Perturbing the Linker Regions of the α-Subunit of Transducin

A NEW CLASS OF CONSTITUTIVELY ACTIVE GTP-BINDING PROTEINS

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The GDP-GTP exchange activity of the retinal G protein, transducin, is markedly accelerated by the photoreceptor rhodopsin in the first step of visual transduction. The x-ray structures for the α subunits of transducin (αT) and other G proteins suggest that the nucleotide-binding (Ras-like) domain and a large helical domain form a “clam shell” that buries the GDP molecule. Thus, receptor-promoted G protein activation may involve “opening the clam shell” to facilitate GDP dissociation. In this study, we have examined whether perturbing the linker regions connecting the Ras-like and helical domains of Go subunits gives rise to a more readily exchangeable state. The sole glycine residues in linkers 1 and 2 were individually changed to proline residues within an αG/αT chimera (designated αGαT). Both αG linker mutants showed significant increases in their basal rates of GDP-GTP exchange when compared either to retinal αT or recombinant αT. The αT linker mutants were responsive to aluminum fluoride, which binds to α-GDP complexes and induces changes in Switch 2. Although both linker mutants were further activated by light-activated rhodopsin together with the βγ complex, their activation was not influenced by βγ alone, arguing against the idea that the βγ complex helps to pry apart the helical and Ras-like domains of Go subunits. Once activated, the αG linker mutants were able to stimulate the cyclic GMP phosphodiesterase. Overall, these findings highlight a new class of activated Gα subunits (αGαT) by stimulating its GDP-GTP exchange reaction. Activated GTP-bound αT then stimulates PDE activity by altering the positions of its regulatory γ subunits (γPR and γPRγ) to hydrolyze and consequently decrease cellular cGMP concentrations. This causes cation-specific cGMP-gated ion channels in the outer segments to close, leading to the hyperpolarization of rod outer segment membranes and the generation of the visual response. There is an amplification of the signal at every step of this cascade such that a single photon of light can lead to the inhibition of $10^{-10} \text{Na}^+$ ions from entering the rod cell.

Members of the family of large G proteins are made up of three distinct subunits: α (39–46 kDa), β (37 kDa), and γ (8 kDa), with the β and γ subunits being in a tightly associated complex, under all nonadenaturing conditions. The N-terminal region of Go subunits, together with the C-terminal region of Gγ subunits, have lipid modifications and are involved in membrane attachment (1–3). G proteins are inactive and unable to propagate signals when their α subunits are in the GDP-bound state. They become activated by receptor-catalyzed guanine-nucleotide exchange, resulting in the dissociation of GDP and the binding of GTP to the Go subunit. This leads to dissociation of the βγ complex from the GTP-bound Go subunit, freeing both the Go and βγ subunits to engage downstream effectors. Thus, in a sense, the γ phosphate of GTP acts as a molecular switch that relays information in the form of a conformational change. The rate-limiting step for G protein activation is GDP release. Compared with other large G proteins, including close family members (Goα1), the retinal G protein transducin has an extremely low basal nucleotide exchange rate, although it undergoes a very rapid receptor-catalyzed nucleotide exchange reaction (4). The low level of background noise exhibited by transducin in the absence of a signal from light-activated rhodopsin is consistent with its role in visual phototransduction, where low biochemical noise levels and extremely tight regulation of signaling are required.

One of the fundamentally important, but still unanswered, questions regarding the mechanism of G protein function is to understand the structural and molecular changes that need to occur within the α subunits (Goα) to activate them for signal propagation. The overall importance of G protein activation is demonstrated by the severe biological consequences ranging from developmental disorders to malignant transformation that can occur when heptahelical receptors or their G protein targets become hyperactive as an outcome of mutagenesis. Both the guanine nucleotide exchange and GTP hydrolytic activities of G proteins need to be tightly regulated, because the loss of regulation can have dire biological consequences. For example, point mutations in the Goα subunit that accelerate GDP dissociation (A366S) or inhibit GTP binding to a nucleo-

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‡ The abbreviations used are: PDE, cGMP-phosphodiesterase; GTPγS, guanosine-5′-O-(3-thiotriphosphate); R*, light-activated rhodopsin.
tide-depleted protein (R231H) give rise to an endocrine disorder (testotoxicosis) and to Type I pseudohypoparathyroidism, respectively (5).

A particularly interesting and perhaps puzzling feature of the activation mechanism of large G proteins is the observation that the heptahelical G protein-coupled receptors do not seem to directly contact either the nucleotide-binding site or the bound nucleotide (6). The x-ray crystal structures of different Go subunits show that their helical and Ras-like GTPase domains act like a clam shell to bury the nucleotide and its associated Mg$_2^+$ ion (1, 7–10). These two domains are connected by linkers (in the case of $\alpha_T$, linker 1 consists of residues 54–58 and linker 2 includes residues 173–179) that may help to open and close the clam shell. The helical domain is thought to act as a rigid lid over the nucleotide-binding site, and hence it is believed that for a heptahelical receptor to stimulate nucleotide exchange and Go activation, there must be a conformational change that opens the cleft (7, 8, 11).

In this study, we decided to investigate the role of the two linkers in controlling the basal nucleotide exchange rate of Go subunits. In the case of $\alpha_T$ (and virtually all Go subunits), each of the linkers has a single glycine residue; Gly$^{179}$ in linker 1 and Gly$^{179}$ in linker 2. Each glycine was mutated to a proline in the background of an $\alpha_T$/G$_{11}$ chimera that contains wild-type $\alpha_T$ residues except in the Switch 3 domain (see “Experimental Procedures”). This chimera, designated from here on as $\alpha_T^*$, was used because unlike wild-type $\alpha_T$, it can be readily expressed and purified from Escherichia coli. The assumption was that the Gly $\rightarrow$ Pro substitutions would decrease the flexibility and consequently increase the rigidity of the linkers. Our results indicate that both $\alpha_T^*$ linker mutants show constitutive nucleotide exchange in the absence of light-activated receptor and full stimulation of PDE activity and thereby represent a novel class of dominant-active Go subunits.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—**Sense and antisense oligonucleotide primers were synthesized with the desired mutated base in the center of the primer. The QuikChange site-directed mutagenesis kit (Stratagene) was used to carry out the PCR-based mutagenesis on miniprep plasmid DNA. The parental DNA template was digested using DpnI endonuclease and the nicked vector DNA with the desired mutation was transformed into Epicurian Coli XL-1 Blue supercompetent cells. DNA was purified from single colonies using the QiaQuick plasmid mini-prep kit. The DNA was sequenced at the Bio Resource Center of Cornell University.

**Construction of the $\alpha_T^*$ Chimera—**The $\alpha_T$ subunit, when expressed in E. coli, is not functional, whereas Go$_{11}$ is fully functional. Therefore, we created a chimera in which the Cterminal domain of Go$_{11}$ was fused in-frame to the Nterminal domain of $\alpha_T$ (12). We used a construct designated pHis6Chi6 that was obtained from Prof. Heidi Hamm at Vanderbilt University. This chimera, which has the corresponding region from $\alpha_1$ inserted between residues 215 and 294 of $\alpha_T$, is unable to stimulate PDE activity, although it is capable of binding to the $\gamma$PDE subunit in a GDP-dependent manner. The mutation of residues 244 (Lys $\rightarrow$ His) and 247 (Asp $\rightarrow$ Asn) to the original residues in $\alpha_T$ restores the ability to stimulate PDE activity to about 50% of the levels obtained with retinal $\alpha_T$. From here on, the $\alpha_T^*$ chimera (previously known as Ch6) that contains these mutations at positions 244 and 247 will be referred to as $\alpha_T^*$. The linker mutations described in this study have been made in the $\alpha_T^*$ background. There, the Go subunits containing the C-terminal to proline substitutions in the linker regions are designated as $\alpha_T^*$ (G56P) and $\alpha_T^*$ (G179P).

**Protein Expression and Purification—**The recombinant $\alpha_T^*$ and the $\alpha_T^*$ linker mutants were expressed in BL21(DE3) supercompetent cells and purified in the presence of 50 $\mu$M GDP as described in Ref. 12. The protein was further purified by gel filtration chromatography on a HiLoad Superdex 75 HR26/60 column equilibrated with a buffer containing 20 mM Na-Hepes, pH 7.5, and 10% glycerol. The samples were aliquoted, snap-frozen, and stored at $-80^\circ$C. The final yield of each subunit ranged from 3–5 mg of pure protein/300 retina. Urea-washed rod outer segment membranes were prepared as described in Ref. 16.

$^{[35S]}$GTP-$\gamma$S Binding Assays—Rhodopsin, together with the $\alpha_T^*$ and $\beta$ complexes, was incubated in HMDN buffer (20 mM Hepes, pH 7.5, 5 mM MgCl$_2$, 1 mM dithiothreitol, and 100 mM NaCl) for 20 min at room temperature (in room light). $^{[35S]}$GTP-$\gamma$S (final concentration 1 mM, specific activity, 1 Ci/mmol) was added to initiate the reaction, and the samples were incubated for the required time period. The reaction was quenched by direct application to prewetted nitrocellulose filters (Schleicher & Schuell; pore size, 0.45 $\mu$m) on a suction manifold. The filters were washed twice with HBM buffer (20 mM Hepes, pH 7.5, 5 mM MgCl$_2$, and 100 mM NaCl), added to scintillation liquid (30% LSC Scintisafe Mixture), and counted in a scintillation counter (LS6500 Multipurpose Scintillation counter).

**Measurements of cGMP PDE Activity—**The analysis of cGMP hydrolysis by the retinal PDE was carried out as described in Ref. 17. A pH microelectrode was used to measure the decrease in pH (in millivolts) from the release of one proton for each molecule of cGMP hydrolyzed by PDE. In a typical assay, $\alpha_T^*$ was preincubated with $\beta_T$, light-activated rhodopsin (R*), GTP-$\gamma$S, and PDE in a buffer containing 5 mM Hepes, pH 7.4, 2 mM MgCl$_2$, and 100 mM NaCl, at room temperature and in room light for 30 min. The reaction was initiated by addition of the substrate, cGMP, to a final concentration of 5 mM, and the change in pH was monitored over time. The assays were initiated by the addition of $[\gamma^3P]GTP$ (final concentration, 0.5 $\mu$M; specific activity, 13 Ci/mmol). Aliquots (40 $\mu$L) were removed at specific time points and added to 1 ml of 5% activated charcoal (neutralized) in 50 mM Na$_2$PO$_4$. The samples were centrifuged at 35 °C, and then 100 $\mu$L of supernatant was added to scintillation fluid and counted in a scintillation counter (LS6500 Multipurpose Scintillation counter).

**Fluorescence Measurements—**The binding of aluminum fluoride (AlF$_4^-$) as well as GTP-$\gamma$S was measured by monitoring the intrinsic tryptophan fluorescence of $\alpha_T^*$ upon excitation at 300 nm and emission at 345 nm (18, 19). In a typical experiment, 300 nM $\alpha_T^*$ (as determined by $^{[35S]}$GTP-$\gamma$S binding activity) was preincubated in HM buffer (20 mM Hepes, pH 7.5, 5 mM MgCl$_2$) at room temperature. In the AlF$_4^-$ binding assays, fluorescence enhancement was read until saturation after the addition of AlF$_4^-$ (the AlF$_4^-$ complex was formed by mixing 5 mM NaF and 100 mM AlCl$_3$). When complete, the samples were centrifuged at 35 °C, and then 100 $\mu$L of supernatant was added to scintillation fluid and counted in a scintillation counter.

**RESULTS**

The $\alpha_T$ Mutants Exhibit Receptor-independent Guanine Nucleotide Exchange Activity—The x-ray crystal structure of $\alpha_T^*$ shows that its helical domain and Ras-like GTPase domain may act like a clam shell to bury the bound guanine nucleotide (Fig. 1 and Refs. 1 and 7). These domains are connected by two linkers (linker 1, residues 54–58, and linker 2, residues 173–179). The helical domain is thought to cover the guanine nucleotide-binding site, such that for a heptahelical receptor (e.g. rhodopsin) to stimulate nucleotide exchange and Go activation,
there must be a conformational change that opens the cleft (7, 8, 11). Thus, we asked whether by perturbing the juxtaposition of the helical domain relative to the Ras-like GTPase domain and thereby opening the cleft between them, can we generate a Ga subunit that mimics the receptor-activated state? In particular, we suspected that the linkers connecting the two domains of Ga subunits may help to open and close the clam shell and thus control the basal nucleotide exchange rate. We therefore assumed that by altering the “rigidity” of the linkers, it may be possible to open the cleft between the two domains and increase the intrinsic rate of guanine nucleotide exchange on a Ga subunit (e.g. αT).

Many of the residues in both linkers 1 and 2 are highly conserved among the heterotrimeric Ga subunits, suggesting that these residues may be critical for participating in or maintaining interdomain interactions. For example, each of the linkers contains a single glycine residue; Gly56 in linker 1 and Gly179 in linker 2 of αT. Glycine, being the amino acid lacking a side chain, shows the greatest conformational flexibility. Using PCR-based mutagenesis, each flexible glycine was mutated to a proline, the amino acid with the least conformational flexibility. The assumption was that the Gly → Pro substitutions would decrease the flexibility and consequently increase the rigidity of the linkers and thereby potentially trap the clam shell formed by the helical and Ras-like domains of αT in an open state. To facilitate expression in E. coli, these point mutations were prepared in the background of an αT/α1 chimera (see “Experimental Procedures”), which we refer to as αT*.

Fig. 2 shows the results of [35S]GTPγS binding assays that led us to conclude that the linker mutants can indeed exchange nucleotide without receptor. The αT* subunit showed the expected requirement of light-activated rhodopsin (R*) and the βγ complex for GDP-GTP exchange and consequent activation (Fig. 2A). Similar to what has been observed with the native retinal αT, the E. coli recombinant αT* subunit shows little detectable nucleotide exchange in the absence of R* and βγ, and only relatively weak exchange activity when incubated with either R* or βγ alone. On the other hand, the αT*(G56P) (Fig. 2B) and αT*(G179P) (Fig. 2C) mutants exhibited significant nucleotide exchange when assayed in the absence of R* and βγ, although in both cases, the rate of nucleotide exchange was further enhanced in the presence of R* and βγ.

Fig. 3 compares the rates of receptor-independent GDP-GTPγS exchange for αT*, αT*(G56P), and αT*(G179P), as assayed by the real time changes in the fluorescence of Trp207, which accompanies the GTP-induced “activating conformational change” within the Switch 2 domain. At room temperature (25 °C), a very slow enhancement in the Trp207 fluorescence of αT* occurred upon the addition of GTPγS to the cuvette, similar to the results obtained with the native retinal αT subunit (Fig. 3, right panel). However, both the αT*(G56P) and αT*(G179P) mutants exhibited a significantly enhanced rate of change in Trp207 fluorescence, reflecting a much more rapid GDP-GTPγS exchange reaction. The same was true when comparing these fluorescence changes at 35 °C. Although under these conditions, the basal rate of nucleotide exchange activity for αT* was higher than that measured at room temperature, as well as being greater than the basal rate of GDP-GTPγS exchange for retinal αT as reported earlier (12), the corresponding rates for
nucleotide exchange on the α7 linker mutants were still significantly greater than that of α7T (Fig. 3, left panel). Taken together, the results presented in Figs. 2 and 3 demonstrate that perturbing the regions that link the helical and Ras-like domains of α7 do indeed give rise to constitutive GDP-GTP exchange activity.

Both the α7G(G56P) and α7G(G179P) Mutants Respond to AlF4—G protein α subunits, when in the GDP-bound state, undergo an activating conformational change upon the formation of a Ga-GDP-AlF4−complex (18, 19). Similar to the case for GTPγS-induced activation of α7 (shown in Fig. 3), the AlF4−-promoted activation event, which requires the presence of GDP in the nucleotide-binding site, can be monitored in real time by changes in Trp207 fluorescence. The AlF4−-induced enhancement of the Trp207 fluorescence of α7 occurs within seconds (Fig. 4). The α7G(G56P) mutant shows virtually identical kinetics for the AlF4−-induced conformational change as that observed for α7T, whereas the α7G(G179P) mutant shows a similar but slightly slower rate for the corresponding AlF4−-induced change. Overall, these results indicate that the nucleotide-binding pocket of the α7 linker mutants is not empty following their expression and purification but rather contains bound GDP, as is required for the binding of AlF4−. The AlF4−-induced changes in Trp207 fluorescence also verify that these linker mutants are capable of undergoing the necessary structural changes in Switch 2 that normally accompany the activation event.

βγ Dependence—In the absence of receptor, the rate of nucleotide exchange for the α7 linker mutants shows little dependence on the concentration of βγ. The exact role that the βγ complex of transducin plays in the rhodopsin-stimulated nucleotide exchange reaction is still debatable. The βγ subunit complex has been shown to increase the affinity of α7 for rhodopsin (20) and in this way may work together with the photoreceptor to stimulate GDP dissociation and nucleotide exchange (21). It has also been proposed that receptors use the βγ subunit complex as a lever to pull a flexible lip of Ga subunits (comprised of Switch 1 and the β3/α2 loop) away from its normal position of blocking an exit from the nucleotide-binding site, thereby setting the stage for the release of GDP (5, 22). The βγ lever model (5) is compatible with the flexibility of the linkers connecting the helical and Ras-like domains of Ga subunits and their ability to function as a hinge (23). Given that the presence of light-activated rhodopsin is essential for the activation of the α7 subunit, it has been difficult to tweeze apart the effects of βγ versus the photoreceptor on the guanine nucleotide exchange reaction. However, because the α7 linker mutants exhibit constitutive nucleotide exchange, they provide the opportunity for examining whether the βγ complex can directly influence nucleotide exchange within a Ga subunit.

Fig. 5A shows the kinetics of the rhodopsin-stimulated binding of [35S]GTPγS to α7 in the absence of βγ and in the presence of 350 and 700 nM βγ. As expected (Fig. 1A), the α7 subunit exhibited a slower rate of rhodopsin-stimulated nucleotide exchange in the absence of βγ, compared with the rate of exchange measured in the presence of both R7 and βγ. Moreover, βγ acted in a dose-dependent manner to increase the rhodopsin-stimulated nucleotide exchange activity of α7 (Fig. 5A). In a similar fashion, the βγ complex enhanced the R7-stimulated nucleotide exchange activity of the α7G(G56P) mutant (Fig. 5B). However, the nucleotide exchange activity for α7G(G56P), which occurred in the absence of R7, was much less sensitive to βγ (Fig. 5C). The same was true when assaying the nucleotide exchange activities of the α7T(G179P) mutant (Fig. 5, D and E).

Overall, these findings suggest that the βγ complex does not play the role of a guanine nucleotide dissociation inhibitor (24–26), at least for the case of the recombinant α7 and likely not for the retinal α7 subunit. This apparently differs from other large G proteins like Ga and Go, where the corresponding βγ complexes markedly inhibit GDP release (10) and might be explained by the fact that α7 (as well as retinal α7) holds the
nucleotide much more tightly than other Go subunits because it has a smaller nucleotide-binding cleft (27) and hence may not require a guanine nucleotide dissociation inhibitor to maintain the basal GDP-bound state. The above results may also hold some interesting implications with regard to the βγ lever arm model. Although they do not definitively disprove the model for the case of transducin, they show that the βγ complex does not help the αT linker mutants to adopt a more open state (in terms of the relative juxtaposition of the helical domain versus the Ras-like domain), because the simple binding of βγ to these mutants does not give rise to an additional detectable enhancement in the overall rate of nucleotide exchange.

Effects of Light-activated Rhodopsin on the Rate of Nucleotide Exchange Exhibited by the αT Linker Mutants—Fig. 6 shows the relative amounts of [35S]GTPγS bound to the αT subunit (after 10 min) in the presence of a fixed concentration of βγ, as a function of different concentrations of R*. Under these assay conditions, in the absence of R*, the αT subunit exhibited negligible basal exchange of GDP for radiolabeled GTPγS and thus little [35S]GTPγS binding activity (i.e. ~6% of maximal binding), whereas upon the addition of light-activated rhodopsin, the rate of nucleotide exchange was markedly accelerated. Maximal binding of [35S]GTPγS to αT occurred at ~10 nM R*, which highlights the ability of R* to act catalytically in promoting the activation of multiple αT subunits, because the αT concentration was set at 700 nM.

Somewhat different results were obtained with the αT(G56P) and αT(G179P) mutants. Although the αT linker mutants exhibited detectable basal [35S]GTPγS-binding activity after 10 min (~33% and ~20–25% of maximal binding, respectively), the binding of GTPγS to the linker mutants was further stimulated by R* and the βγ complex. However, comparisons of the dose responses for R* obtained with the αT linker mutants show that for the αT(G56P) mutant, maximal binding was still not reached when using 50 nM R*. The differences were even more dramatic with the αT(G179P) mutant, where half-maximal binding of [35S]GTPγS was just being approached when assaying with 50 nM R*. These results suggest that the αT linker mutants exhibit a reduced affinity for the photoreceptor complex compared with the αT subunit, where the juxtaposition of the helical domain relative to the Ras-like domain has not been perturbed.

The Turnover Numbers for GTP Hydrolysis Rates Are Slower in the Linker Mutants—GTP hydrolysis serves as an internal clock that controls the activation lifetime of Go subunits. In the presence of R* and the βγ subunit complex, the αT subunit was shown to undergo a GTP hydrolytic reaction (following the exchange of GDP for GTP) that was linear over 2 h (Fig. 7A), with a turnover number of ~0.16 (±0.04) mol 32P per mol αT per minute. Note that the ability of R* and the βγ complex to stimulate the GTP hydrolytic activity of αT simply reflects the fact that the binding of GTP by αT, as an outcome of GDP-GTP exchange, is enhanced under these conditions. The αT linker mutants, i.e. αT(G56P) (Fig. 7B) and αT(G179P) (Fig. 7C), were also capable of GTP hydrolytic activities that were linear over several hours. The fact that the αT linker mutants show a significantly greater intrinsic GTP hydrolytic activity, compared with αT, is consistent with the abilities of these mutants to constitutively exchange GDP for GTP. How-
ever, the turnover numbers for GTP hydrolysis catalyzed by the linker mutants, in the presence of R* and without R* or D. Thus, once the $\alpha_T$ linker mutants have undergone GDP-GTP exchange, the GDP-GTP-bound forms of these $\alpha_T$ mutants are able to bind to the PDE and stimulate its activity as effectively as the wild-type $\alpha_T$ subunit. This indicates that perturbing the juxtaposition of the helical and Ras-like domains through these linker mutations does not compromise binding and regulation of the target/effector.

**DISCUSSION**

During the past several years, a good deal of effort has been directed toward understanding the molecular basis by which heterotrimeric G proteins serve as molecular switches in sensory response signaling pathways. The successful determination of the x-ray crystal structures of Gs subunits in both their GDP-bound (signaling off) and GTPyS-bound (signaling on) states has been particularly revealing in terms of the molecular basis by which G proteins act as signal transducers (7, 8). Such structural comparisons have highlighted three conformationally sensitive regions on Gs subunits that are suspected to play essential roles in signal propagation. Two of these regions, designated as Switch 1 and Switch 2, correspond to the conformationally sensitive regions that were originally identified from structural work on the small GTP-binding protein Ras and the bacterial protein EF-Tu (1). A third conformationally sensitive region (Switch 3) appears to be important in translating the binding of an activated Gs subunit to its target/effector into the stimulation of effector activity (8, 28). Additional x-ray structures for Gs-GDP bound to the $\beta\gamma$ complex (9, 10), and for activated $\alpha$ subunits associated with their target/effectors (29, 30), have further added to the picture regarding the necessary molecular steps by which activated Gs subunits disengage from their upstream heptahelical receptors and $\beta\gamma$ subunit cohorts and bind and regulate their downstream effectors.

Still, despite this accumulation of structural and mechanistic information, a major question remains regarding the molecular basis by which heptahelical G protein-coupled receptors trigger the key first step in their signaling cascade. Namely, how do these receptors stimulate the dissociation of GDP from Gs subunits within the heterotrimeric G protein, so that GTP binding and the necessary activating conformational changes within the Gs subunits can ensue? This represents a particularly challenging question because heptahelical receptors, as they sit in the plasma membrane, are located a significant distance away from the bound GDP molecule on the Gs subunits that they activate (6, 26). Nonetheless, various mechanisms have been proposed regarding the necessary conformational changes for the receptor-mediated activation event. One has involved a receptor-induced perturbation of the $\alpha_5$ helix of the Gs subunit, because this serves as a link between the $\alpha_5$-$\beta_6$ loop, which is adjacent to and contacts the guanine ring of GDP, and the receptor-contact site at the C-terminal tail of Gs (5). In fact, mutations within the $\alpha_5$-$\beta_6$ loop have been shown to result in accelerated rates of GDP dissociation from Gs subunits (31).

A second mechanistic route has been proposed to begin with a receptor-promoted change in the juxtaposition of the Gs and $\beta\gamma$ complex, which then allows the $\beta\gamma$ complex to act as a lever to pull what has been referred to as a flexible lip (which entails the Switch 1 region and the $\beta_3$-$\alpha_2$ loop of the Ras-like domain of Gs subunits) away from a possible exit site for GDP (5). This proposal, in part, stemmed from the mechanism used by the guanine nucleotide exchange factor, EF-Ts, to bind and stimulate the rate of GDP dissociation from EF-Tu, because the $\beta\gamma$ subunit complex appears to engage Gs subunits in a manner analogous to the functional coupling of EF-Ts to EF-Tu. It has also been reported that mutations of Gs subunits that do not
compromise binding to Gα subunits nonetheless interfere with receptor-promoted G protein activation (5). More recent studies have shown that by mutating the N-terminal end of the Gα subunit, so that its juxtaposition relative to the βγ complex was tilted, made the Gα subunit responsive to βγ-induced GDP dissociation (22).

Bearing in mind these possible activating mechanisms, an important piece of the puzzle that remains to be understood involves the juxtaposition of the large helical domain of Gα subunits, relative to the Ras-like domain. The helical domain essentially fits over the nucleotide-binding site within the Ras-like domain and thereby impedes the release of GDP. This has led to the description of Gα subunits as resembling a clamp shell and represents the major distinguishing structural feature from the members of the small GTP-binding protein family, which lack the large helical domain. It also suggests that helical G protein-coupled receptors, which functionally are analogous to the GEFs for small GTP-binding proteins, will likely need to perturb the juxtaposition of the helical and Ras-like domains on Gα subunits and in effect open the clamp shell, as a means of catalyzing GDP release. If this were indeed the case, it should be possible to generate a constitutively active Gα subunit (i.e. capable of spontaneous GDP-GTP exchange) by altering the normal juxtaposition of the helical and Ras-like domains through mutagenesis. This general idea was likely the basis for a series of mutagenesis experiments aimed at disrupting potential contact sites between the helical and Ras-like domains of Gα, (32), with the conclusion of that study being the perturbation of the interactions between the helical and Ras-like domains did not significantly change the intrinsic nucleotide exchange activity of Gα subunits. However, in the present work, we have taken a different approach and altered the inherent flexibility of the linker regions that connect the helical and Ras-like domains of the Gα subunit with the idea being to generate a more open state for the clamp shell and thereby facilitate GDP-GTP exchange. Previous molecular dynamic studies have suggested that the helical domain of Gα moves toward the Ras-like domain, facilitated by a hinge bending motion that is centered on residues Glyγ86 and Glyγ179 (33, 34). Indeed, we have found that individually changing these conserved glycine residues in linkers 1 and 2 to prolines yielded Gα mutants (i.e. Gα(G56P) and Gα(G179P)) that showed a significant capability for undergoing GDP-GTP exchange in the absence of light-activated rhodopsin and the βγ subunit complex.

Thus, we feel that we have generated a new type of constitutively active Gα mutant, which, because it is able to exist in a more open state than the wild-type protein, can more rapidly dissociate GDP and provide access to the nucleotide-binding site for cellular GTP. Once bound, GTP is then able to induce the necessary conformational changes in these Gα mutants to allow them to dissociate from the βγ complex and to bind and activate the cyclic GMP PDE.

The ability to generate new types of constitutively active Gα subunits raises a number of questions and possible mechanistic implications. One interesting question concerns the exact role of the βγ complex, which appears to vary from one G protein heterotrimer to another. The ability of the βγ complex to directly influence nucleotide exchange on Gα subunits may be linked to the βγ complex playing a role analogous to the guanine nucleotide dissociation inhibitor proteins, which inhibit GDP dissociation from certain members of the superfamily of small GTP-binding proteins. In particular, it has been shown that in the case of Gαs, the βγ complex slows the dissociation of GDP from the Gα subunit and in effect stabilizes the GDP-bound state (10, 24, 25). It is likely that in heterotrimeric G proteins like Gαs, Gαo, and Gαq, the βγ complex inhibits the rate of GDP release by interacting with and stabilizing the Gα Switch 1 and 2 regions. However, in contrast, the retinal Gα subunit, as well as the recombinant Gα subunit characterized in this study, exhibits an extremely high affinity for GDP, even in the absence of βγ. Therefore, their relatively slow GDP dissociation rate is seemingly unaffected by addition of βγ (4).

On the other hand, an underlying tenet of the βγ-lever hypothesis is that upon the binding of an activated receptor to the heterotrimeric G protein, the juxtaposition of the α and βγ subunits would be altered, thereby reversing the inhibition of GDP dissociation and allowing the βγ complex to promote further the separation between the subunits and thereby fully stimulate GDP release (5). We initially suspected that if in fact the βγ complex were playing a direct role in the activation of Gα subunits (e.g. working as a lever to help create an exit route for bound GDP), then the addition of βγ to the Gα(G56P) or Gα(G179P) mutant would further promote its receptor-independent GDP-GTP exchange activity. However, that clearly did not turn out to be the case, thus demonstrating that the βγ complex is likely not involved in helping to pry open the Gα clamp shell, at least in the case of transducin.

Thus, it may be that for Gα and perhaps other Gα subunits,
a primary role of the receptor is to alter the juxtaposition of the helical and Ras-like domains (as depicted in Fig. 9) and that P by does not significantly contribute to this role. Rather, in these cases, Pγ may function to increase the affinity of the receptor for the Gα subunit, as originally suggested for transducin (21). In fact, earlier studies with the phototransduction system showed that when sufficient amounts of light activated rhodopsin were added to reconstituted systems, full activation of Gα could be achieved in the absence of any detectable Pγ (20). Still, it is clear that in the case of Gα, the photoreceptor must be doing other things to stimulate GDP-GTP exchange because the Gα linker mutants do not fully recapitulate the rate of activation achieved in the presence of rhodopsin and Pγ, and the linker mutants can be further stimulated by these components.

The results presented here highlight some other potentially interesting mechanistic issues. For example, it appears that both Gα linker mutants have a reduced affinity for rhodopsin. Thus, during the normal course of the receptor-promoted activation of G proteins, if indeed an important, intermediate step in the receptor-induced perturbation of the juxtaposition of the helical and Ras-like domains, it may be that this intermediate state for the Gα subunit has a weakened affinity for the receptor. Ultimately, upon the dissociation of GDP, a stable, high affinity complex forms between the receptor and the nucleotide-depleted Gα subunit. However, the Gα linker mutants would be effectively locked in the intermediate state that binds rhodopsin more weakly than the nucleotide-depleted form of Gα.

We also find that the Gα linker mutants have significantly reduced turnover numbers for GTP hydrolysis. This likely reflects a perturbation of the essential arginine 174 (i.e. the “arginine finger”) that is in linker 2. However, perhaps most interesting, it appears that perturbations of the juxtaposition of the helical domain relative to the Ras-like domain do not influence the nature of the GTP-induced activating conformational changes, at least in the Switch 2 domain, and do not alter Gα-PDE interactions. It has been suggested that the helical domain may play an important role in coupling the binding of Gα to the PDE with the stimulation of the effector activity (35). By altering the linker region and perturbing the juxtaposition of the helical and Ras-like domains, we suspected that this might alter the effective activation of the PDE if in fact the helical domain were involved. Although we cannot definitively rule out the possibility that the helical domain has a regulatory role in effector activation, our findings would limit the ways that GTP binding to the Ras-like domain might be communicated to the helical domain, because perturbing the linker regions apparently does not abrogate such communication.

Clearly, an important challenge for the future will be to generate an αT mutant that fully mimics the rhodopsin-stimulated activation state, such that it spontaneously exchanges GDP for GTP at a comparable rate to that for nucleotide exchange stimulated by the photoreceptor. We have made some attempts to achieve this by generating α mutants that contain substitutions in the linker regions as well as at the C-terminal alanine (Ala122) residue (where changes were previously shown to accelerate the off-rate for GDP (31)) and at aspartic acid 268, which is part of the conserved NKXD motif that binds to the guanine ring. However, thus far these α mutants have not proven to be sufficiently stable to allow detailed characterization. It may be that different and perhaps more subtle changes in combination with the linker mutations will be necessary to generate a constitutively active αT mutant that fully mimics the rhodopsin-stimulated activation state.

The development of activated Gα mutants that are both stable and capable of constitutive GDP-GTP exchange should provide valuable tools for studying G protein-coupled signaling activities in cells. In particular, this will allow for interesting comparisons between the cellular signaling events triggered by constitutively active G proteins that are capable of receptor-independent nucleotide exchange versus those cellular responses stimulated by the more conventional dominant-active, GTPase-defective Gα mutants. When such types of comparisons were made with different mutants of the Cdc42 GTP-binding protein, new signaling endpoints for Cdc42 were identified, as mutants that were capable of constitutive GDP-GTP exchange, but still hydrolyzed GTP, showed much more powerful effects on cell growth and transformation compared with mutants that were incapable of catalyzing GTP hydrolysis (36). Thus, it should prove interesting to examine the cellular effects of Gα mutants that capable of constitutive GTP-GTP exchange, especially in those cases where the full range of responses that are regulated by a particular GTP-binding protein have not been completely defined (e.g. Gα12 and Gα13).

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Perturbing the Linker Regions of the α-Subunit of Transducin: A NEW CLASS OF CONSTITUTIVELY ACTIVE GTP-BINDING PROTEINS
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