from PDE6 to activate the enzyme. Pγ was utilized substoichiometrically in comparison with αiGTPγS and Paββ (0.7 Pγ, 1 αi, 1 subunit of Paββ) so that αiGTPγS and Paββ could effectively “compete” in their interactions with Pγ and therefore provide a greater opportunity for revealing a preferential labeling on αiGTPγS or Paββ from a given Pγ position. As shown in the autoradiogram (Fig. 4A) and the data that are summarized as the labeling ratios

action; the C-terminal domain of Pγ contributed the major strength for the interaction with αiGTPγS compared with the remainder of the Pγ molecule (Figs. 1B and 2B).

The C-terminal Domain of Pγ Provides Major Binding Strength for the Pγ Interaction with αiGTPγS, as Does the Central Domain for the Interaction with Paββ—in order to further assess the role of the Pγ C-terminal domain in the interaction with αiGTPγS, a different approach (pull-down) was applied, using Btn-Pγ bound to streptavidin beads. As shown in Fig. 3A, αiGTPγS was specifically pulled down by Btn-Pγ (lane 2), because no αi was detected in the control with no Btn-Pγ (lane 1). Interestingly, the Pγ C-terminal peptide Py(62–87) effectively abolished αiGTPγS pull-down (lane 3), but the N-terminal peptide Py(1–61) of the same concentration did not (lane 4). This result demonstrates a dominant role of the Pγ C-terminal domain in the Pγ-αiGTPγS interaction. This conclusion is also supported by the observation that the C-terminal half of Pγ (Btn-Pγ(46–87)) efficiently pulled down αiGTPγS (Fig. 3D, lane 3), but the N-terminal HisPγ(1–61) peptide did not (Fig. 3C, lane 4). Importantly, the observation that the Pγ C-terminal domain provided the major Pγ-αiGTPγS interaction strength
Complementary nature of the Pβ interaction. The competition between the two targets, with the C-terminal domain favoring PβGTP and the central region preferring Pαβ, we sought to test whether the complementary interactions play an important role in the intermediate transducin-PDE6 complex, using co-immunoprecipitation approaches (Fig. 5, A and B). If the Pγ-PDE6 complex is indeed primarily held together by the Pγ-C-terminal interaction with αGTPγS and the central region interaction with Pαβ (diagramed in Fig. 5C), disrupting either of these interactions using a Pγ peptide should dissociate the αGTPγS-PDE6 complex.

We first observed that αGTPγS was co-immunoprecipitated specifically with the holo-PDE6 that was immobilized to Protein A beads via the anti-Pα antibody (Fig. 5A, lane 2), as compared with the control with no holo-PDE6 added (lane 1). The immunoprecipitation of αγ proved to be GTP-dependent because only a background level of αγGDP was co-immunoprecipitated with holo-PDE6 (lane 5). Moreover, the αγ precipitation diminished in the presence of either Pγ(1–61) (lane 3) or Pγ(62–87) (lane 4), indicative of complementary Pγ interactions with αγ and Pαβ.

for the two targets (Fig. 4B), a distinct preference of photolabel transfer for αGTPγS over Pαβ occurred from the Pγ-C-terminal positions, in particular from position 76. Accordingly, the recent crystal structure (5) showed that whereas the Pγ C terminus bound to the chimeric PDE5/6 catalytic domain, Leu76 pointed away, positioning itself in a direction ready for interaction with αγ. In clear contrast to the C-terminal positions, however, more labeling from the Pγ central positions (Val21-Leu49) was observed on Pαβ than on αGTPγS (Fig. 4B). These experiments of label transfer competition further confirm the complementary nature of the Pγ interactions with αGTPγS and Pαβ, which was first exhibited by the different interaction profiles.

Complementary Pγ Interactions with αγ and Pαβ Constitute the αγ-PDE6 Complex—Given the above evidence that Pγ differentially interacted with its two targets, with the C-terminal domain favoring αGTPγS and the central region preferring Pαβ, we sought to test whether the complementary interactions play an important role in the intermediate transducin-PDE6 complex, using co-immunoprecipitation approaches (Fig. 5, A and B). If the αGTPγS-PDE6 complex is indeed primarily held together by the Pγ-C-terminal interaction with αGTPγS and the central region interaction with Pαβ (diagramed in Fig. 5C), disrupting either of these interactions using a Pγ peptide should dissociate the αγ-PDE6 complex.

We first observed that αGTPγS was co-immunoprecipitated specifically with the holo-PDE6 that was immobilized to Protein A beads via the anti-Pα antibody (Fig. 5A, lane 2), as compared with the control with no holo-PDE6 added (lane 1). The immunoprecipitation of αγ proved to be GTP-dependent because only a background level of αγGDP was co-immunoprecipitated with holo-PDE6 (lane 5). Moreover, the αγ precipitation diminished in the presence of either Pγ(1–61) (lane 3) or Pγ(62–87) (lane 4), indicative of complementary Pγ interactions with αγ and Pαβ.
Transducin-PDE6 Interaction

To further explore the Pγ-dependent nature of the αγPDE6 co-immunoprecipitation, experiments were performed in a reverse manner, detecting co-immunoprecipitation of Paβ with αγGTPγS, which was immobilized on Protein A beads through the anti-αγ antibody (Fig. 5B). Similar to the results shown in Fig. 5A, Paβ was co-immunoprecipitated with αγGTPγS in the presence of Pγ (lane 3). Obviously, Paβ was precipitated by the PγαγGTPγS complex, because no Paβ precipitation was observed when Pγ was absent (lane 4). Paβ precipitation was diminished in the presence of Pγ(1–61) (lane 1) or Pγ(62–87) (lane 2). A significant portion of Paβ was still precipitated with excess Pγ(1–61) present (lane 1), reflecting tight binding of the Pγ N-terminal half with Paβ (see Fig. 3, B and C). These data further confirm the Pγ-mediated complementary interactions.

With the data in Fig. 5 showing Pγ-dependent co-immunoprecipitation of αγGTPγS with PDE6 as well as co-immunoprecipitation of PDE6 with αγGTPγS, we obtained additional evidence (Fig. 6, A–C) supporting the conclusion that complementary and simultaneous binding of Pγ to its two partners constituted the αγPDE6 complex.

The experiments were designed based on the following idea. If Paβ binds Pγ simultaneously along with αγGTPγS (diagramed in Fig. 6D, 1), Paβ should be pulled down by the Btn-Pγ molecules on streptavidin beads that are saturated by excess αγGTPγS, and vice versa. As shown in Fig. 6A, Paβ was readily pulled down by Btn-Pγ, which was bound to the beads and preincubated with αγGTPγS at a 5-fold molar excess (lanes 2 and 4), supporting the notion that co-binding of αγGTPγS and Paβ to Btn-Pγ occurred as depicted in Fig. 6D, 1–4. However, these concerns can be ruled out by detailed analysis of our data. First, Paβ was

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not pulled down by a population of Btn-Pγ that could have become free due to extensive washing of the αγ-saturated beads (see Fig. 6D, 2), because the same amount of Pαβ was pulled down in the presence of excess αγ, that was added back to the αγ-saturated beads (Fig. 6A, lane 4). Second, the addition of Pαγ to αγ-saturated beads did not reduce αγ pull-down (Fig. 6B, compare lane 4 with lane 3), indicating that the Pαβ pull-down did not result from replacement of a portion of αγ by the Pαβ binding to Btn-Pγ (see D3). In this case, replaced αγ should have been washed off and would have led to a lowered intensity of the αγ band. This was also true for the αγ pull-down on Pγ-saturated beads (compare lane 2 with lane 1 in B). Third, Pαβ was not pulled down by directly interacting with αγ (see D4), because αγ was readily pulled down by Btn-Pγ (46–87) but Pαβ was not pulled down together with αγ (Fig. 3D, lane 5). Similarly, α γ was not pulled down together with Pαβ, which was pelleted with His-Pγ(1–61) on nickel beads (Fig. 3C, lane 3).

Finally, Pγ(1–61) in a 1000-fold excess could not compete with the strong full-length Pγ-Pαβ interaction (Fig. 3B, lane 2, and Fig. 6C, lane 2) but could do so in the presence of αγ,GTPγS (Fig. 6C, lane 3). The simplest explanation is that simultaneous binding of αγ,GTPγS to Btn-Pγ along with Pαβ weakened the Pγ-Pαβ interaction by sequestering the Pγ-C-terminal domain and thus kept Pαβ from a high affinity binding with the full-length Pγ. Taken together, these lines of evidence suggest that Pγ facilitated the formation of the αγ,GTPγS-PDE6 complex by binding to both Pαβ and αγ,GTPγS simultaneously.

DISCUSSION

PDE6 activation mediated by αγ is the central step in the visual transduction cascade. How Pγ interacts with its two targets (αγ and Pαβ) is the key to understanding the molecular mechanism of PDE6 activation, yet it has been difficult to capture a snapshot of this dynamic process through structural biology. We have obtained evidence here supporting the conclusion that complementary interactions (a strong Pγ C-terminal interaction with αγ,GTP and a tight binding of the central region

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to Pαβ) occur in favor of PDE6 activation and at least partly account for the binding force in the intermediate αγ-PDE6 complex.

It is known that the Pγ central region and the C-terminal domain are the two primary binding sites not only for αγ,GTPγS but also for Pαβ (8, 10, 16–18, 29, 37, 38), but it is not yet clear how the two Pγ domains differentiate their interactions with αγ and Pαβ (9) when both targets are involved during PDE6 activation.

The observation of complementary Pγ domain interactions with αγ,GTPγS and Pαβ was based on comparison of the interaction profiles with the two targets, both obtained from systematic mapping of the entire interaction interface. A stronger αγ,GTPγS interaction with the Pγ C-terminal domain than with the Pγ central region (Figs. 1B and 2B) was in interesting contrast to a stronger Pαβ interaction with the Pγ central region than with the Pγ C-terminal domain (28), as also indicated by the pull-down experiments (Fig. 3). More significantly, this complementary feature was also observed when both of the targets were present competing for Pγ interaction (Fig. 4).

The complementary Pγ interactions with its two targets may have important implications for the molecular mechanism of PDE6 activation. The significance is 2-fold. First, because the αγ-interacting C-terminal domain (Thr<sup>82</sup>–Ile<sup>87</sup>) includes the PDE6-inhibiting region, a strong Pγ C-terminal interaction with αγ,GTP is essential to compete with the Pγ-Pαβ interaction in order to displace the Pγ C terminus from the Pαβ catalytic site (5, 8, 20). Accordingly, a weak interaction between the Pαβ catalytic domain and the Pγ C-terminal domain should therefore provide αγ,GTP a stronger competitive edge over Pαβ, ensuring an efficient PDE6 activation. Moreover, tight binding of the Pγ C-terminal domain to αγ,GTP helps keep Pγ from reinitiating PDE6 before the visual signal is adequately amplified (20).

Second, a greater binding strength of Pαβ for the Pγ central region keeps it sequestered by Pαβ. Because it is the complex formed by αγ,GTP and the full-length Pγ that can be readily recognized by RGS9-1-Gβ5 to maximally fulfill the GAP function (32), sequestration of the Pγ N-terminal half by Pαβ may prevent visual signaling from being terminated too early. An extremely high rod visual sensitivity is thus achieved not only because of an exceptionally high efficiency of the activated PDE6 but also for its extended lifetime (1).

Furthermore, the complementarity of the Pγ-αγ interaction and the Pγ-αγβ interaction also reveals some insights with regard to the molecular topology of the αγ-PDE6 complex. αγ,GTPγS was co-immunoprecipitated by holo-PDE6, and Pαβ was co-immunoprecipitated by αγ,GTPγS, all in a Pγ-dependent fashion (Fig. 5). This indicates a molecular organization

**FIGURE 7. Schematic diagram of the complementary Pγ interactions with its two targets during PDE6 activation.** The molecules involved in PDE6 activation are represented by different shapes. Egg, Pα and Pβ; circle, αγ; ribbon, Pγ; square, GDP; oval, GTP; triangle, cGMP. For simplicity, the three domains of Pα and Pβ are shown as segments separated by the dotted red lines. Our previous studies revealed that the Pγ Phe<sup>10</sup> region preferred binding to Pγ, whereas the Ser<sup>98</sup> region favored binding to Pβ, suggesting simultaneous Pγ interactions with Pα and Pβ (16, 28). Because PDE6 can only be efficiently activated 50% by transducin (26, 46), it is highly likely that only one Pγ is displaced by αγ,GTP during PDE6 activation in the mammalian retina, whereas the other Pγ (on the opposite side) stays tightly bound to Pαβ (1). A movement of the Pγ(78–87) segment from Pαβ to αγ,GTP was suggested by Baner et al. (5), based on the crystal structure of the Pγ(70–87):PDE6/5 catalytic domain complex.
such that the C-terminal domain of Pγ binds tightly with αiGTPγS while the central region forms a strong interaction with Paβ, thus “gluing” αiGTPγS and Paβ into the PDE6 activation complex, or “transducisome.” This proposed organization is also supported by the data from co-pull-down of αiGTPγS and Paβ by Btn-Pγ (Fig. 6). Thus, the complementary Pγ interactions may explain a long held puzzle; although the Paβ-binding regions and αi-binding regions overlap on Pγ (1, 9), an intermediate transducin-PDE6 complex could still occur during visual transduction (22–24). Our data do not exclude the possibility that in the PDE6 activation complex, the Pγ central region may also be involved in binding with αiGTP, albeit probably through weak interactions. A mutagenesis study showed that Lys41, Lys44, and Lys45 on the C-terminal side of the Pγ polyarginine region were involved in the interaction with αi but not with Paβ (39), raising the possibility of simultaneous non-competitive αiGTPγS and Paβ binding to the Pγ central region.

Early studies using rod disc membranes suggested that direct αi-Paβ interaction accounted for an important binding force in the αi-PDE6 complex (24, 27). In the current study, however, αiGTPγS and Paβ were not co-immunoprecipitated with each other in the absence of Pγ (Fig. 5). Moreover, when either αiGTPγS or Paβ was bound to Btn-Pγ (46–87) or HisPγ (1–61) on affinity beads, the other was not co-pulled down (Fig. 3). These results indicate a lack of direct αi-Paβ interaction in the αi-PDE6 complex under our experimental conditions. Because no disc membranes were involved in our experiments, we suggest that the disc membranes used in the early studies may have played a role in organizing the proteins in such a way that αi and Paβ make direct contacts that further tighten the αi-PDE6 complex. In support of this proposition, increasing evidence indicates that disc membranes enhance protein functions in phototransduction (40–42). Nevertheless, our data indicate that the complementary Pγ interactions with its two targets, at least in part, account for the binding force in the αi-PDE6 complex.

Based on the data presented herein and evidence from previous studies, a possible scenario of protein-protein interactions during PDE6 activation is depicted in Fig. 7. GTP-bound αi may initially engage PDE6 by making contacts with the Pγ residues Trp70 and Leu76, which are not involved in intimate interactions with the Paβ catalytic site (5, 8, 11), and may also interact via part of the polyarginine region (19, 39) (Figs. 1B and 2B). These initial αi contacts with Pγ may trigger a conformational change that results in a rigid body movement of the Pγ C-terminal region away from the Paβ catalytic site with Leu76 serving as a “hinge.” The Pγ C-terminal domain can now bind tightly with αi and PDE6 is deinhibited (5). At this stage, an intermediate complex containing αiGTPγPaβ, or transducisome, probably exists due to the complementary binding of Pγ to αiGTP and Paβ (Figs. 4–6), with the Pγ C-terminal domain tightly bound to αiGTP and the central region bound to the Paβ GAF domain with high affinity. The Pγ central region could stay bound to the Paβ GAF domain until the binding is weakened by lowered cGMP levels, through a mechanism of positive cooperativity of Pγ and non-catalytic cGMP in binding to the GAF domain (1, 14, 26, 43, 44).

Thus, the duration of the αiGTPγPaβ transducisome is probably subject to regulation by the cGMP occupancy in the Paβ GAF domain. However, the activity of the GAP complex may have a more significant effect on the lifetime of the transducisome, because GTP hydrolysis in αi, accelerated by RGS9-1 is the rate-limiting step of the rod photoresponse (45). Interesting questions hereby arise as to how Pγ dynamically and differentially interacts with RGS9-1/Gβ5, αi, and Paβ upon a transition from its role in the PDE6 activation complex to that in the GAP complex and how the cGMP binding in the GAF domain regulates this process. Because disruption of Pγ interactions with its partners in phototransduction causes impaired visual functions (9), systematic investigations of these interactions will advance our understanding of the molecular mechanisms of the related retinal diseases.

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