Gain-of-function screen of α-transducin identifies an essential phenylalanine residue necessary for full effector activation

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Two regions on the α subunits of heterotrimeric GTP-binding proteins (G-proteins), the Switch II/α2 helix (which changes conformation upon GDP–GTP exchange) and the α3 helix, have been shown to contain the binding sites for their effector proteins. However, how the binding of Gα subunits to their effector proteins is translated into the stimulation of effector activity is still poorly understood. Here, we took advantage of a reconstituted rhodopsin-coupled phototransduction system to address this question and identified a distinct surface and an essential residue on the α subunit of the G-protein transducin (αγ) that is necessary to fully activate its effector enzyme, the cGMP phosphodiesterase (PDE). We started with a chimeric G-protein α subunit (αγ*) comprising residues mainly from α2 and a short stretch of residues from the Gαi subunit (α1i), which only weakly stimulates PDE activity. We then reinstated the α2 residues by systematically replacing the corresponding αγ residues within α2γ* with the aim of fully restoring PDE stimulatory activity. These experiments revealed that the αG/α4 loop and a phenylalanine residue at position 283 are essential for conferring the α2γ* subunit with full PDE stimulatory capability. We further demonstrated that this same region and amino acid within the ααi subunit of the Gαi protein (αi) are necessary for full adenyl cyclase activation. These findings highlight the importance of the αG/α4 loop and of an essential phenylalanine residue within this region on Gα subunits α2 and α3 as being pivotal for their selective and optimal stimulation of effector activity.

The X-ray crystal structures of a number of Gα subunits identified two major domains (1–4), referred to as the Ras-like GTPase domain and the helix-rich (helical) domain. Bound GDP or GTP is cradled between the two domains and is occluded from solvent. These structures have also highlighted the conformational changes underlying the transition of Gα subunits from their GDP-bound signaling inactive states, to their GTP-bound signaling active states, necessary for regulating their downstream effector proteins (1, 5). Three regions on Gα subunits undergo significant conformational changes induced by GDP–GTP exchange and are commonly designated as Switch I, II, and III. Upon GTP binding, the conformational changes in Switch I and II are transmitted to Switch III through a series of ionic interactions. These three switch regions represent common structural elements of every Gα subunit, as they are essential for coupling to G-protein-coupled receptors (GPCRs) (6), as well as for associating with Gβγ subunit complexes (7, 8), and play an important role in regulating effector proteins (9–11).

Structural studies have shown that Switch II needs to undergo conformational changes upon GDP–GTP exchange in order for the Gβγ subunit complexes to dissociate from their Gα subunit partners. Changes in the conformation of Switch II also enable activated Gα subunits to then directly engage their effector proteins (i.e. contacts made through Switch II and the α3/β5 loop). This was shown in detail in the X-ray crystal structure of ααi-GTPγS complexed to portions of the cytoplasmic domains of adenyl cyclase (10), as well as in the structure of an AlF4–-activated chimeric ααi/αi subunit bound to a C-terminal fragment of PDEγ (9). Still, the mechanisms by which the binding of activated Gα subunits to effector enzymes are translated into the regulation of their enzymatic activity are not fully understood.

Previous studies have suggested that the C-terminal regions of the Gα subunits, including the α4 helix and the α4/β6 loop, play a role in the activation of their cognate effectors (12–15). For example, a synthesized C-terminal peptide of α4 (corresponding to residues 293–314) was shown to be capable of stimulating PDE activity (15). Other studies showed that replacing amino acid residues within the α4 helix and the α4/β6 loop of a chimeric α4 subunit, with the corresponding residues from α2, markedly attenuated its ability to stimulate adenyl cyclase activity (12). Taken together, these data suggest that conformational changes in Switch II and III are somehow com-

4 The abbreviations used are: GPCR, G-protein–coupled receptors; PDE, phosphodiesterase; CRE, cAMP-response element; SEAP, secreted alkaline phosphatase; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; HMDM buffer, 20 mM Hepes, pH 7.5, 5 mM MgCl2, and 0.01% (w/v) dodecylmaltoside; HM buffer, 20 mM Hepes, pH 7.5, and 5 mM MgCl2.
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To further delineate the mechanistic basis by which Gα subunits activate their biological effectors, we took advantage of the vertebrate phototransduction system that is responsible for vision in dim light (16). The primary components of this signaling system are the GPCR, rhodopsin, the heterotrimeric G-protein, transducin, and the effector enzyme, the cGMP phosphodiesterase (PDE) (17). Upon GDP–GTP exchange catalyzed by light-activated rhodopsin, the heterotrimeric G-protein transducin dissociates into a GTP-bound α subunit (αT) and a βγ (βγT) complex. The GTP-bound αT subunit interacts with the regulatory γ subunits (PDEγ) of the PDE, altering their orientation relative to the larger PDE subunits (PDEα, PDEβ), thereby providing substrate (cGMP) access to the enzyme catalytic site. Activated PDE hydrolyzes cytosolic cGMP, causing the closure of cGMP-gated cation channels and the hyperpolarization of the rod cell membrane, which transmits the visual signal to secondary nerve cells.

Because each of the primary components of the rhodopsin/transducin-coupled signaling pathway has been purified, biochemically characterized, and their interactions reconstituted, this system provided a good starting point toward determining the underlying mechanisms that translate the GTP-dependent interaction between a Gα subunit and its biological effector into a specific regulation of effector activity. However, to explore key sites on Gα subunits for effector regulation by targeted mutagenesis, it was necessary to use a chimeric Gα subunit, which contains the majority of residues from αT and a limited number of residues from the closely related αi, because native αT cannot be expressed in Escherichia coli (18). Within this chimera (designated αT-Chi8), two fragments of αT, residues 216–227 and 237–294, are replaced with the corresponding residues from αi. The E. coli–expressed αT–Chi8 chimera has greatly facilitated biochemical and crystallographic structural studies (18–22), although, in its original form, it was unable to stimulate PDE activity. The restoration of two residues in the α3 helix of αT (i.e. His-244 and Asn-247) within the αT–Chi8 background, yielding the chimeric subunit from here on referred to as αT*, partially restored PDE-activating capability (19, 23). With this partial gain-of-function αT* construct as a starting point, we then set out to delineate the necessary sites and residues within αT that convert its binding to PDE into the full activation of its enzymatic activity.

Based on these studies, we show that the chimeric αT* subunit regained full PDE-stimulatory activity upon the restoration of the αG/α4 loop from αT. Within this fully active chimera (designated αT*-SFD), we further demonstrate that a single amino acid residue, Phe-283, is essential for conferring maximum stimulatory capability upon the retinal Gα subunit. Moreover, we show that a similar region, and the same conserved amino acid residue, are essential for the ability of the Go subunit to fully stimulate its biological effector, adenylyl cyclase, thus demonstrating the fundamental importance of these Gα sites in mediating effector regulation.

Results

The αG/α4 loop plays an important role in Gα-dependent effector activation

The available X-ray crystal structures of the Gα subunits of transducin (αT) and Gs (αs) reveal a unique surface on the same face of the protein that is distinct from the regions necessary for the binding of the Gβγ subunit complex, and GPCRs such as rhodopsin and the β-adrenergic receptor (3, 9, 10, 24). This surface is comprised of the conformationally sensitive Switch II and III domains, as well as the α3 helix, the α3/β5 loop, the α4 helix, and the α4/β6 loop (highlighted in blue in Fig. 1A). The Switch II domain (α2 helix), together with the α3 helix and the α3/β5 loop, have been shown to enable activated αT to directly engage PDEγ and activated αs to bind to portions of the cytoplasmic loops of adenyl cyclase (9–11). However, how GTP-bound (activated) Gα subunits, upon binding to their biological effectors, regulate effector activity (e.g. how αT and αs fully activate PDE and adenylyl cyclase, respectively) remains an important question. Here we have set out to address this question by first using a reconstituted rhodopsin-coupled phototransduction signaling pathway that culminates in the stimulation of PDE activity, and by taking advantage of a chimeric αT/α1 subunit (αT-Chi8) (Fig. 1, B and C) that can be expressed in high yields in E. coli (unlike native αi). This chimeric subunit is fully capable of interacting with light-activated rhodopsin and the βγT subunit complex. Native retinal αT, as well as αT–Chi8, binds the nonhydrolyzable GTP analog, GTPγS, in the presence of light-activated rhodopsin and the βγT subunit complex (i.e. upon GDP–GTPγS exchange) (19, 25). Retinal αT bound to GTPγS is capable of fully activating PDE, whereas, the αT–Chi8 is unable to activate PDE under the same conditions (Fig. 2, A and B). The restoration of just two residues from αi into the α3 helix generated a new chimera, referred to as αT* (Fig. 1, B and C), that is significantly more effective than αT–Chi8 at activating PDE but still much less effective than retinal αT (Fig. 2, A and B) (19). We reasoned that by systematically restoring amino acids from native αT into the proposed PDE-interacting surface within αT*, it might be possible to generate a Gα subunit which would be able to fully stimulate PDE activity.

We began by making substitutions within the αG/α4 loop of αT* (residues 275–291) (Fig. 2, C and D). One specific chimera, designated αT*-SFD, when bound to GTPγS, was capable of stimulating PDE activity to levels comparable to and even better than retinal αT (Fig. 2, A and B).

The αT*-SFD chimera was generated by substituting three αT residues (Ser-280, Phe-283, and Asp-285) into the αG/α4 loop of αT* (Fig. 2, C and D). Aluminum fluoride (AlF4–) binds to the GDP–bound form of α subunits in the position occupied by the γ-phosphate of GTP and is thought to resemble the transition state for GTP hydrolysis. This binding of AlF4– results in a concomitant increase in intrinsic tryptophan fluorescence, similar to what is seen upon binding of nonhydrolyzable GTP analogs. This increase in tryptophan fluorescence is because of the change in the environment of a conserved tryptophan residue (Trp-207 in retinal αT) in the Switch II region of Gα subunits. The αT*-SFD chimera showed a robust increase in tryptophan fluorescence (blue line, Fig. 2E) that was similar to that
observed with retinal αT (black line) and αT*-SFD (red line). The relative importance of each of the individual residues that were mutated to create αT*-SFD was then examined for the contribution to the stimulation of PDE activity. This was carried out by individually mutating each residue to the corresponding one in αT and assaying its ability to activate PDE when bound to GTPγS. Introduction of either Ser-280 or Asp-285 had a minimal effect on the ability of αT*-SFD to activate PDE (data not shown).

In contrast, the introduction of Phe-283 into the αT*- background (αT*-Y283F) was sufficient to restore an effector stimulatory capability matching that of αT* (Fig. 2, B and D).

**Identifying a site on Gα subunits for effector regulation**

As a complement to the studies assaying effector activation, outlined above, we examined the relative abilities of activated, GTPγS-bound retinal αT, recombinant αT*, and αT*-SFD to bind to the PDEγ subunit, labeled at its sole cysteine residue (Cys-68) with the fluorescent reporter group IAEDANS. The binding of GTPγS-bound αT to the IAEDANS-labeled PDEγ subunit (IAEDANS-PDEγ) resulted in a saturable increase in the fluorescence emission of the IAEDANS moiety, providing a direct readout for this binding interaction (Fig. 3A).

The enhancement in IAEDANS fluorescence that occurred with increasing concentrations of αT*, when fit to a bimolecular binding model, yielded an apparent Kd value of 2 nM for the interaction of retinal αT with IAEDANS-PDEγ. Binding titrations performed with GTPγS-bound αT* and αT*-SFD yielded apparent Kd values of 8 and 4 nM, respectively. Because GTPγS-bound αT*-Y283F was capable of stimulating PDE to levels similar to that of αT*, its binding to IAEDANS-PDEγ was examined, yielding a Kd of 5 nM, similar to that for αT*-SFD. We also measured the direct binding of αT-Chi8 and αT*-Chi8-SFD to IAEDANS-labeled PDEγ. These two chimeric αT subunits contain the αT subunit residues Lys-244 and Asp-247 within the α3 helix, instead of the αT residues His-244 and Asn-247, and show little ability to stimulate PDE activity. They also exhibited a weaker binding affinity for PDEγ, with apparent Kd values in the range of 15–20 nM (Fig. 3A, right panel).

We then examined the binding of these different αT subunits to the intact holo-PDE complex. This first required removing the native PDEγ subunits from holo-PDE by limited trypsin treatment, yielding the heterodimeric PDEαβ subunit complex, which contains the catalytic site for cGMP hydrolysis. The PDEαβ subunits were then reconstituted with purified IAEDANS-labeled PDEγ (IAEDANS-PDEγ) to generate a fluorescently labeled, IAEDANS-PDEγ-associated holo-PDE complex, which was titrated with the different GTPγS-bound αT subunits. The various αT subunits showed the same relative abilities to activate the reconstituted, fluorescent-labeled holo-PDE as observed for native unlabeled PDE (e.g. the αT*-SFD chimera was as effective as native retinal αT at stimulating the labeled holo-effector enzyme (data not shown)). The addition of increasing concentrations of GTPγS-activated, native retinal αT and other chimeric αT/α1 subunits resulted in a saturable increase in the fluorescence emission of the IAEDANS moiety on holo-PDE, as shown in Fig. 3B. The data were fit to a bimolecular binding model and yielded apparent Kd values of 21 nM for retinal αT, 56 nM for αT*, 27 nM for αT*-SFD, and 28 nM for αT*-Y283F. These data indicate that these αT subunits exhibit a somewhat weaker affinity for the holo-enzyme compared with the purified PDEγ subunit, although their relative Kd values show a trend similar to that observed when assaying their interactions with PDEγ. The binding of the αT-Chi8-SFD and αT*-Chi8 subunits to holo-PDE was also examined and yielded apparent Kd values of 42 nM and 114 nM, respectively.
Figure 2. Construction of a fully active chimeric transducin α subunit. A, dose-dependent activation of PDE by αT subunits. The bovine α subunit of transducin (αT), the chimeras αT-Chi8 or αT*, or mutant αT* subunits were activated in the presence of 200 nM light-activated rhodopsin, 1 μM βγ, and 100 μM GTPγS in PDE assay buffer (10 mM Tris, pH 8, 2 mM MgCl2, 100 mM NaCl) for up to 45 min at room temperature. The activated αT subunits were incubated with holo-PDE (50 nM) and the voltage was monitored with a pH meter. Subsequently, 5 mM cGMP was added and the increase in voltage was recorded for 150 s. NaOH (400 nmol) was added to determine the buffer capacity. The αT subunits were assayed at varying concentrations (active concentrations determined by GTPγS binding) as indicated by the graph. Data are shown as mean ± S.E. (error bars) of at least three replicates. B, PDE stimulation by αT subunits. The bovine α subunit of transducin (αT), the chimeras, αT-Chi8 or αT*, or mutant αT* subunits were assayed as in (A). The concentration of the αT subunits used in this experiment was 1 μM. The percent rates of cGMP hydrolysis of the αT* subunits were plotted relative to the levels of cGMP hydrolysis stimulated by native retinal αT. Data represent mean ± S.E. (error bars) of at least three replicates; *** p < 0.001. C, the crystal structure of αT (PDB ID: 1TND) modeled with PDEγ (PDB ID: 1FQJ) highlighting both reported (His-244, Asn-247) and putative (Ser-280, Phe-283, and Asp-285) effector-interacting residues. The αT subunit is colored light gray and PDEγ is shown in charcoal. Reported PDE-interacting regions are shown in blue and the nucleotide, GTPγS, is colored green as in Fig. 1. D, sequence alignment of the Gα4 loop region between αT, αT-Chi8, αT*, and αT*-SFD. The αT-Chi8 residues mutated to αT residues in αT* are shown in bold-faced type generating αT*-SFD. The tyrosine mutated in αT-Chi8 generating αT*- (Y283F) is colored red. The residues were either mutated individually or in groups. E, changes in the intrinsic tryptophan fluorescence of various αT subunits induced by AlF4−. αT (black), αT* (red), or αT*-SFD (blue) (400 nM) was incubated in 1 ml of HMDM buffer. Then 20 μl of a premixed aliquot containing 250 mM NaF and 2.5 mM AlCl3 to generate AlF4− was added and the enhancement in tryptophan fluorescence (excitation: 280 nm; emission: 340 nm) was monitored.
in both sets of binding assays, the SFD substitution within the $\alpha_\gamma$ background yielded an increased affinity for the effector, it is difficult to definitively conclude that this enhanced binding capability adequately accounts for the advantage it provides in activating the PDE, as the dose response profiles for $\alpha_\gamma$-stimulated effector activity are consistently and significantly shifted (to the right) compared with the binding titrations. Thus, a weaker secondary interaction is apparently necessary for full effector activation, and therefore the activation dose response might represent the main contribution provided by the SFD site (see “Discussion”).

A similar region and essential residue are required for the activation of adenyl cyclase by $\alpha_\gamma$

Given the findings described in the preceding sections, we were interested in seeing whether the $\alpha_G/\alpha_4$ loop, and a single essential residue within this region, played similarly important roles in other $G\alpha$–effector interactions. Therefore, we examined the stimulation of adenyl cyclase activity by the $\alpha$ subunit of $G_s$ ($\alpha_s$). Comparing the amino acid sequences between $\alpha_T$ and $\alpha_s$ within the $\alpha_G/\alpha_4$ loop showed that Phe-283, which we found to be essential for full PDE stimulatory activity, is conserved in $\alpha_s$ (i.e. Phe-312) (Fig. 4A). Moreover, the positions of this conserved phenylalanine within the $\alpha_G/\alpha_4$ loops of $\alpha_T$ and $\alpha_s$ are similar (Fig. 4B). The $\alpha_G/\alpha_4$ loop residue Phe-312 in the $\alpha_s$ subunit corresponds to Tyr-287 in $\alpha_T$. We therefore examined whether changing Phe-312 of $\alpha_s$ to tyrosine, yielding the $\alpha_s$ (F312Y) mutant, reduced the stimulatory capability of $\alpha_s$ for adenyl cyclase.

Bovine $\alpha_s$ WT has a conserved tryptophan at position 234, analogous to Trp-207 in retinal $\alpha_T$, recombinant WT $\alpha_s$ ($\alpha_s$ WT), as well as $\alpha_s$ (F312Y), displayed an enhancement in intrinsic tryptophan fluorescence upon addition of either $\text{AlF}_4^-$ (Fig. 4C) or GTP-$\gamma$S (Fig. 4, D and E, red lines). The latter changes reflect the ability of the $\alpha_s$ subunit to exhibit measurable intrinsic GDP–GTP-$\gamma$S exchange. These data suggest that the $\alpha_s$ (F312Y) mutant can transition into the activated form in a manner similar to that of $\alpha_T$ WT. In addition, it has been suggested that the $\beta_2\gamma_3$ subunit can reduce the rate of GDP dissociation from $G\alpha$ subunits, which is the rate-limiting step for nucleotide exchange (26, 27). As expected, the addition of the $\beta_2\gamma_3$ subunit slowed the rate of GTP-$\gamma$S binding to $\alpha_s$ WT (Fig. 4D, blue line), as well as to $\alpha_s$ (F312Y) (Fig. 4E, blue line).
This indicates that the substitution at position 312 does not alter the ability of the βγ subunit to interact with α4.

Gα4 stimulates adenyl cyclase activity, leading to the phosphorylation of the cAMP-response element-binding protein (CREB-P) by protein kinase A (Fig. 5A). CREB-P then docks onto the cAMP-response element (CRE) regulating the transcription of downstream genes. Using this pathway as a model, we took advantage of a fluorescent plate assay for measuring the...
relative levels of adenylyl cyclase activity in mammalian cells under different conditions. A vector containing CRE and a secreted alkaline phosphatase (SEAP) reporter group was co-transfected with vectors containing \( \alpha_5 \) constructs into HEK-293 cells. The relative adenylyl cyclase activity for a particular condition was determined by the amount of SEAP protein secreted into the cellular growth medium (Fig. 5A). Two \( \alpha_5 \) constructs were designed for the assay: the first, \( \alpha_5 \) QL, a constitutively active form of \( \alpha_5 \) containing the GTP hydrolysis–defective (Q227L) mutation, was used as a positive control to fully stimulate adenylyl cyclase activity. The second \( \alpha_5 \) construct, \( \alpha_5 \) QL (F312Y), encoded an \( \alpha_5 \) mutant where the parental \( \alpha_5 \) G/\( \alpha_5 \) 4 loop residue Phe-312 was changed to the tyrosine found in \( \alpha_5 \).

HEK-293 cells were transfected with the profiling vector and either pcDNA3, \( \alpha_5 \) WT, \( \alpha_5 \) QL, or \( \alpha_5 \) QL (F312Y) (Fig. 5B). Cells transfected with \( \alpha_5 \) QL (F312Y) exhibited a significant reduction in adenylyl cyclase activation, as readout by SEAP levels, compared with cells transfected with \( \alpha_5 \) QL (Fig. 5, C and D), thus demonstrating the essential role of the conserved phenylalanine residue within the G/\( \alpha_5 \) 4 loop of \( \alpha_5 \) for fully stimulating effector activity.
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Discussion

Heterotrimeric G-proteins act as molecular switches that are converted to a signaling-active state as a result of GDP–GTP exchange catalyzed by GPCRs. The activated GTP-bound Gα subunits then interact with a specific biological effector, which is often an enzyme whose catalytic activity is stimulated as an outcome of this interaction. The X-ray crystal structure of an AlF₄⁻-activated α₇/αi chimeric subunit bound to a peptide from the C-terminal region of the inhibitory PDEγ subunit (residues 45–87), and the RGS domain of RGS9, shows that the peptide contacts residues in the Switch II domain, the α3 helix (which is immediately downstream from Switch II), and the α3/β5 loop (9). Moreover, the X-ray crystal structure of the GTPγS-bound αs subunit complexed to a soluble form of adenyl cyclase, comprised of portions of its two major cytoplasmic domains, shows that homologous regions in αs are involved in contacting adenyl cyclase (10, 11).

Here we set out to gain new insights into the molecular mechanisms by which these Gα-effector–binding interactions are translated into the regulation of effector activity. We began by examining how the αT subunit of transducin fully activates holo-PDE, by making use of a chimeric αT* subunit that is capable of undergoing GDP–GTP exchange as catalyzed by the GPCR rhodopsin, but only weakly stimulates PDE activity (18, 19).

Given that the αT* chimera contained the contact residues for PDEγ, it was not immediately obvious why an activated αT* subunit was unable to activate holo-PDE as effectively as native retinal αT. Previously we had shown that introducing two residues from the α3 helix of αT, His-244 and Asn-247, into αT-Chi8 (αT*) provided a partial restoration of PDE activation, further highlighting the importance of the α3 helix in effector binding (19, 23). Still, these findings indicated that an additional region(s) is required to achieve full effector activity. This additional region is shown here to be the αG/α4 loop.

The αG/α4 loop of the chimeric αT* subunit has several residues that differ from those in native retinal αT; among these are αT Ser-280, Phe-283, and Asp-285 (Fig. 4A). These residues were introduced to create a new chimeric αT* subunit, referred to as αT*-SFD, which was found to stimulate the activity of PDE as effectively as native retinal αT. A systematic analysis creating single and double substitutions within the αG/α4 loop then led to the identification of Phe-283 to be the critical αT residue allowing for full PDE activation.

Alignment of the primary sequence of various Gα subunits indicates that both αs and αT have this conserved phenylalanine residue present within their αG/α4 loops (Phe-283 in αT and Phe-312 in αs). Consistent with its importance for full effector activation, we found that mutagenesis of Phe-312 in αs to a tyrosine residue (αs-F312Y) resulted in a protein that was impaired in its ability to fully stimulate adenyl cyclase activity. Therefore, these findings demonstrate that the αG/α4 loop and, specifically, a single amino acid within that stretch of residues, are necessary for Gα subunits to be fully effective in stimulating the enzymatic activity of their biological effectors.

Comparisons of the X-ray crystal structures for the signaling-inactive (GDP-bound) versus the signaling-active (GTP- bound) forms of Gα subunits fail to provide an obvious explanation for the role of the αG/α4 loop in effector activation (1, 2, 5, 24). These structures show no discernable difference in the αG/α4 loop conformation upon GDP–GTP exchange. In fact, the X-ray crystal structure for the AlF₄⁻-activated α7/αi chimeric subunit bound to PDEγ and RGS9 suggests that the αG/α4 loop is on the opposite side of the subunit from the α3/β5 loop that directly contacts PDEγ (9).

Similarly, the X-ray crystal structure of the GTPγS-bound αs subunit complexed to portions of the cytoplasmic domains of adenyl cyclase would indicate that the αG/α4 loop does not directly contact these regions of the effector enzyme (10). Because limit domains were used in the X-ray structures for both the PDEγ subunit (i.e. the C-terminal half of this subunit) and adenyl cyclase (portions of the two major cytoplasmic loops), it is possible that the αG/α4 loop and its essential phenylalanine residue contact other as yet unidentified sites within these effector enzymes which are important for triggering a full stimulation of their activity.

Our direct binding experiments indicate that the αG/α4 loop may contribute to a modest increase in the binding affinity of αT for the PDEγ subunit. However, when directly comparing the dose response profiles for αT-stimulated PDE activity, and the direct binding of αT to free PDEγ or reconstituted holo-PDE as readout by changes in reporter group fluorescence, it appears that following a high-affinity interaction between αT and PDEγ, a second weaker interaction is required for PDE activation, as reflected by the αT dose response for maximum αT-stimulated cGMP hydrolysis. One plausible explanation for this observed difference in dose responses is that the high- and low-affinity interactions represent the binding of activated αT subunits to each of the two PDEγ subunits on holo-PDE. A previous study from our group suggested that the two PDEγ subunits on the holo-PDE enzyme are not functionally identical (28). The high-affinity interaction may involve the α2 (Switch II) helix, the α3 helix, and the α3/β5 loop of αT (depicted as Site I on the left side of Fig. 6) with the C-terminal region of one of the PDEγ subunits on holo-PDE. This represents the binding interaction that is observed in the X-ray crystal structure for the AlF₄⁻-activated α7/αi chimeric subunit bound to PDEγ and RGS9 (9). The translation of this binding into a partial activation of PDE requires the αT contact residues His-244 and Asn-247, as shown when comparing the effector stimulatory capabilities of αT-Chi8 and αT*. In this view, the initial high-affinity αT–PDEγ interaction would induce site–site communication between the PDEα and PDEβ subunits, thereby enabling a second, weaker αT interaction to be brought into play at the second PDEγ site, such that the αG/α4 loop and the essential Phe-283 residue of αT (depicted as Site II on the left side of Fig. 6) interact with the PDEγ, giving rise to the maximum stimulation of effector enzyme activity. The αG/α4 loop flexibility is restricted in the αT-Chi8 subunit, where the X-ray crystal structure shows Tyr-283 is constrained in its movement by intramolecular interactions that prevent the αG/α4 loop from undergoing a stimulatory interaction with the PDE. Although our model proposes that this constraint is eliminated by the Y283F substitution, allowing for an additional stimulatory contact with the effector, we cannot rule out the possibility that this mutation
induces subtle conformational changes in \( \alpha_T \), at sites distant from the \( \alpha_G/\alpha_4 \) loop that impact \( \alpha_T/PDE \) interactions and stimulation. A recent study by Qureshi et al. (29) has indeed proposed a model that is consistent with our data. This model suggests that the high-affinity binding of the first \( \alpha_T \) introduces an asymmetry in the holo-PDE enzyme, thus priming the enzyme. The subsequent, low-affinity binding of a second \( \alpha_T \) is required for the full activation of the holo-PDE enzyme.

A peptide from the \( \alpha_T \) subunit (residues 293–314) encompassing the \( \alpha_4 \) helix, which is adjacent to the \( \alpha_G/\alpha_4 \) and \( \alpha_4/\beta_6 \) loops, was reported to stimulate PDE activity (15). Five residues within this peptide were shown to be important for PDE activation (30), and cross-linking studies have suggested that the PDE-\( \gamma \) subunit binds to residues within the \( \alpha_4/\beta_6 \) loop (31). Homologous regions of the \( \alpha_T \) subunit have been shown to be critical for the stimulation of adenylyl cyclase activity (12). In our model, the interaction of the \( \alpha_G/\alpha_4 \) loop and in particular, the Phe-283 residue (Fig. 6, left side, Site II), with the second PDE-\( \gamma \) subunit on holo-PDE, enables the \( \alpha_4 \) helix and the \( \alpha_4/\beta_6 \) loop to interact with proximal regions of the effector resulting in full enzyme activation.

An important area for future studies will be to obtain a more detailed understanding of the structural basis of the role of the essential phenylalanine residue within the \( \alpha_G/\alpha_4 \) loop of the \( \alpha_T \) subunit in effector activation. What is the relationship between the role of this phenylalanine residue and the changes that occur in the switch regions upon GDP–GTP exchange? We hypothesize that changes in Switch II and III (32) enable a high-affinity \( \alpha_T/PDE\gamma \) interaction, that precedes a weaker interaction by a second \( \alpha_T \) subunit involving its \( \alpha_G/\alpha_4 \) loop and the phenylalanine residue at the second PDE\( \gamma \) site on holo-PDE, giving rise to full effector activation. Similarly, because the conserved phenylalanine residue on the \( \alpha_s \) subunit, Phe-312, is also essential for maximum stimulation of adenylyl cyclase activity, it will be important to better understand the relationship between this site and the high-affinity interactions of Switch II, the \( \alpha_3 \) helix, and the \( \alpha_3/\beta_5 \) loop on \( \alpha_s \) (Fig. 6, right side) with the cytoplasmic regions of adenylyl cyclase, as visualized in the X-ray crystal structure of this complex (10, 11). This again implies multiple interactions between an activated \( G\alpha \) subunit and its effector enzyme, which in the case of adenylyl cyclase, is consistent with the evidence purporting dimeric and even multimeric forms of this effector enzyme having functional importance, and the cooperativity that has been observed when assaying its activation by \( \alpha_s \) (11, 31–35).

**Experimental procedures**

**Expression and purification of \( \alpha_T \) and \( \alpha_s \) subunits**

A chimeric \( \alpha_T \) construct, referred to as \( \alpha_T{-}\)Chi8 (18) was obtained from Dr. Heidi Hamm’s laboratory at Vanderbilt University. This chimera was generated by substituting two regions of \( \alpha_{11} \) (residues 216–227 and 237–294) for the corresponding residues of native retinal \( \alpha_T \) to facilitate its expression in *E. coli*. A modified chimera was created by mutating residues K244H and D247N in \( \alpha_T{-}\)Chi8, resulting in a chimeric \( G\alpha \) subunit referred to as \( \alpha_{T*} \).

Recombinant \( \alpha_{T*} \) and its mutants were expressed in BL21 (DE3) supercompetent cells and purified as described previously (19). The yields of \( \alpha_{T*} \) and its mutants ranged from 0.2 to 2 mg per liter of bacterial culture. The proteins were further purified by ion exchange chromatography on a HiTrap Q FF Sepharose column (GE Healthcare Life Sciences), using a buffer containing 50 mM Tris, pH 8.0, 5 mM MgCl₂, and a linear con-
concentration gradient of NaCl from 20 mM to 1 m. The purified chimeric $\alpha_T^*$ proteins were snap frozen and stored at $-80^\circ$C. Protein concentrations were determined by using absorbance at 280 nm and the calculated molar extinction coefficient. The molar extinction coefficient for each protein was calculated from their sequence using the ExPASy ProtParam tool (https://web.expasy.org/protparam/).

The $\alpha_T$ construct was obtained from Dr. Catherine Berlot (Geisinger Medical Center). Recombinant $\alpha_T$ or $\alpha_T$ (F312Y) was expressed in E. coli JM109 competent cells and purified using a protocol similar to that for the $\alpha_T$ subunits, with the exception of adding 25 $\mu$M GDP in all the buffers to enhance protein stability. The proteins were further purified by size-exclusion chromatography on a HiLoad Superdex 75 gel filtration column (GE Healthcare), using a buffer containing 20 mM Na-HEPES, pH 7.5, 5 mM MgCl$_2$, 100 mM NaCl, 10% glycerol, and 1 mM DTT. The purified proteins were snap frozen and stored at $-80^\circ$C. Protein concentrations were determined by using absorbance at 280 nm and the calculated molar extinction coefficient. The molar extinction coefficient for each protein was calculated from their sequence using the ProtParam tool (https://web.expasy.org/protparam/).

Purification of retinal proteins

Bovine rod outer segment membranes were isolated using a sucrose gradient as described previously (36). Urea-washed disc membranes were prepared as described and served as the source of light-activated rhodopsin (37). PDE and transducin were isolated by washing rod outer segment membranes with hypotonic buffer in the absence and presence of GTP. The $\alpha_T$ subunit and $\beta_\gamma$ subunit complex of transducin were resolved on a HiTrap Blue Sepharose column (GE Healthcare). The column was first washed with a buffer containing 10 mM Na-HEPES, pH 7.5, 6 mM MgCl$_2$, 1 mM DTT, 100 mM KCl, and 10% glycerol to elute the $\beta_\gamma$ complex. To elute $\alpha_T$, a buffer containing 10 mM Na-HEPES, pH 7.5, 6 mM MgCl$_2$, 1 mM DTT, 500 mM KCl, and 10% glycerol was subsequently applied onto the column. All transducin subunits and holo-PDE were loaded on HiLoad Superdex 75 and Superdex 200 gel filtration columns (GE Healthcare), respectively, and eluted with a buffer containing 20 mM Na-HEPES, pH 7.4, 5 mM MgCl$_2$, 100 mM NaCl, and 1 mM DTT. The pure retinal proteins were concentrated to $\sim 20 \mu$M, snap frozen, and stored at $-80^\circ$C.

Determination of active concentrations of $\alpha_T$ subunits

Active $\alpha_T$ subunit concentrations were determined by measuring the increase in intrinsic tryptophan fluorescence upon the rhodopsin-catalyzed exchange of GTP$\gamma$S for GDP (38). Fluorescence was measured on a Cary Eclipse fluorescence spectrometer with the excitation wavelength set at 300 nm and the emission wavelength set at 345 nm. All experiments were carried out at 25°C using a circulating water bath connected to the cuvette holder. The assay was performed in 1 ml HMDM buffer (20 mM Hepes, pH 7.5, 5 mM MgCl$_2$, and 0.01% (w/v) dodecylmaltoside). Light-activated rhodopsin (50 nM), $\beta_\gamma$ (300 nM), and an $\alpha_T$ subunit (500 nM) were added to the cuvette. The increase in fluorescence was monitored upon addition of two successive additions of GTP$\gamma$S, at sub-stoichiometric amounts, to final concentrations of 100 nM and 200 nM. The slope ($m$) of a linear fit of the fluorescence change as a function of the concentration of added GTP$\gamma$S was obtained. Subsequently, GTP$\gamma$S was added to a final concentration of 10 $\mu$M. Under this condition all the $\alpha$ subunits would have bound GTP$\gamma$S and provide the maximal change in fluorescence ($F_{max}$). The active concentration of $\alpha$ subunits in the sample was calculated by dividing $F_{max}$ by the slope, $m$.

Fluorescence measurements of AlF$^{4-}$ and GTP$\gamma$S binding

Fluorescence assays reading out the binding of aluminum fluoride (AlF$^{4-}$) to $\alpha_T$, chimeric $\alpha_T$/$\alpha_T$, or $\alpha_T$ (F312Y) subunits were performed on a Cary Eclipse fluorescence spectrometer. The binding of AlF$^{4-}$ was measured by monitoring the intrinsic tryptophan fluorescence of $\alpha$ subunits with the excitation wavelength set at 280 nm (bandwidth = 2.5 nm) and the emission wavelength set at 340 nm (bandwidth = 10 nm). $\alpha_T$, $\alpha_T^*$, or $\alpha_T^*$-SFD (400 nm) was incubated in 1 ml of HMDM buffer. $\alpha_T$ or $\alpha_T$ (F312Y) (400 nm) was incubated in 1 ml of HM buffer (20 mM Hepes, pH 7.5, and 5 mM MgCl$_2$). Tryptophan fluorescence emission was monitored continuously for up to 2 min. Subsequently, 20 $\mu$L of a premixed aliquot containing 250 mM NaF and 2.5 mM AlCl$_3$ to generate AlF$^{4-}$ was added and the enhancement in tryptophan fluorescence was monitored in real time. All measurements were performed at room temperature. GTP$\gamma$S binding to $\alpha_T$ or $\alpha_T$ (F312Y) was measured by monitoring tryptophan fluorescence (excitation: 300 nm; emission: 345 nm) in real time. $\alpha_T$ or $\alpha_T$ (F312Y) (250 nm) was incubated in the presence or absence of 250 nM $\beta_\gamma$ (250 nm). $\beta_\gamma$ in 1 ml of HM buffer. Tryptophan fluorescence emission was monitored continuously for 2 min. Subsequently, 10 $\mu$L GTP$\gamma$S was added and the enhancement in tryptophan fluorescence was recorded.

Purification and labeling of PDE$\gamma$

The recombinant PDE$\gamma$ subunit was expressed and purified as described previously (39). The purified PDE$\gamma$ was loaded onto a PD-10 desalting column (GE Healthcare) for removal of DTT in the storage buffer. The desalted protein solution was incubated with a 30-fold molar excess of IAESENS at room temperature in the dark for 1 h. To terminate the modification reaction, 60 mM 2-mercaptoethanol was added to the reaction mixture. IAESENS-labeled PDE$\gamma$ was then separated from free probe on a PD-10 desalting column using a buffer containing 20 mM Hepes, pH 7.5, and 100 mM NaCl. The concentration of labeled protein was determined by measuring the absorbance at 336 nm using a molar extinction coefficient of 5700 M$^{-1}$ cm$^{-1}$. The stoichiometry of labeling was determined to be 1.03 ± 0.02 mol per PDE$\gamma$.

Fluorescence assays reading out the binding of $\alpha_T$ and chimeric $\alpha_T$/$\alpha_T$ subunits to IAESENS-labeled PDE$\gamma$ were performed on a Cary Eclipse fluorescence spectrometer. In a stirred quartz cuvette, 200 nM IAESENS-labeled PDE$\gamma$ in a buffer containing 10 mM Tris, pH 8.0, 2 mM MgCl$_2$, and 100 mM NaCl, was incubated in either the presence or absence of 100 nM trypsinized PDE. The excitation and emission wavelengths were set at 336 nm (bandwidth = 10 nm) and 490 nm (bandwidth = 10 nm), respectively. Aliquots of activated $\alpha_T$ mutants were added in 30 nM increments and the increase in fluorescence...
cence emission was monitored in real time. The $\alpha_{\text{G}}$ subunits were activated by incubating the proteins with $100 \mu M$ GTP$\gamma$S in the presence of light-activated rhodopsin ($200 \text{ nm}$) and $\beta_{1}\gamma_{1}$ ($1 \text{ mM}$) at room temperature for up to 45 min. The ability of a catalytic level of rhodopsin/$\beta_{1}\gamma_{1}$ to effect nucleotide exchange was not diminished by any of the $\alpha_{\text{G}}$ subunit mutations used to monitor PDE binding or activity (subsequent section). The binding isotherms obtained from the titration experiments were fit to a simple two-state binding model.

**Measurement of PDE activity**

cGMP hydrolysis by PDE was measured and analyzed as described previously (40). Typically, in $200 \mu l$ of reaction buffer containing $10 \text{ mM}$ Tris, pH 8.0, $2 \text{ mM}$ MgCl$_2$, and $100 \text{ mM}$ NaCl, GTP$\gamma$S-loaded $\alpha_{\text{G}}$ subunits, at varying concentrations, were incubated with either holo-PDE or reconstituted PDE (50 nm) in a 48-well plate. The pH (in millivolts) was monitored in real time and once a stable baseline was achieved, 5 nm cGMP was added and the decrease in pH was recorded for 150 s. The buffering capacity of the assay buffer (mV/nmol) was determined from the ratio of the initial slope of the pH record (mV/s) and the buffering capacity of the assay buffer (mV/nmol).

**Cell culture**

HEK-293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37 °C and 5% CO$_2$. HEK-293 cells were transiently transfected with the pcDNA3 vector alone or with the same vector containing distinct $\alpha_{\text{G}}$ constructs using Lipofectamine and PLUS reagents (Invitrogen).

**Cell-based $\alpha_{\text{G}}$ plate assay**

The WT $\alpha_{\text{G}}$ construct was obtained from Dr. Catherine Berlot, as indicated above. The Q227L and F312Y point mutations were generated by site-directed mutagenesis and confirmed by sequencing at the Biotechnology Resource Center (BRC), Cornell University. For the SEAP assays, HEK-293 cells were transiently transfected with pCRE-SEAP (Clontech) and either pcDNA3 (vector-alone), $\alpha_{\text{G}}$ WT, $\alpha_{\text{G}}$(Q227L), or $\alpha_{\text{G}}$(Q227L, F312Y) expression constructs in 10-cm dishes. After 24 h, cells were serum starved in DMEM and the buffering capacity of the media was determined. A plate reader (BioTek, Synergy HT) with excitation and emission wavelengths set at 340 nm and 460 nm, respectively, was used to detect SEAP levels in the media of the plate. Each sample was examined in triplicate. The HEK-293 cells from the transfections were harvested and the protein expression of the various $\alpha_{\text{G}}$ mutants was examined by Western blot analysis. The antibody against $\alpha_{\text{G}}$ was obtained from Santa Cruz Biotechnology (Dallas, TX). The vinculin antibody was purchased from Cell Signaling Technology (Danvers, MA).

**Statistical analysis**

SigmaPlot and GraphPad software was used to carry out statistical analysis. The data are presented as mean ± S.E. Statistical significance was evaluated using the unpaired Student’s $t$ test. Comparisons with $p$ values less than 0.05 were considered to be statistically significant.

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