

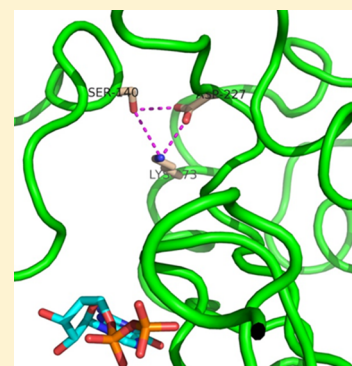
Comparative Analysis of Cone and Rod Transducins Using Chimeric $G\alpha$ Subunits

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S Supporting Information

ABSTRACT: The molecular nature of transducin- α subunits ($G\alpha_t$) may contribute to the distinct physiology of cone and rod photoreceptors. Biochemical properties of mammalian cone $G\alpha_{t2}$ subunits and their differences with rod $G\alpha_{t1}$ are largely unknown. Here, we examined properties of chimeric $G\alpha_{t2}$ in comparison with its rod counterpart. The key biochemical difference between the rod- and cone-like $G\alpha_t$ was ~ 10 -fold higher intrinsic nucleotide exchange on the chimeric $G\alpha_{t2}$. Presented mutational analysis suggests that weaker interdomain interactions between the GTPase (Ras-like) domain and the helical domain in $G\alpha_{t2}$ are in part responsible for its increased spontaneous nucleotide exchange. However, the rates of R^* -dependent nucleotide exchange of chimeric $G\alpha_{t2}$ and $G\alpha_{t1}$ were equivalent. Furthermore, chimeric $G\alpha_{t2}$ and $G\alpha_{t1}$ exhibited similar rates of intrinsic GTPase activity as well as similar acceleration of GTP hydrolysis by the RGS domain of RGS9. Our results suggest that the activation and inactivation properties of cone and rod $G\alpha_t$ subunits in an *in vitro* reconstituted system are comparable.



The two types of photoreceptors in the vertebrate retina, rods and cones, mediate vision at dim and bright light, respectively. Cones produce small rapidly decaying responses and are much less sensitive to light than rods. The molecular basis for the differences in physiology of rods and cones are poorly understood. The phototransduction cascades in rods and cones utilize homologous components including visual pigments, heterotrimeric G proteins (transducins), and cGMP-phosphodiesterases (PDE6).^{1–3} In rods, a robust amplification in the signaling cascade is achieved due to a high rate of transducin activation by photolyzed rhodopsin (Meta II or R^*) and rapid hydrolysis of cGMP by transducin-activated PDE6. Consistent with the low sensitivity of cones, the signal amplification in mouse cone phototransduction was found to be lower.⁴ The molecular differences between major cone and rod signaling proteins have been examined as a probable origin of the physiological differences.^{5–9} Current evidence does not implicate the signaling properties of rod and cone visual pigments. Expression of rhodopsin and red cone pigment in *Xenopus* cones and rods, respectively, yielded responses identical to those of native *Xenopus* photoreceptors.⁵ Recent physiological analysis points to an important role of transducin α -subunits in shaping specific photoresponses.⁷ Rods of GNAT2C transgenic mice with substitution of rod $G\alpha_{t1}$ for cone $G\alpha_{t2}$ displayed decreased sensitivity, reduced rate of activation, and accelerated recovery characteristic of cone photoreceptors.⁷ Reduced rate of cascade activation may result from a slower rate of R^* -dependent activation of the $G\alpha_{t2}$ complex with rod-specific $G\beta_1\gamma_1$ or less efficient activation of PDE6 by $G\alpha_{t2}$.¹⁰ Accelerated recovery of GNAT2C rods suggests faster GTP hydrolysis in the transition complex of $G\alpha_{t2}$ with PDE6 and the RGS9-1 GTPase accelerating protein

(GAP) complex.¹¹ Thus, $G\alpha_{t2}$ may have a higher intrinsic rate of GTP hydrolysis and/or a greater GTPase potentiation by the GAP complex than $G\alpha_{t1}$.

In contrast to well-characterized rod $G\alpha_{t1}$, biochemical properties of $G\alpha_{t2}$ have not been investigated. Native $G\alpha_{t1}$ is readily available for biochemical analyses, whereas the sparsity of cones in mammalian retina impedes isolation of native $G\alpha_{t2}$. In addition, a wealth of information about $G\alpha_{t1}$ properties, including its interaction with R^* , PDE6, and RGS9, was developed using robust bacterial expression of transducin-like $G\alpha_{t1}/G\alpha_{i1}$ chimeras.^{12–14} Here, we applied a chimera approach to analyze key signaling properties of $G\alpha_{t2}$. A $G\alpha_{t2}/G\alpha_{i1}$ chimera ($G\alpha_{t2}'$) was produced and investigated in comparison to analogous $G\alpha_{t1}/G\alpha_{i1}$ chimera Chi8,¹³ termed hereafter $G\alpha_{t1}'$. $G\alpha_{t2}'$ and $G\alpha_{t1}'$ are $\sim 94\%$ identical to $G\alpha_{t2}$ and $G\alpha_{t1}$, respectively, which is significantly greater than the degree of homology between $G\alpha_{t2}$ and $G\alpha_{t1}$ ($\sim 82\%$). $G\alpha_{t2}'$ showed a large ~ 10 -fold increase in spontaneous nucleotide exchange rate in comparison to $G\alpha_{t1}'$. A series of chimeric and mutant $G\alpha_t$ subunits delineated $G\alpha_{t2}'$ residues responsible for high intrinsic nucleotide exchange rate. In contrast, the rates of R^* -dependent nucleotide exchange were comparable for $G\alpha_{t2}'$ and $G\alpha_{t1}'$ reconstituted with $G\beta_1\gamma_1$. Furthermore, $G\alpha_{t2}'$ and $G\alpha_{t1}'$ exhibited similar rates of intrinsic GTPase activity as well as similar acceleration of GTP hydrolysis by RGS9.

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EXPERIMENTAL PROCEDURES

Materials. Guanosine 5'-[γ - 35 S]thiotriphosphate triethylammonium salt (GTP γ S; 1100 Ci/mmol), guanosine 5'-[γ - 32 P]-triphosphate triethylammonium salt (~5000 Ci/mmol), and nicotinamide adenine dinucleotide ([32 P]NAD; 800 Ci/mmol) were from PerkinElmer. Pertussis toxin was from Sigma. Bovine rod outer segment (ROS) membranes were prepared as described.¹⁵ Urea-washed ROS membranes (uROS) were prepared according to a published protocol.¹⁶ Recombinant G $\beta_1\gamma_1$ complex was expressed using the baculovirus/sf9 cell system and isolated as described.¹⁷ The RGS-domain of RGS9 (RGS9d, aa 284–461) was expressed and purified as previously described.¹⁴

Cloning, Expression, and Purification of Chimeric G α_t Subunits. To obtain chimera G α_{t2} '-2 (Figure 1), the full-

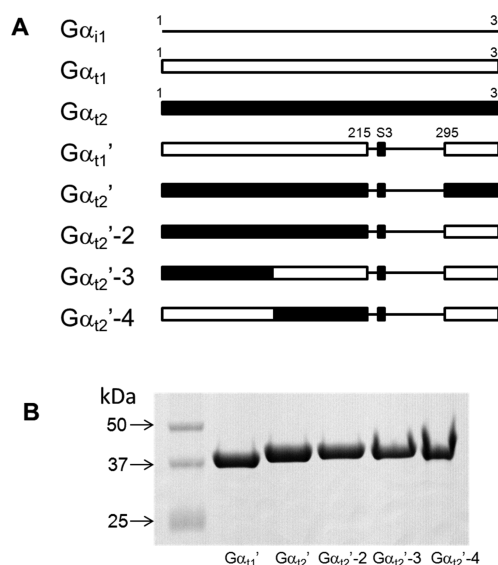


Figure 1. (A) Schematic representation of G α_t chimeras. The switch III region (S3, G α_{t1} -228–236) (black) is identical in G α_{t1} and G α_{t2} . (B) Coomassie blue-stained SDS-gel showing purified G α_t chimeras. Samples represent equal fractions of the preparations from 0.5 L bacterial cultures.

length G α_{t1} ' (Chi8) sequence was PCR-amplified from the plasmid¹³ with a pair of sequence-specific primers (reverse primer contained *Bam*HI site) and cut with *Bam*HI to isolate a 430-bp fragment coding the C-terminal portion of G α_{t1} '. This fragment was inserted into the large fragment of the pET15b-G α_{t2} vector⁹ digested with *Bam*HI, thereby replacing the C-terminal portion of G α_{t2} with the corresponding fragment of G α_{t1} '. Correct orientation of the inset was selected by sequencing the construct. G α_{t2} ' was generated from G α_{t2} '-2 by replacing three G α_{t1} -specific residues, Val301, Glu305, and Arg310, with the corresponding G α_{t2} -specific residues Ser305, Asp309, and Lys314. The triple mutant was produced using the QuikChange mutagenesis protocol (Stratagene). The same mutagenesis procedure was used to generate the A144S mutant of G α_{t2} ' and the following single, double, and triple mutants of G α_{t1} ': S140A, K117P/S120V, S153N/D154Q, and V159T/T160D/G162E.

Chimeras G α_{t2} '-3 and G α_{t2} '-4 were produced by swapping the residues 1–116 and 117–215 of G α_{t1} ' with residues 1–120 and 121–219 of G α_{t2} , respectively. The G α_{t2} '-1–120 sequence was amplified from the G α_{t2} '-template using a 5'-primer

containing an *Nco*I site and a 3'-primer with a flanking G α_{t1} ' sequence. The resulting PCR product was paired with a 3' primer containing a *Hind*III site in PCR amplification from the G α_{t1} ' template to produce the G α_{t2} '-3 sequence, which was then cloned into the *Nco*I/*Hind*III sites of the pHis6 vector.¹³ To produce G α_{t2} '-4, the G α_{t1} '-1–116 sequence was amplified from the G α_{t1} ' template using a 5'-primer containing an *Nco*I site and a 3'-primer with a flanking G α_{t2} ' sequence. The resulting PCR product was paired with a 3' primer containing a *Xho*I site in PCR amplification from the G α_{t2} '-2 template to produce the G α_{t2} '-4 sequence, which was then cloned into the *Nco*I/*Xho*I sites of the pET15b vector. Upon sequence verification, protein expression of G α_{t1} ', G α_{t2} ', their derivatives, and mutants was induced in BL21(DE3) codonPlus competent cells with 30 μ M IPTG at 16 °C overnight. His₆-tagged proteins were purified on an ion-exchange chromatography on Uno-Q1 column (Bio-Rad). Fractions containing functional G α -subunits were dialyzed against buffer containing 40% glycerol overnight and stored at –20 °C.

GTP γ S Binding Assay. Chimeric G α_t subunits (1 μ M) alone, or mixed with 1 μ M G $\beta_1\gamma_1$ and uROS membranes (0.25, 1, or 2 μ M rhodopsin), were incubated for 2 min at 25 °C in the presence of light. Binding reactions were started with the addition of 5 μ M or 100 nM [35 S]GTP γ S. Aliquots of 15 μ L were withdrawn at the indicated times, mixed with 1 mL of ice-cold 20 mM Tris-HCl (pH 8.0) buffer containing 130 mM NaCl, 2 mM MgSO₄, and 1 mM GTP, passed through Whatman cellulose nitrate filters (0.45 μ m), and washed three times with 3 mL of the same buffer without GTP. The filters were dissolved in 5 mL of a xylene-based 3a70B counting cocktail (RPI Corp.), and [35 S]GTP γ S was measured in a liquid scintillation counter.¹⁸ The k_{app} values for the binding reactions were calculated by fitting data with equation %GTP γ S bound = 100(1 – e^{– k_t}). Groups of measurements were compared with two-tailed unpaired *t* test.

GTPase Activity Assays. Single-turnover GTPase activity measurements were carried out in suspensions of uROS membranes (10 μ M rhodopsin) reconstituted with chimeric G α_t subunits (1 μ M) and G $\beta_1\gamma_1$ (1 μ M) in 10 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl and 4 mM MgSO₄.^{18,19} Where indicated, RGS9d (3 or 6 μ M) was added. After incubation for 5 min at 25 °C, 100 nM [γ - 32 P]GTP was added to the mixtures to initiate the reaction. GTPase hydrolysis was quenched at the indicated times by mixing 10 μ L aliquots with 100 μ L of 6% (v/v) perchloric acid. Nucleotides were precipitated with 700 μ L of 10% (w/v) charcoal suspension in phosphate-buffered saline, and free [32 P]_i was measured by liquid scintillation counting. GTPase rate constants were calculated by fitting the data equation % GTP hydrolyzed = 100(1 – e^{– k_{cat}}), where k_{cat} is the rate constant for GTP hydrolysis.

Pertussis Toxin-Catalyzed ADP-Ribosylation. Chimeric G α_t subunits (0.5 μ M each) were mixed with G $\beta_1\gamma_1$ (0.5–2 μ M) in 50 μ L of 20 mM Tris-HCl (pH 8.0) buffer containing 2 mM MgSO₄, 2 mM dithiothreitol, 1 mM EDTA, 10 μ M GDP, and 5 μ g/mL pertussis toxin (preactivated with 100 mM dithiothreitol and 0.25% SDS for 10 min at 30 °C). The reaction was started by addition of 5 μ M [32 P]NAD and allowed to proceed for 1 h at 25 °C. Reaction mixtures were diluted with 1 mL of ice-cold 20 mM Tris-HCl (pH 8.0) buffer containing 100 mM NaCl and filtered through Whatman cellulose-nitrate filters. The filters were washed four times with

the same buffer and counted in a liquid scintillation counter. Aliquots (10 μ L) were withdrawn from reaction mixtures, mixed with sample buffer for SDS-PAGE, and analyzed by SDS-PAGE and autoradiography.

RESULTS

Increased Spontaneous Nucleotide Exchange in Chimeric $G\alpha_{t2}$ Subunits. To examine the biochemical properties of cone transducin- α , we generated a chimeric $G\alpha_{t2}$ -subunit, which is a counterpart of previously characterized $G\alpha_{t1}$ / $G\alpha_{i1}$ chimera Chi8 or $G\alpha_{t1}$ ¹³ (Figure 1A). This choice of a chimeric template is based on the efficient bacterial expression of $G\alpha_{t1}$ and the fact that it is 94% identical to $G\alpha_{t1}$ and appears to recapitulate its essential signaling characteristics. Indeed, expression of functional $G\alpha_{t2}$ in *E. coli* was similarly robust, and the protein is readily purified (Figure 1B).

In comparison to $G\alpha_{i1}$, native $G\alpha_{t1}$ and $G\alpha_{t1}$ / $G\alpha_{i1}$ chimeras containing 215 N-terminal residues of $G\alpha_{t1}$ have been shown to have very slow rates of spontaneous guanine nucleotide exchange rate.¹³ In agreement, the basal rate of GTP γ S-binding to $G\alpha_{t1}$ under our experimental conditions was $0.0050 \pm 0.0006 \text{ min}^{-1}$. In contrast to $G\alpha_{t1}$, $G\alpha_{t2}$ showed markedly higher intrinsic GTP γ S-binding rate ($0.048 \pm 0.003 \text{ min}^{-1}$) (Figure 2). Next, we tested chimeric $G\alpha_{t2}$ -2, containing 219 N-

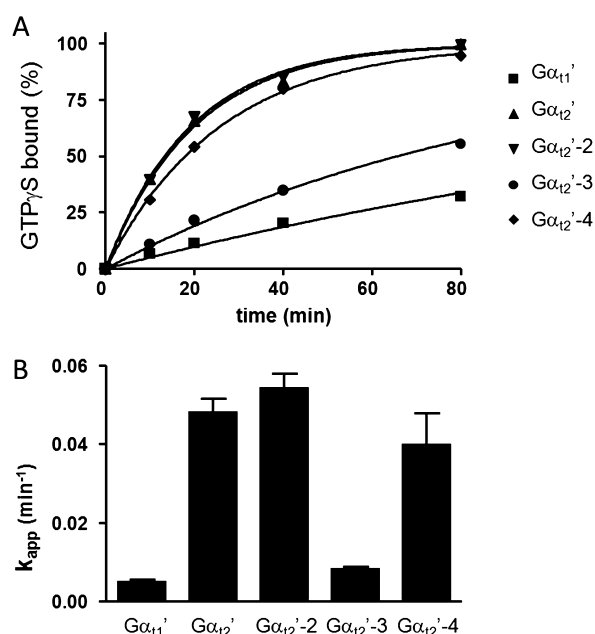


Figure 2. (A) Spontaneous GTP γ S binding to $G\alpha_{t1}$ and $G\alpha_{t2}$ chimeras. The binding of GTP γ S to $G\alpha_{t1}$ and $G\alpha_{t2}$ chimeras (1 μ M each) was initiated with the addition of 5 μ M [³⁵S]GTP γ S. $G\alpha$ -bound GTP γ S was counted by withdrawing aliquots at the indicated times and passing them through Whatman cellulose nitrate filters. Results from one of four similar experiments are shown. (B) k_{app} values (min⁻¹) (mean \pm SE) of intrinsic GTP γ S binding to chimeric $G\alpha$ proteins were calculated from four experiments such as shown in (A).

terminal residues of $G\alpha_{t2}$ (Figure 1A). The basal GTP γ S-binding rate for $G\alpha_{t2}$ -2 was similar to that of $G\alpha_{t2}$ (Figure 2), suggesting that the N-terminal part of $G\alpha_{t2}$ is responsible for the increased nucleotide exchange. Chimeras $G\alpha_{t2}$ -3 and $G\alpha_{t2}$ -4 were produced by swapping the residues 1–116 and 117–215 of $G\alpha_{t1}$ with the corresponding residues of $G\alpha_{t2}$ (Figure 1A). The rate of GTP γ S binding to $G\alpha_{t2}$ -4 was nearly as high

as that for $G\alpha_{t2}$, whereas $G\alpha_{t2}$ -3 demonstrated relatively small \sim 1.7-fold increase in the binding rate over $G\alpha_{t1}$ ($p = 0.016$) (Figure 2). The $G\alpha_t$ determinants of the spontaneous nucleotide exchange within the $G\alpha_{t1}$ (117–215) region were further probed by mutational analysis of $G\alpha_{t1}$. This region contains only a few residues that are different between rod and cone $G\alpha_t$ but strongly conserved within each transducin family (Supporting Information Figure 1). The following single, double, and triple mutations replacing rod-specific residues with their cone-specific counterparts were introduced into $G\alpha_{t1}$: S140A, K117P/S120 V, S153N/D154Q, and V159T/T160D/G162E (Figure 3A). The double and triple mutations did not significantly alter the GTP γ S-binding rate of $G\alpha_{t1}$, while the S140A substitution led to a moderate 1.8-fold increase in the k_{app} value for GTP γ S binding ($p = 0.001$) (Figure 3B). We then examined if the reverse mutation A144S in $G\alpha_{t2}$ would alter its nucleotide exchange kinetics (Figure 3C,D). Indeed, the GTP γ S-binding rate of the A144S mutant was 1.7-fold lower than that of $G\alpha_{t2}$ ($p = 0.004$) (Figure 3D).

Binding of GTP γ S to $G\alpha$ subunits with high spontaneous nucleotide exchange such as $G\alpha_i$ or $G\alpha_s$ can often be inhibited by $G\beta\gamma$ subunits.^{20,21} $G\beta_1\gamma_1$ had no effect on the basal rate of GTP γ S binding to $G\alpha_{t2}$ (Figure 3E).

Pertussis Toxin-Catalyzed ADP-Ribosylation. Binding of $G\beta_1\gamma_1$ subunits to $G\alpha_{t1}$ facilitates pertussis toxin-catalyzed ADP-ribosylation at Cys347 of $G\alpha_{t1}$.^{22–24} We utilized this reaction to assess the interactions of $G\alpha_{t1}$ and $G\alpha_{t2}$ with $G\beta_1\gamma_1$. Pertussis toxin-catalyzed ADP-ribosylation of $G\alpha_{t1}$ and $G\alpha_{t2}$ was carried out in the absence or presence of increasing concentrations of $G\beta_1\gamma_1$. The basal level of ADP-ribosylation in the absence of $G\beta_1\gamma_1$ was somewhat higher for $G\alpha_{t2}$ compared to $G\alpha_{t1}$ (Figure 4). The dose dependencies of $G\beta_1\gamma_1$ -supported ADP-ribosylation were comparable for $G\alpha_{t1}$ and $G\alpha_{t2}$, suggesting similar submicromolar K_d values for $G\beta_1\gamma_1$ binding (Figure 4).

Rhodopsin-Catalyzed Activation of Chimeric $G\alpha_{t2}$ and $G\alpha_{t1}$ Reconstituted with $G\beta_1\gamma_1$. The efficiencies of activation of $G\alpha_{t2}$ and $G\alpha_{t1}$ by R^* were measured using a GTP γ S-binding assay and reconstitution of the $G\alpha$ subunits with $G\beta_1\gamma_1$ and uROS. Suspensions of uROS containing 0.25 and 1 μ M R^* were used to ensure that the GTP γ S binding rates are submaximal. In the presence of uROS containing 0.25 μ M R^* , the rates of R^* -dependent GTP γ S binding to $G\alpha_{t2}$ (k_{app} $0.0045 \pm 0.0005 \text{ s}^{-1}$ or 0.27 min^{-1}) and $G\alpha_{t1}$ (k_{app} $0.0048 \pm 0.0008 \text{ s}^{-1}$ or 0.29 min^{-1}) were similar and much greater than the unstimulated rates (Figure 5). In the presence of uROS containing 1 μ M R^* , the GTP γ S binding rates to $G\alpha_{t2}$ and $G\alpha_{t1}$ were higher still (\sim 0.011 s^{-1} or 0.7 min^{-1}) (Figure 5). No significant differences were seen in the activation kinetics of the two $G\alpha_t$ proteins.

GTPase Activity of Chimeric $G\alpha_{t2}$ and $G\alpha_{t1}$ and the Effects of RGS9d. The catalytic rates of GTP hydrolysis for $G\alpha_{t2}$ and $G\alpha_{t2}$ -2 were determined in comparison to $G\alpha_{t1}$ using a single turnover assay (GTP = 100 nM \ll $G\alpha\beta_1\gamma_1$ = 1 μ M) and a high concentration of uROS (10 μ M R^*). Using uROS containing 2 μ M R^* and 100 nM GTP γ S, the rates of GTP γ S-binding to $G\alpha_{t1}$ and $G\alpha_{t2}$ were significantly faster than the measured rates of GTP hydrolysis (Supporting Information Figure 2). Thus, the guanine nucleotide binding rates did not limit GTPase reactions under our experimental conditions. The intrinsic GTPase activity of $G\alpha_{t2}$ ($k = 0.024 \pm 0.001 \text{ s}^{-1}$) was not significantly different from that of $G\alpha_{t1}$ ($k = 0.023 \pm 0.001$

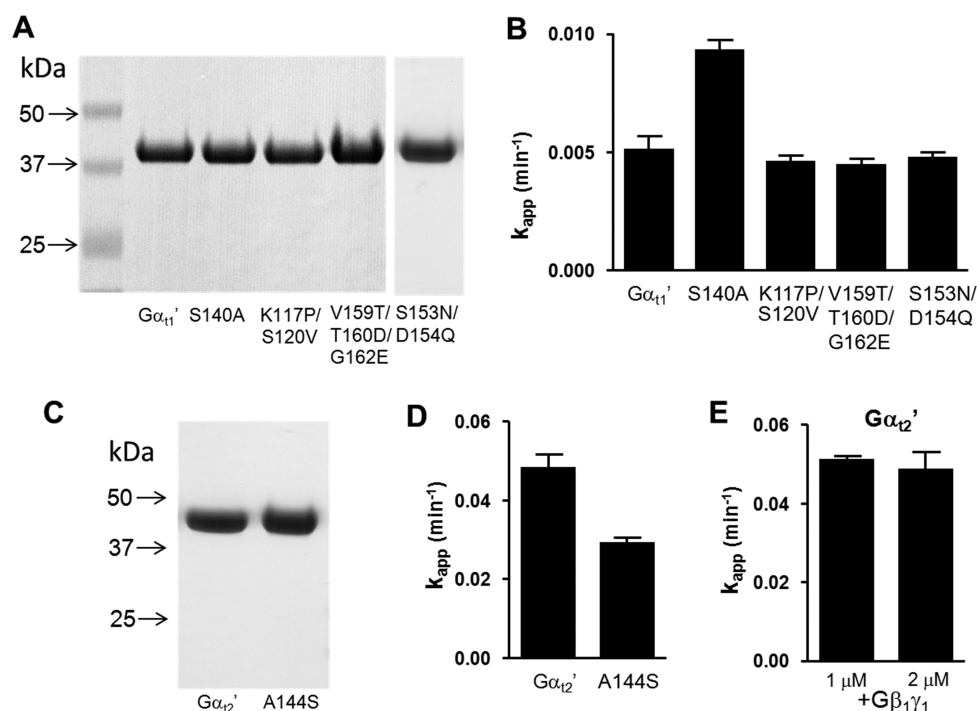


Figure 3. (A, C) Coomassie blue-stained SDS gel showing purified mutant $G\alpha_{t1}'$ and $G\alpha_{t2}'$ subunits. Samples represent equal fractions of the preparations from 0.5 L bacterial cultures. (B, D) k_{app} values (min^{-1}) (mean \pm SE) of intrinsic GTP γ S binding to mutant $G\alpha_{t1}'$ and $G\alpha_{t2}'$ calculated from four binding experiments for each mutant. (E) k_{app} values (min^{-1}) (mean \pm SE) of intrinsic GTP γ S binding to mutant $G\alpha_{t2}'$ (1 μM) in the presence of 1 and 2 μM $G\beta_1\gamma_1$.

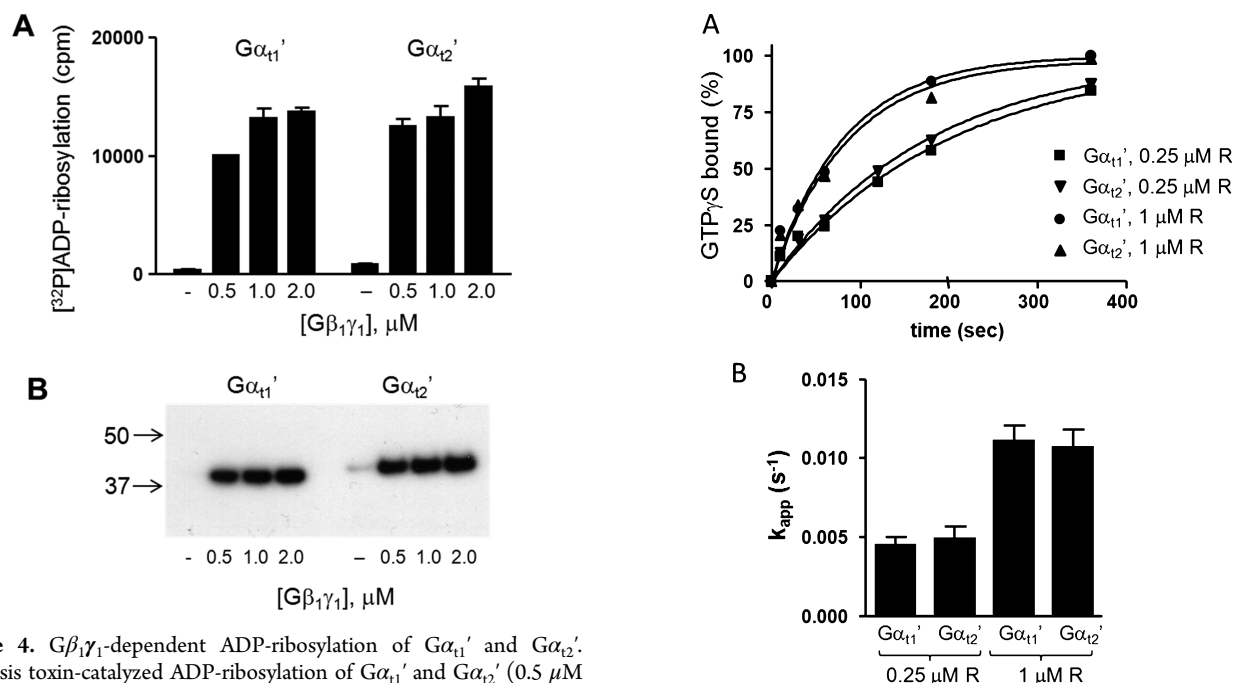


Figure 4. $G\beta_1\gamma_1$ -dependent ADP-ribosylation of $G\alpha_{t1}'$ and $G\alpha_{t2}'$. Pertussis toxin-catalyzed ADP-ribosylation of $G\alpha_{t1}'$ and $G\alpha_{t2}'$ (0.5 μM each) was carried out in the presence of increasing concentrations of $G\beta_1\gamma_1$. (A) $[^{32}\text{P}]\text{ADP-ribose}$ of $G\alpha_{t1}'$ and $G\alpha_{t2}'$ is analyzed by liquid scintillation counting (mean \pm SE, $n = 3$). (B) Aliquots from the ADP-ribosylation reaction mixtures were analyzed by SDS-PAGE followed by autoradiography.

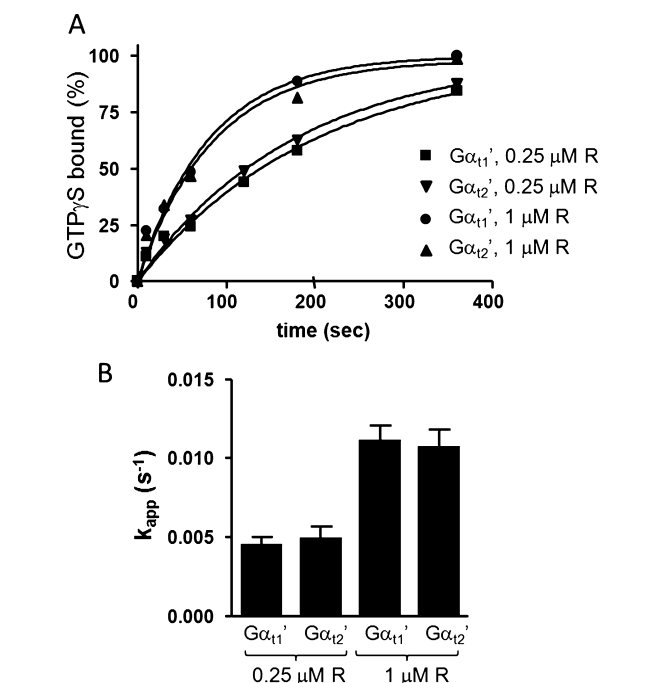


Figure 5. (A) Rhodopsin-catalyzed GTP γ S binding to $G\alpha_{t1}'$ and $G\alpha_{t2}'$. The binding of GTP γ S to $G\alpha_{t1}'$ and $G\alpha_{t2}'$ (1 μM each) in the presence of 1 μM $G\beta_1\gamma_1$ and uROS membranes (0.25 or 1 μM rhodopsin) was initiated with the addition of 5 μM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. $G\alpha$ -bound GTP γ S was counted by withdrawing aliquots at the indicated times and passing them through Whatman cellulose nitrate filters. Results from one of four similar experiments are shown. (B) k_{app} values (s^{-1}) (mean \pm SE) of intrinsic GTP γ S binding to chimeric $G\alpha_i$ proteins were calculated from four experiments such as shown in (A).

s^{-1}) (Figure 6). The rate of GTP hydrolysis by $G\alpha_{t2}'$ -2 was also similar to that of $G\alpha_{t1}'$ (Supporting Information Figure 3).

The ability of the RGS9-284-461 domain to stimulate GTPase activity of $G\alpha_{t2}'$ and $G\alpha_{t1}'$ was measured at two RGS protein concentrations, 3 and 6 μM . RGS9-284-461 com-

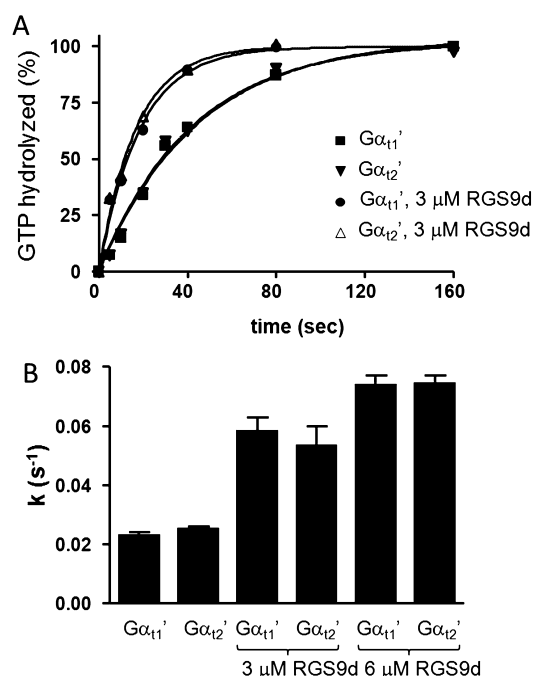


Figure 6. Single turnover GTPase assays of $G\alpha_{t1}'$ and $G\alpha_{t2}'$. Effects of RGS9d. (A) Single-turnover GTPase activity measurements were carried out in suspensions of uROS membranes (10 μ M rhodopsin) reconstituted with chimeric $G\alpha_t$ subunits (1 μ M) and $G\beta_1\gamma_1$ (1 μ M). Where indicated, RGS9d was added. Reactions were started with the addition of 100 nM [γ - 32 P]GTP, and free 32 P_i was measured by liquid scintillation. Results from one of three similar experiments are shown. (B) The k_{cat} values (s⁻¹) (mean \pm SE) for GTP hydrolysis by chimeric $G\alpha_t$ proteins were calculated from three experiments such as shown in (A).

parably stimulated GTPase activities of the cone and rod chimeric $G\alpha$ subunits. The levels of GTPase activity of $G\alpha_{t2}'$ and $G\alpha_{t1}'$ were \sim 2.2–2.5 and \sim 3-fold higher in the presence of 3 and 6 μ M RGS9d, respectively (Figure 6B).

DISCUSSION

The role of transducin- α subunit in setting the sensitivity and kinetics of light responses of rods and cones has been actively debated.^{6,7,25} Electrophysiological recordings from mouse cones indicate that in mouse S- and M-cones the amplification of phototransduction is 2–3-fold lower and the inactivation is considerably faster than in rods.⁴ In view of the similar catalytic efficiencies of rod and cone PDE6⁹ and the smaller size of cone outer segment, the lower amplification implies at least 5-fold lower rate of PDE6 activation per activated pigment molecule in mouse cones compared to rods.⁴ Replacing the rod $G\alpha_{t1}$ with the cone $G\alpha_{t2}$ reduced the amplification in mouse rods by \sim 2-fold and speeded up the recovery phase by \sim 2-fold, suggesting that the differences in the rod and cone responses might be attributed to a significant extent to the nature of $G\alpha_t$.⁷ We sought to determine the biochemical basis underlying the physiological changes induced by $G\alpha_{t2}$ in *GNAT2C* rods. Although the functional GTP γ S-bound form of $G\alpha_{t2}$ can be isolated following transducin expression in *E. coli*,⁹ this preparation cannot be used to study the activation of Gt by R* or $G\alpha_{t2}$ GTPase activity. Therefore, we produced and examined chimeric $G\alpha_{t2}$ -like proteins.

The key biochemical difference between rod-like $G\alpha_{t1}'$ and its cone counterpart $G\alpha_{t2}'$ was found to be markedly higher intrinsic nucleotide exchange of $G\alpha_{t2}'$. The region primarily responsible for the increased nucleotide exchange was mapped to residues $G\alpha_{t2}$ (121–219). $G\alpha$ subunits of heterotrimeric G proteins are composed of two domains: a GTPase (Ras-like) domain and a helical domain. GDP (or GTP) is buried between the two domains.^{26,27} A network of interactions between the two domains is involved in control of basal nucleotide exchange. Mutations disrupting the interdomain interactions increased the basal nucleotide exchange in $G\alpha_t$.²⁸ Within $G\alpha_{t2}$ (121–219), a conserved cone-specific residue Ala144 corresponds to the conserved rod-specific Ser140. Ser140 from the helical domain interacts with Asp227 and Lys273 from the Ras-like domain.^{26,27} The model of the Ser \rightarrow Ala substitution using

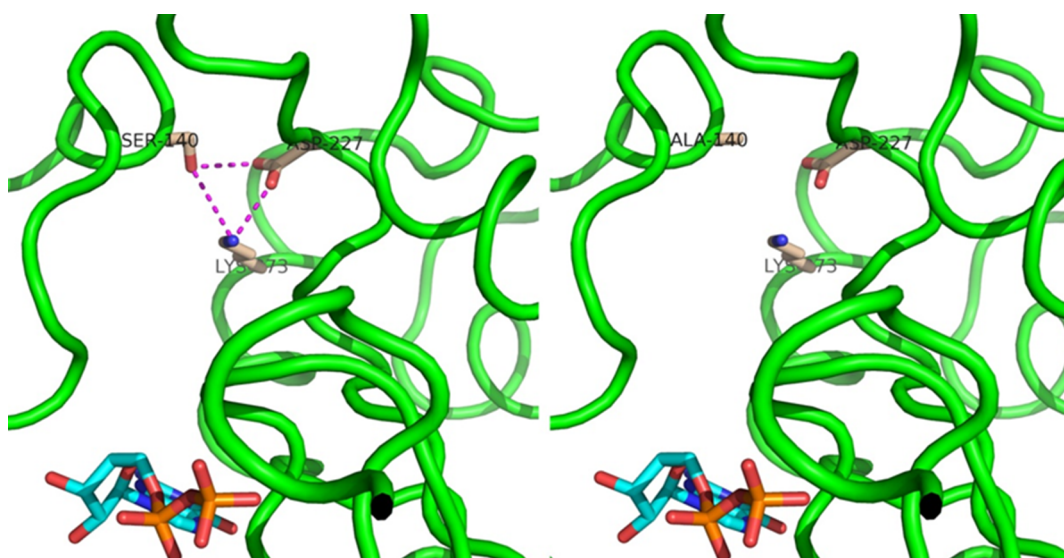


Figure 7. Interdomain interactions of Ser140 in rod $G\alpha_{t1}$ are lost in the S140A mutant. In the structure of $G\alpha_{t1}$ GDP,²⁴ $G\alpha_{t1}$ Ser140 from the helical domain interacts with Asp227 and Lys273 from the Ras-like domain (left). The S140A mutation was introduced into the structure of $G\alpha_{t1}$ GDP using the Swiss-PdbViewer (v.4).⁴⁴ The mutation disrupts the interdomain interactions in $G\alpha_{t1}$ GDP (right). The images were produced using PyMOL 1.4.1.⁴⁵

the structure of $G\alpha_tGDP^{27}$ demonstrates that the interdomain interactions involving Ser140 are disrupted (Figure 7). Previous study found no evidence for the role of Ser140 in controlling $G\alpha_{t1}$ nucleotide exchange.²⁹ However, a trypsin-protection assay as readout of $G\alpha_{t1}$ activation is severely limited in terms of the kinetic resolution. Our results suggest that the Ser → Ala substitution in $G\alpha_{t2}$ contributes to its high intrinsic nucleotide rate. The $G\alpha_{t1}$ 'Ser140Ala mutation increased, whereas the $G\alpha_{t2}$ 'Ala144Ser mutation decreased the nucleotide exchange rates of the respective $G\alpha$ subunits. The effects of these mutations on the basal $GTP\gamma S$ binding rates were moderate, suggesting the involvement of other residues. $G\beta_1\gamma_1$ did not inhibit the high nucleotide exchange rate of $G\alpha_{t2}$. This agrees with the impairment of the interdomain contacts in $G\alpha_{t2}$ as the cause of its high nucleotide exchange rate. $G\beta$ binds only to the N-terminus and the Ras-like domain of $G\alpha$, and thus, may not influence the interdomain interface.¹² The finding that the $GTP\gamma S$ -bound $G\alpha_{t2}$ can be isolated through the spontaneous nucleotide exchange supports the notion that native $G\alpha_{t2}$ also has high intrinsic nucleotide exchange rate.⁹ The functional significance of this phenomenon, if any, remains to be determined. Spontaneous activation of G_{t2} in cones may elevate basal activity of PDE6, potentially altering the sensitivity and kinetics of cone light responses.³⁰

Our experiments revealed no significant differences in the binding of $G\beta_1\gamma_1$ to $G\alpha_{t1}'$ and $G\alpha_{t2}'$ (Figure 4), nor in the efficiency of $G\alpha_{t1}'$ and $G\alpha_{t2}'$ activation by R^* in the presence of $G\beta_1\gamma_1$ (Figure 5). However, the rates of activation of non-N-acylated recombinant $G\alpha$ in the R^* , $G\beta_1\gamma_1$ -reconstituted system observed previously and in this study are well below the rates of activation of native Gt under similar conditions.^{13,31,32} Thus, our activation paradigm may not be capable of detecting small differences in the R^* -Gt coupling for native $G\alpha_{t2}$ and $G\alpha_{t1}$. Moreover, the potential role of cone-specific $G\beta_3\gamma_8$ to transducin/ R^* coupling has not been investigated in this study. The interdomain interactions in $G\alpha$ -subunits appear to be essential to receptor-mediated activation of G proteins. Mutant $G\alpha_s$ -subunits with disrupted interdomain interactions showed decreased ability for activation by the β -adrenergic receptor.³³ The relatively weak interdomain interface in $G\alpha_{t2}$ may potentially reduce the $G\alpha_{t2}$ coupling to R^* . Our results seem to favor the idea that the lower amplification in GNAT2C rods is linked to the lower efficiency of $G\alpha_{t2}$ coupling to rod PDE6 rather than to R^* . Yet, the potency of rod PDE6 activation by $G\alpha_{t2}GTP\gamma S$ in solution is not significantly lower than the activation by $G\alpha_{t1}GTP\gamma S$.⁹ Nonetheless, rod PDE6 activation by $G\alpha_{t1}$ on the membrane is markedly more potent than in solution.³⁴ Alternatively, small reductions in both couplings, $R^*/G\alpha_{t2}$ and $G\alpha_{t2}/PDE6$, may result in the combined 2-fold lower amplification in GNAT2C rods. In cones, however, a short lifetime of photoactivated cone pigments due to rapid spontaneous decay and pigment phosphorylation, and a lower rate of G_{t2} activation, may dictate the activation phase of photoresponses.^{8,35,36}

The intrinsic GTPase activities of $G\alpha_{t2}'$ and $G\alpha_{t1}'$ were found to be similar. The k_{cat} for the unstimulated GTP hydrolysis by $G\alpha_{t1}'$ is equivalent to the previously reported k_{cat} values of native $G\alpha_{t1}$.^{14,37} Furthermore, the intrinsic GTPase activities of $G\alpha_{t1}$ and $G\alpha_{t1}$ are comparable.¹⁴ Thus, the chimeric $G\alpha_t$ subunits appear to faithfully reflect intrinsic GTPase activities of $G\alpha_{t2}$ and $G\alpha_{t1}$, which, in all probability, are similar. The GTPase activity of transducins *in vivo* is far greater than its intrinsic activity owing to the GAP activity of the membrane-

bound RGS9 protein complex with the cooperative input from the effector PDE6.^{38,39} The faster rate of transducin inactivation in cones compared to rods may result from the greater potency or the higher expression levels of the RGS9 complex.^{14,40} The former possibility is suggested by the finding that RGS9 is a much more potent GAP for $G\alpha_{t1}$ compared to $G\alpha_{t1}$, and the selectivity determinants reside in the $G\alpha_{t1}$ helical domain.¹⁴ Nonetheless, the lack of the difference in the effects of the RGS9d on the GTPase activities of $G\alpha_{t2}$ and $G\alpha_{t1}$ indicates that the efficacies of RGS9 toward cone and rod transducins are not grossly different. This result is consistent with the strong conservation of RGS9-contact residues of transducin⁴¹ and supportive of RGS9 protein levels as a determining factor in transducin inactivation. The rate of transducin inactivation is regulated by the membrane attachment of the RGS9 GAP complex.^{42,43} Therefore, further studies with the use of the N-acylated $G\alpha_t$ subunits are needed to probe the selectivity of the RGS9 GAP complex.

■ ASSOCIATED CONTENT

● Supporting Information

Figure S1: sequence alignment of $G\alpha_{t1}$, $G\alpha_{t2}$, $G\alpha_{i1}$, and chimeric $G\alpha_{t1}'$ and $G\alpha_{t2}'$; Figure S2: the kinetics of $GTP\gamma S$ binding to $G\alpha_{t1}'$ and $G\alpha_{t2}'$ under the conditions of the single-turnover GTPase assay; Figure S3: GTPase activity of $G\alpha_{t2}'$ -2 in the single-turnover assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

$G\alpha_{t1}$, rod transducin α -subunit; $G\alpha_{t2}$, cone transducin α -subunit; $G\alpha_{t2}'$, $G\alpha_{t2}/G\alpha_{i1}$ chimera; PDE6, photoreceptor phosphodiesterase-6; R^* , photoexcited rhodopsin; RGS9, regulator of G-protein signaling 9; $GTP\gamma S$, guanosine 5'-O-(3-thiotriphosphate).

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