Concentration dependence of Gαt on rod sensitivity

Functional Comparison of Rod and Cone Gαt on the Regulation of Light Sensitivity*

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Background: A similar phototransduction cascade confers different light sensitivity in rods and cones.

Results: Rod and cone Gαt are similar with respect to coupling to visual pigments and light-induced translocation.

Conclusion: Rod and cone Gαt are equivalent functionally.

Significance: Reduced sensitivity in cones does not result from reduced coupling efficiency between the GPCR and G-protein or a lower concentration of G-protein in cones.

SUMMARY
The signaling cascades mediated by G-protein coupled receptors (GPCRs) exhibit a wide spectrum of spatial and temporal response properties to fulfill diverse physiological demands. However, the mechanisms that shape the GPCR’s signaling response are not well understood. In this study we replaced cone transducin α (cTα) for rod transducin α (rTα) in rod photoreceptors of transgenic mice, which also express S opsin, to evaluate the role of Gα subtype on signal amplification from different GPCRs in the same cell; such analysis might explain functional differences between retinal rod and cone photoreceptors. We showed that ectopically expressed cTα: 1) forms a heterotrimeric complex with rod Gβγ, 2) substitutes equally for rTα in generating photoresponses initiated by either rhodopsin or S-cone opsin, and 3) exhibited similar light-activated translocation as endogenous rTα in rods and endogenous cTα in cones. Thus, rTα and cTα appear functionally interchangeable. Interestingly, light sensitivity appeared to correlate with the concentration of cTα when expression is reduced below 35% of normal. However, quantification of endogenous cTα concentration in cones showed a higher level to rTα in rods. Thus reduced sensitivity in cones cannot be explained by reduced coupling efficiency between the GPCR and G-protein, or a lower concentration of G-protein in cones versus rods.

Two photoreceptor cell types, rods and cones, and the retinal circuitry that carries their signals to higher visual centers collectively allow our vision to report luminance information over the course of night and day. Rods are exquisitely sensitive and can detect single photons (1,2), while cones are ~100-fold less sensitive and do not saturate under bright light (3,4). Furthermore, the kinetics of the photoreponse in cones is 5-fold faster than rods, allowing increased temporal resolution (5). These two types of photoreceptors utilize a similar G-protein signaling cascade for phototransduction, but the differences in the cascades that produce these functional distinctions are just beginning to be understood. Some components of the cascades are identical in
both cell types, while others, including the visual pigments and G proteins, have distinct rod and cone isoforms. Thus, functional differences between rods and cones may arise from: 1) different levels of expression of transduction components, or 2) different functional properties of their respective transduction proteins. For example, a higher expression level of rhodopsin kinase (6,7), or RGS9-1 (8), has been proposed to allow for faster recovery of the photoresponse in cones. Higher guanylyl cyclase activity and a faster cGMP turnover in cones may also contribute to speeded responses in cones compared to rods (9). Differences in rod/cone sensitivity likely arise from multiple mechanisms, and a full understanding of what sets the differences requires approaches to quantify the contribution of individual phototransduction components.

Previous biochemical (7,10) and physiological (11-13) evidence suggest that a lower amplification gain in cones may be a primary contributor to lower sensitivity. To test whether this alteration arises from cell-type specific Tα, we expressed mouse cTαα (GNAT2) in rTα (GNAT1) knockout mice. By crossing these mice with those whose rods also express the S-cone pigment (14), we also compared the coupling efficiencies between rod and cone visual pigments with cTα and evaluated the influence of Tα concentration on visual sensitivity. We obtained two lines of mice that expressed cTα at 15% and 35% of endogenous rTα in rods. Suction electrode recordings showed that these rods were less sensitive to light. However, the decreased sensitivity correlated with the reduced Tα concentration. Normalized photoresponses from cTα rods displayed similar rising and recovery phases. Because lowered Tα concentration leads to lowered sensitivity, we investigated whether endogenous cones express a lower concentration of cTα. To the contrary, we found that the cTα concentration in cones is higher than rTα concentration in rods. We also provide evidence that rTα and cTα are indistinguishable biochemically, as evidenced by their similar activation by both rod and S-cone pigments. Lastly, we observed that endogenous cTα in cones translocated away from the outer segment in response to light, a property that was recapitulated by ectopically expressed cTα in rods.

EXPERIMENTAL PROCEDURES

All experimental procedures involving the use of mice were performed in compliance with regulations set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research, with protocols approved by the University of Southern California’s Institutional Animal Care and Use Committee.

Generation of cTα transgenic mice— The pBKS-cTα transgenic construct contained the ~1.3 kb mouse cone transducin α subunit cDNA flanked by the 4.4 kb fragment of mouse rod opsin promoter (15) at the 5’ end and a 0.6 kb polyadenylation signal from the mouse protamine gene at the 3’ end that also provided a splice site (Fig. 1A). The 1.3 kb cTα cDNA coding sequence was synthesized by reverse transcription-PCR with primers cTα_F (CCGCTCGAGTCTCAAGGCAAGGTAGGC) and cTα_R (GAAGATCTCTCATCAACAGGATGGG) using mRNA prepared from mouse retinas. The pBKS-cTα plasmid was purified by CsCl2 gradient and digested with XhoI and XbaI to yield the 6.3 kb insert fragment, which was then purified by QIAEXII gel extraction kit (Qiagen, Hilden, Germany) and Elutip-d column (Schleicher and Schuell Bioscience, Keene, NH). The DNA fragment was microinjected into fertilized eggs from C57BI/6J and DBA/2J F1 strains according to standard procedures. The cTα transgenic mice were identified by PCR of DNA obtained from tail biopsies. To increase cTα dosage, the cTα transgene was bred to homozygosity (cTαα+). Both cTα+ and cTα++ transgenic mice were subsequently crossed with rTα knockout mice (GNAT1−/−) (16) to produce cTα+/rTα+/− and cTα++/rTα−/− mice, respectively. Hereafter these lines are referred to as cTα+ and cTα++ mice to simplify the nomenclature. In addition, the cTα+ transgenic mice were crossed with S-opsin−/− mice, which expresses cone short wave S-opsin in mouse rods in the rod opsin knock-out background (14) to obtain cTα+/S-opsin−/− mice.

Immunoblot analysis and quantification of transgene expression— Each isolated retina was homogenized in 200 μl of buffer [80 mM Tris, pH 8.0, 4 mM MgCl2 and protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN)]. The samples were incubated with the addition of 30 units of DNase I (Roche Diagnostics) at room
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For quantification of endogenous rTa, each C57BL/6J retina was homogenized in 120 µl buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.1% dodecyl maltoside and protease inhibitor mix), from this 2 µl was further diluted to 80 µl containing the indicated amount of chi8 and either 10 µl or 15 µl of the samples were loaded on SDS-PAGE, blotted and probed with an anti- GT$\alpha$ common region antibody (KENLKDCGLF, Meridian Life Science, Inc.) as described above. To quantify endogenous cTa, two retinas from GNAT1-/- mouse were combined and homogenized in 120 µl buffer. Indicated amount of chi8 was added to 5 µl of this homogenate and separated by SDS-PAGE. The gels were blotted and probed with an antibody that targets a highly divergent sequence in cTa (sc-390, Santa Cruz Biotechnology). Signals were visualized and quantified as described in the previous section. The values were plotted and fitted using Excel software.

Biochemical analysis of ADP ribosylation in mouse ROS membranes– Mice were dark-adapted overnight and rod outer segment (ROS) membranes were isolated under dim red light as described previously (19). Rhodopsin (Rho) concentrations were determined using the difference in absorbance at 500 nm before and after photobleaching. The procedures of pertussis toxin catalyzed ADP ribosylation was similar to the method described by Kerov et al. (20). ROS membranes containing 1 µM Rho per sample were used. Pertussis toxin was pre-activated by 15 min incubation at 30°C with 100 mM dithiothreitol and 0.2% SDS. ROS samples were then incubated with 3 µg/ml of pertussis toxin and 5 µM $[^{32}\text{P}]$ nicotinamide adenine dinucleotide ($[^{32}\text{P}]$NAD) for 1 h at 25°C in the darkness or under light exposure. Meanwhile, 30 µM AlCl$_3$ and 10 mM NaF were added to half of the samples of each condition. The reactions were stopped by SDS-loading buffer and analyzed by 12% SDS-PAGE gels followed by autoradiography of dried gels.

Light-dependent translocation and light calibration– Mice were dark-adapted for at least 12 hr before light exposure. For the dark condition, mice were euthanized and the eyes were emulsified and fixed with 4% paraformaldehyde under infrared illumination. To see light-dependent translocation of Ta in wildtype, rTa/- and cTa++ mice, their eyes were treated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride to dilate the pupils prior to exposure to diffuse white light (2000 lux) for 1 h. For calibrated light exposure, mice were anesthetized and immobilized, and one of the eyes was dilated and exposed to light for 30 min. The light source was a 100-watt quartz tungsten halogen lamp connected to a fiber optic guide (Oriel Instruments, Stratford, CT) and a narrow bandwidth interference filter (10 nm FWHM, Oriel Instruments) with peak center wavelength at 500 nm; light intensity was adjusted using neutral density filters and measured with a calibrated photodiode (United Detector Technology Sensors, Inc., Hawthorne, CA). The delivered photon flux was calculated using the following equation:

$$\theta_{sec} = \frac{1}{I} \cdot \frac{l}{0.16} \cdot \frac{\pi r_{pupil}^2}{4 \times 10^{-19} \cdot \frac{4 \pi r_d^2}{\theta}}$$

where $\theta$ is the number of photons, the current (I) was measured from the calibrated photodiode (Table 1), $r_{pupil}$ is the radius of mouse’s pupil which is ca. 1 mm, $r_d$ is the distance from the light source to the mouse pupil (5 mm). The bleached rhodopsin (R*) can be further estimated by:
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$$R^*/rod\cdot sec = \frac{\theta/sec}{6.4\times10^5 rod/retina \times efficiency}$$

where one mouse retina is estimated to contain approximately $6.4\times10^5$ rod cells (21); and the quantum efficiency, or the probability that the adsorption of a photon initiates rhodopsin activation, is 0.67 (22). In this manner, the rate of $R^*/rod\cdot sec$ was determined for the different neutral density filters, as shown in Table 1.

<table>
<thead>
<tr>
<th>Neutral Density Filter</th>
<th>Current ($\mu$A)</th>
<th>$R^*/rod\cdot sec$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 2.3</td>
<td>2.26</td>
<td>3.3 x $10^4$</td>
</tr>
<tr>
<td>OD 2.6</td>
<td>1.18</td>
<td>1.7 x $10^4$</td>
</tr>
<tr>
<td>OD 2.8</td>
<td>0.67</td>
<td>9.7 x $10^3$</td>
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<tr>
<td>OD 3.0</td>
<td>0.417</td>
<td>6.1 x $10^3$</td>
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<tr>
<td>OD 3.3</td>
<td>0.214</td>
<td>3.1 x $10^3$</td>
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Immunocytochemistry– The superior pole of the mouse eye was marked by cauterization before enucleation. Cornea and lens were removed, and the remaining eyecups were fixed in 4% paraformaldehyde, infiltrated with 30% sucrose overnight, and embedded in O.C.T. (Tissue-Tek, Sakura Finetech, Torrance, CA) as described previously (23). Ten µm thick frozen retinal sections were obtained using a cryostat (Leica, Nussloch, Germany) at -20°C. Sections were air-dried and treated with 0.1mg/ml proteinase K (Roche, Germany). After blocking with PBS containing 1% BSA, 5% normal goat serum, and 0.3% Triton X-100, the sections were incubated with 1:200 dilution of TF15 recognizing a common peptide on both rT$\alpha$ and cT$\alpha$ (Cytosignal, San Diego, CA). Rhodopsin 1D4 antibody (gift from Robert Molday) was biotinylated following manufacturer’s protocols (Pierce Biotechnology). Proteins were visualized with FITC or Texas Red-conjugated secondary antibodies at 1:400 dilutions, or Texas Red Avidin D (Vector Laboratories, Burlingame, CA). Images were acquired on an Axiosplan2 microscope (Zeiss, Oberkochen, Germany). All images for each section were taken at the same detection gain unless indicated.

Expression and Purification of Recombinant Rod T$\alpha$– His$_6$-tagged $G_{\alpha t}/G_{\alpha i1}$ chimeric construct previously characterized for functional activity and solubility in E. coli expression system (24) (chi8, obtained from N. Artemyev) was transformed into E. coli BL21(DE3) and grown in 2X YT media containing 100 µg/ml ampicillin at room temperature up to OD$_{600}$ of 0.5, and induced with 30 µM IPTG for 18 h at 18 °C. The cell pellet was resuspended in 1:20 of the original cell culture volume with a buffer containing 50 mM Tris-HCl, pH8.0, 50 mM NaCl, 5 mM MgCl$_2$, 50 µM GDP, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 5 mM β-mercaptoethanol, and then disrupted by sonication. The cell homogenate was centrifuged at 45,000 x g for 1 h at 4°C. Solid ammonium sulphate was slowly added to the supernatant to a final concentration of 30% with continued stirring at 4°C for 30 min. After centrifugation at 10,000 x g for 20 min, the pellet was resuspended in 20 mM Tris-HCl, pH8.0, 450 mM NaCl, 6 M urea, 0.1% Triton X-100, 1 mM β-mercaptoethanol, and 10 mM imidazole. This crude sample was loaded onto a Ni-NTA agarose resin (Invitrogen) column, washed, and eluted with loading buffer containing 100 mM imidazole. The elution fraction was dialyzed against 20 mM Tris-HCl, pH8.0, 50 mM NaCl, and 0.1 mM PMSF, concentrated using Aquacide II (Calbiochem), and dialyzed against the same buffer. The sample was then subjected to preparative Tris-glycine SDS-polyacrylamide gel electrophoresis, and the gels were subsequently stained with SYPRO® Ruby (Invitrogen) in 250 mM KCl, 1 mM DTT. The recombinant chi8 was excised from the gel. The gel slices were minced and homogenized using a Kontes pestle in passive elution buffer (50 mM Tris pH 7.9, 150 mM NaCl, 0.1% SDS, 1 mM EDTA). The sample was dialyzed extensively against 25 mM ammonium bicarbonate and lyophilized. To quantify the amount of chi8, an aliquot was subjected to amino acid analysis in triplicates by the Biopolymer Laboratory at the University of California, Los Angeles.

Electroretinography (ERG)– The protocol used for ERG measurements was similar to previously described (25). Briefly, six week-old mice were dark-adapted overnight. The procedures were carried out under dim red light the following day. Mice were anesthetized with an intraperitoneal injection of 100 mg ketamine and 10 mg xylazine per kg of bodyweight. Pupils were dilated with 0.5% tropicamide (Tropicacyl®,
Akorn Co., Buffalo Grove, IL) and 2.5% phenylephrine hydrochloride (Akorn Co.). A drop of GonaK™ (2.5% hypromellose ophthalmic solution, Akorn) placed on the cornea facilitated electrical contact with the corneal electrode and kept the eye moist during the recording session. A steel needle placed subcutaneously near the eye served as the reference electrode. The light source was a xenon arc lamp, and 10 ms flashes of light at 500 nm were presented to the eye. A series of neutral density filters were used to control light intensity. ERG signals were amplified by an AC/DC differential amplifier (A-M Systems, Inc., Carlsborg, WA), bandpass-filtered at 0.1-1000 Hz, sampled at 2000 Hz, and digitized with a Digidata 1322A using pClamp software (Axon Instruments, Union City, CA).

Single-cell recordings and analysis—Light-evoked currents from mouse rod photoreceptors were measured with suction electrodes from finely chopped pieces of retinal tissue under recording conditions that have been described previously (26). Briefly, clusters of cells with the outer segments protruding were targeted and an individual outer segment was drawn gently into a suction electrode containing Ames’ media (Sigma-Aldrich) buffered with 10 mM HEPES to pH 7.4. The tissue was bathed with 35-37 °C bicarbonate-buffered Ames’ media that was equilibrated with 5% CO₂/95%O₂. Families of light-evoked currents were measured following 10 ms flashes from an LED ($\lambda_{\text{max}} \approx 470$ nm, FWHM ~ 30 nm; $\lambda_{\text{max}} \approx 571$ nm, FWHM ~ 10 nm; $\lambda_{\text{max}} \approx 367$ nm, FWHM ~ 10 nm; Optodiode Corporation, Newbury Park, CA) or 30 ms flashes from a tungsten-halogen source passed through an interference filter ($\lambda_{\text{max}} \approx 500$ nm, FWHM ~ 15 nm). In experiments where UV light (i.e. $\lambda_{\text{max}} \approx 367$ nm) was used to stimulate the preparation, light was delivered to the recording chamber through a quartz fiber optic (Newport Corporation, Irvine, CA). Families of responses to flashes of increasing intensity were collected from rods for each of the lines of mice described above. The elementary response of the rod, or the response to a single absorbed photon, was estimated from linear range responses by scaling the average response by a factor proportional to the ratio between the time-dependent variance and the mean (1). Currents were low-pass filtered at 20 Hz with an 8-pole Bessel filter, and digitized at 1 kHz.

RESULTS

Expression of cTα in rod photoreceptors of transgenic mice—Cone transducin alpha (GNAT2; here referred to as cTα) cDNA was reverse-transcribed from retinal RNA extracted from C57/B6 mice and cloned downstream of the mouse rod opsin promoter (Fig. 1A). Two independent lines were obtained; both showed similar expression level and pattern, and both lines were used interchangeably in this study. The cTα mice were crossed with the rod transducin alpha (GNAT1; here referred to as rTα) knockout mice (16) to replace cTα for rTα (Fig. 1B). Western blot of retinal extracts showed a noticeable over-expression of cTα in the cTα+ line when compared to the endogenous level of cTα expressed in cones (Fig. 1B, upper panel). For some experiments the cTα+ transgene was also bred to homozygosity to increase expression level (cTα++). Relative expression levels of rTα and cTα was quantified using an antibody that recognized a common epitope on both isoforms (KENLKDCGLF, Meridian Life Science, Inc.), as shown in Fig. 1C. The relative level of ectopically expressed cTα+ to endogenous rTα in rods was 15 ± 1.2%. Doubling the gene dosage increased the level to 35 ± 2.0%. Swapping cTα for rTα appeared to have no discernible effect on the expression level of other transduction proteins that interact with Tα (Fig. 1D), nor on retinal morphology (data not shown).

The subunit composition for Tα in rods is rTαβγγ₁ whereas for cones is cTαβγγ₈ (27). The βγ subunit plays an important role in presenting the α subunit to the GPCR and enhances the coupling efficiency (28-30). To determine whether cTα forms a heterotrimeric unit with βγγ₁ in these rods, we used the pertussis toxin assay in which the toxin catalyzes ADP ribosylation of the α-subunit when it is complexed with βγ. Control experiments show rTα labeled strongly in the dark-adapted sample, and this labeling was reduced by light exposure (Fig. 1E). Application of AlF₄⁻, which activates Tα-GDP by mimicking the γ-phosphate of GTP, dissociates the subunits, effectively reducing ADP ribosylation in both dark and light-exposed samples (Fig. 1E). Reduced ADP ribosylation was also observed in the cTα samples following light exposure and AlF₄⁻ application, albeit the signal was weaker due to the low
expression level of cTα (Fig. 1E). These results indicate that cTα forms a heterotrimeric unit with rod βγγ.

**Rhodopsin and S-opsin activate cTα with similar efficiency**—Having discerned that cTα forms a heterotrimeric complex with Gβγγ, we sought to determine whether cTα can substitute for rTα to drive rod photoresponses. Furthermore, we sought to compare the efficiencies by which rhodopsin and a cone pigment (S-opsin) activate cTα. Previously Shi et al. (14) showed that S-opsin and rhodopsin, when co-expressed in the same rod, generated single-photon responses through rTα with similar amplitudes and kinetics. In other words, S-opsin and rhodopsin activated endogenous rTα with similar efficiency. We generated Sop+,Rh+/-,cTα+ mice that coexpressed cone S-opsin (~12%) and rhodopsin (~88%) in the rod outer segment, as well as the substitution of cTα for rTα, to compare the activation of cTα by the two visual pigments in the same rod cell. The spectral sensitivities of S-opsin and rhodopsin peak near 360nm and 500nm, respectively (31). Using this spectral separation, we compared the light-evoked responses produced mostly by S-opsin activation at 367nm, and almost completely by rhodopsin activation at 571nm. For 367 nm stimuli, the absorption of S-opsin was near its maximum and rhodopsin absorption was <1/5 of its peak value; whereas the response for 571 nm stimuli was generated almost completely by rhodopsin (14). The photoresponses and derived single photon response from rhodopsin coupled to cTα elicited by 571 nm (green trace) or 367 nm (blue trace) is shown in Fig. 2A and B, respectively (Rh+/-,cTα+). These results were similar to that obtained from Sop+,Rh+/-,cTα+ rods (Fig. 2C, D). Furthermore, the results showed that the amplitude of the elementary responses of for 367nm and 571nm stimuli was indistinguishable in these rods (Fig. 2D). Thus, we find that cTα is activated by S-opsin and rhodopsin similarly well, and the inactivation of cTα expressed in rods is similar to rTα inactivation in rods.

**Tα concentration affects sensitivity**—Suction electrode recordings form ROS showed that photoresponses from single allele cTα+ mice displayed similar single photon response amplitude and time course to WT rods (Fig. 3A, B), indicating that cTα is capable of functionally replacing rTα in the rod phototransduction cascade. However, these rods were about 10-fold less sensitive than WT rods (Fig. 3C). The half-maximal flash strength was about 27 photons/μm² in WT rods, but was about 330 photons/μm² in cTα+ rods. This decrease in flash sensitivity was also observed in ERG recordings, where the light sensitivity of cTα+ mice was in between that of wildtype and rTα-/- mice, whose responses arise exclusively from cones (Fig. 3D). A similar dependence was also observed in mice expressing half the native rhodopsin (Rh+/-; Fig. 3E). Rh+/- rods displayed a half-maximal flash strength of about 66 photons/μm², while on the same Rh+/- background the half-maximal was about 490 photons/μm² in cTα+ rods and about 140 photons/μm² in cTαα+ rods. Thus the light sensitivity appears to correlate with the expression level of Tα within this concentration range. Along with the similar activation and deactivation phase of responses in rods expressing either cTα or rTα (Fig. 4A), these results indicate that the difference in light sensitivity between rods and cones are not likely due to a cell-specific Tα.

**The concentration of endogenous rTα and cTα in rods and cones**—The dependence of sensitivity on Tα concentration prompted us to compare the concentration of endogenous rTα in rods and cTα in cones. To quantitatively measure the concentration of endogenous Tα, we obtained a Gαi/Gαi4 chimeric construct (chi8) which has been used for functional (24) and crystallographic studies (32), and substituted cTα sequences in the chimeric construct so the recombinantly expressed protein can serve as standards. Chi8 was expressed and purified to apparent homogeneity as visualized by SYPRO®-Ruby stained polyacrylamide gel (Fig. 4A). The trace amount of higher molecular weight bands appears to be higher order aggregates, whereas the lower molecular weight bands are likely degradation products because these bands also appeared in the western blot (Fig. 4B). The concentration and purity of chi8 was determined by amino acid analysis. Tα in rods (rTα) was quantified using an antibody that recognizes the carboxyl-terminal sequence, KENLKDGCGLF (Meridian Life Science, Inc.) which is conserved between rTα and cTα. However, this antibody showed cross-reactivity with Gi that co-migrated with cTα (but not rTα),

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therefore a different antibody that recognized a highly divergent epitope on cTα (I-20, Santa Cruz Biotechnology) was used to quantify cTα. Because in the retina rTα is expressed only in rods and cTα is expressed only in cones, we can use the value obtained from whole retinal homogenates to quantify the amount of each Tα in these cells. It is known that detection efficiency in western blots can be strongly influenced by protein complexity of the sample. To circumvent this issue, known quantities of chi8 were mixed with whole retinal homogenates prepared from C57/B6 mice or rTα-/− mice as an internal standard. The His-tagged recombinant protein migrated slower on SDS-PAGE, allowing it to be distinguished from the endogenous proteins (Fig. 4C and D). The fluorescence signal from chi8 was plotted as a standard curve and the best polynomial fit was obtained from whole retinal homogenates to determine whether endogenous cTα also translocates in cones, we studied rTα+/− mice where only cTα is expressed (Fig. 5, rTα+/− panels). Their identity as cones was verified using rhodamine-conjugated peanut agglutinin (Fig. 5, bottom panels), a known cone marker (45). Upon light exposure we found that cTα moved to the cone inner segment compartments in a pattern that is qualitatively similar to endogenous rTα in WT and transgenic cTα in rods. These results show that ectopically expressed cTα is functionally similar to endogenous rod and cone Tα with respect to its ability to move between photoreceptor outer and inner segments following light exposure.

The light threshold for cTα translocation did not correspond to Tα concentration—The ability of Tα to translocate depends on its level of activation; when the concentration of Tα-GTP exceeds that of the GTPase complex, it moves to the compartments in the rod inner segment (46,47). Because cTα+ and cTα++ rods display reduced Tα concentration compared to WT rods, a simplistic expectation is that there should be a corresponding increase in the threshold for translocation. Under these circumstances more R* would be required to activate the same number of Tα to reach the threshold level to saturate the GTPase complex. We observed in the WT retina that Tα translocation was driven by a light yielding an initial rate of 9.7 x 10^3 R*/rod·sec (2.8
OD, Fig. 6B), whereas translocation in the retina of cTa++ and cTa+ mice was driven by a light yielding 1.7 x 10^4 R*/rod·sec (Fig. 6 F and H, 2.6 OD), a light intensity only 1.7-fold higher than WT mice. In cTa retinas low-level translocation can be observed at similar light threshold as WT retinas (Fig. 6E). Therefore the threshold for translocation does not appear to depend on the relative concentration of Ta.

DISCUSSION

In this study we replaced cTa for rTa in transgenic mouse rods to evaluate how Ga subtype influences signal amplification from different GPCRs, and how this might explain functional differences between rods and cones. We showed that ectopically expressed cTa forms a heterotrimeric complex with Gβγγ1, displays light-triggered translocation, and can substitute equally for rTa in generating photoresponses initiated by either rhodopsin or S-cone opsin. Thus, from a functional standpoint rTa and cTa appear interchangeable. Below we discuss this functional interchangeability.

The role of Ta in setting the properties of the rod and cone photoresponse—A number of studies have investigated whether signal amplification at the first step of visual transduction, between the pigment and G protein, may provide a mechanism that explains the difference in light sensitivity between retinal rod and cones. In support of this notion, Xenopus short-wave cone opsin heterologously expressed in COS-1 cells exhibited lower Ta activation when compared to rhodopsin (10). In addition, biochemical assays performed on purified rods and cones from carp retina revealed a lower amplification of G protein activity, as well as faster visual pigment phosphorylation in cones (6,7,48). We also observed a decrease in sensitivity in mice that expressed cTa in rods. However, this decrease in sensitivity correlated with the level of cTa expression (Fig. 3). A comparison of the normalized single photon response arising from rTa and cTa showed them to be similar in rising phase as well as response recovery, indicating the similar amplification efficiency of rTa and cTa by rhodopsin, and similar deactivation by the GAP complex. Deng et al., who used AAV to introduce cTa into GNAT1-/- rods, also observed a similar sensitivity and response recovery when compared to rTa (49), a result qualitatively similar to our findings.

The co-expression of S-cone opsin in rods allowed us, for the first time, to determine whether cell-type specific Ta interacted preferentially with visual pigment subclasses. We demonstrated that cTa is activated by rhodopsin and S-cone opsin equally when both are expressed in the same cell. These results, together with our previous finding that S-opsin and rhodopsin produced equivalent light responses when coupled to endogenous rTa, suggest strongly that the difference in sensitivity between rods and cones does not lie within rTa and cTa.

Our findings are also similar to a previous study of rods and cones in the tiger salamander, whose green rods and blue cones contain the same S-cone opsin that is coupled to rTa and cTa, respectively. It was found that these cells display similar light sensitivities and response kinetics, indicating that S-cone opsin activates the native rTa and cTa with the same efficiency regardless of the structural features of the photoreceptor outer segment (50). The interchangeability between rTa and cTa may not be totally surprising. Structural studies of the interaction between rTa-GDP and Gβγ between activated rTa and γ PDE, and between activated rTa and the RGS complex found that the interface between these interactions often show complete conservation of the amino acids between rTa and cTa (32,51). One distinction is at the amino terminus, where cTa contains an insert of 4 amino acids, ELAR, in a domain determined to participate in Gβγ as well as receptor binding (32,52,53). Our data show that this insert does not appear to affect these interactions within the rod cell. Given this apparent interchangeability of Ta, it is remarkable that rods and cones express different forms of transducin. Although our results show that Ta in rods and cones is interchangeable, it remains possible that the cell type-specific Gβγ subunits are functionally specific in the phototransduction cascade or perhaps at the synapse (54).

A recent study by Chen et al. (55) used a similar strategy as ours to replace endogenous rTa with cTa (termed GNAT2C) in transgenic mice, and reported a lower amplification of GNAT2C by rhodopsin that resulted in a decrease in light sensitivity and faster response recovery. The level of GNAT2C used in their study was similar to that of endogenous rTa, whereas cTa expression used
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The value of cT was ascertained to be 2.6 ± 0.4 pmol, which when normalized to the number of cones and their smaller outer segment volume, is higher than the concentration of rTα in rods. Therefore, the lower sensitivity of cones cannot be explained simply by a lower Tα concentration. Considering that Gβγ increases the coupling efficiency between R* and Tα (28), the level of endogenous Gβγ8 in cones and their association with cTα may be important determinants for setting sensitivity in cones. Additional factors that are likely to play a role include a faster rate of cone pigment phosphorylation (6,7), regeneration (62), and their faster turnover rate of cGMP (9).

Ectopically expressed cTα translocates normally–Light-induced Tα translocation from the outer segment to the inner compartments decreases the gain of phototransduction and has been proposed to be an adaptation mechanism (35). How translocation is regulated has been a topic of intense investigation, particularly because this process has been reported to be nonlinearly related to light exposure (43). Studies have shown that translocation threshold in rods can be shifted to dimmer or brighter light exposures in the GAP defective mice, or those over-expressing RGS9, respectively, giving rise to the model that Tα translocation ensues only when the concentration of Tα-GTP exceeds that of the GTPase-activating complex (46,47). The same rule was proposed for mouse cones as well, where the failure to observe cTα translocation was attributed to the higher expression level of the GAP complex in cones (63). However in this study we observed that endogenous cTα in cones readily translocated with moderate light exposure (2,000 lux). A similar observation of cTα translocation was also reported by Chen et al. (64). The discrepancy in the observation of cTα translocation by different groups could be due to differences in the tissue preparations, epitope unmasking, and the sensitivity of the antibodies used. The translocation pattern of cTα is consistent with the functional interchangeability of rTα and cTα in rods and cones, and suggests that translocation of endogenous cTα in cones may contribute to light adaptation under prolonged light exposure.

We took advantage of mice expressing different Tα levels to investigate the relationship between Tα concentration and the light threshold that triggers translocation. Because cTα+ and cTα++ rods contain 15% and 35% of Tα concentration compared to WT rods, a simple expectation is to see a corresponding increase in
light threshold to reach the concentration of Tα-GTP necessary to saturate the GAP complex for translocation to ensue. Instead, we observed only a slight increase in light thresholds (Fig. 6). Although the almost two-fold difference in the light threshold did correlate with their relative difference in sensitivity, the relation between translocation threshold and absolute sensitivity is non-linear. The non-linearity observed for light threshold for translocation likely reflects a non-linear relationship between the number of activated transducin generated per R* at bright light levels which are beyond the functional range of rods. Additional candidates that may affect this non-linearity include lipid modifications on Tα (44,47,65,66), lipid microdomains on ROS discs, and protein-protein interactions that restrict their lateral and longitudinal diffusion (67,68).

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Concentration dependence of Gαt on rod sensitivity


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FOOTNOTES

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3The abbreviations used are: rTα, rod transducin alpha; cTα, cone transducin alpha; Tα, transducin alpha; GAP, GTPase accelerating protein; PDE6, phosphodiesterase 6; WT, wild-type; RGS, regulator of G-protein signaling.
4Transgenic mice that expressed cTα were generated by G. Shi and the Norris Transgenic Core Facility.
5Rod transducin alpha knockout mice were provided by J. Lem.

FIGURE LEGENDS

FIGURE 1. Characterization cTα transgene expression. A, Transgene construct to express cTα in rods. B, Western blot analysis of serial dilutions of retinal homogenates. Cone Tα and rTα were recognized by SC390 and Tα1A antibodies, respectively. Actin was used as loading control. C, Relative quantities of both cTα and rTα from western blots (n=7) utilizing TF15 antibody which recognizes a conserved epitope on cTα and rTα. The relative expression levels from different genotypes were normalized to retinal extracts from C57 mice (WT). D, Western analyses of G11, G5 and RGS9-1 from WT, rTα/- and cTα retinas. The G5 antibody recognized both the long and the short isoform of the protein. No changes in these transduction proteins were detected as a consequence of cTα expression. E, Cone Tα associates with rod Gβ1γ1. Pertussis toxin catalyzed heterotrimeric G-protein dependent ADP ribosylation of wildtype rTα and transgenic cTα. ADP ribosylation was inhibited by light or in the presence of AlF4^- ions for both WT and cTα samples.

FIGURE 2. Rod and cone Tα couple to rhodopsin and cone S-opsin with similar efficiency. A, Averaged response families for Rh+/-, cTα+ rods for 10 ms flashes of green light (λmax = 571 nm) with flash strengths of 46, 90, 180, 360, 700, and 1400 photons µm^-2, or UV light (λmax = 367 nm) with flash strengths of 85, 170, 330, 660, 1300, 2600, and 5300 photons µm^-2. B, For Rh+/-,cTα+ mice, the single photon response was determined as the ratio of the time-dependent variance of all dim flash responses (< 25% Rmax) to the mean amplitude for flashes of light from a green LED (λmax = 571 nm; mean ± SEM, n = 5 rods) or a UV LED (λmax = 367 nm; mean ± SEM, n = 6). The mean single-photon response amplitude was 0.32 ± 0.10 pA for the 571 nm stimulus, compared to 0.36 ± 0.09 pA for the 367 nm stimulus. C, Averaged response families for Sop+Rh+/-,cTα+ rods for 10 ms flashes of green light (λmax = 571 nm; n = 17 rods) with flash strengths of 45, 89, 180, 350, 700, 1400, 2800, and 5600 photons µm^-2, or UV light (λmax = 367 nm; n = 11 rods) with flash strengths of 170, 340, 680, 1300, 2700, 5400, and 11000 photons µm^-2. D, Estimated single-photon response for mice expressing Sop+Rh+/-,cTα+ also displays a similar amplitude and kinetics independent of the wavelength of stimulation. The mean single-photon response amplitude was 0.27 ± 0.07 pA for the 571 nm stimulus, compared to 0.21 ± 0.04 pA for the 367 nm stimulus. E, The normalized mean single photon response for Rh+/-,cTα+ (black trace; n = 11 rods) and Sop+,Rh+/-,cTα+ (red trace; n = 28 rods). The normalized single photon responses demonstrate similar kinetics for both Rh+/-,cTα+ and Sop+,Rh+/-,cTα+ in both the rising phase (activation of the cascade) and in the decay (inactivation).

FIGURE 3. Sensitivity of rods expressing cTα is correlated with expression level. A, Representative flash response families from WT and cTα+ rods. 10 ms flashes were delivered at the time indicated by the arrow. B, Population mean single-photon responses from WT (black trace) and cTα+ mice (red trace). The responses have been normalized to compare the kinetics. C, Normalized flash response amplitude is plotted against flash strength for WT and cTα+ rods. Smooth curves are fitted according to the exponential saturation equation: y = 1 - e^-kt. Half-maximal flash strengths for these populations were 27 ±
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1.8 photons/µm$^2$ in WT rods (mean ± SEM; n = 14) and 330 ± 36 photons/µm$^2$ in cTα+ rods (mean ± SEM; n = 12). The ~10-fold shift in this value is similar to the 10-fold reduction in Tα in these rods. D, ERG recorded from 6-week-old WT, cTα+. Responses from rTα-/- were shown to illustrate the cone threshold. Sensitivity was presented as normalized b-wave vs. light intensity (photons/µm$^2$ at the cornea). E, Normalized flash response amplitude is plotted against flash strength for rods expressing a single (+) or double dose (++) of cTα in mice expressing half the normal rhodopsin (Rh +/-). The half-maximal flash strengths were 66 ± 8.0 photons/µm$^2$ in Rh+/- rods (mean ± SEM; n = 7), 490 photons/µm$^2$ in Rh+/-,cTα+ rods (mean ± SEM; n = 18), and 140 photons/µm$^2$ in Rh+/-,cTα++ rods (mean ± SEM; n = 14). Thus, in the Rh+/- background, a dependence of half-maximal flash strength on Tα concentration remains. F, Relative sensitivity of WT, cTα+ and rTα-/- retinas as measured by the electroretinogram. Sensitivity measurements were based on normalized b-wave amplitudes. The difference in sensitivity between WT, where the response is largely contributed by rods, and rTα-/- where the response arise exclusively from cones, spanned a little over two logarithmic units. The value of cTα+ lies approximately midway between these two responses.

FIGURE 4. Quantification of endogenous rTα and cTα proteins using recombinant protein chi8. A, Purified chi8 was visualized by SYPRO-Ruby stained polyacrylamide gel. At 0.1 µg, chi8 appeared as a single band whereas bands of higher and lower molecular weights were detected when 1 µg was loaded (arrow). B, Western blot of purified chi8 protein probed with a Gtα antibody which recognizes a common epitope in rTα and cTα (Meridian Life Science). Aside from the band corresponding to the full length protein (arrow), similar band of higher and lower molecular weights as in A were also present, indicating higher aggregates of chi8 as well as its degradation products. The concentrations of endogenous rTα (C) and cTα (D) proteins were measured by western blots of retinal homogenates from C57BL/6 mice containing known quantities of chi8. Serial dilutions were loaded onto each gel and proteins were detected using a Gtα antibody (Meridian Life Science) for rTα and sc390 (Santa Cruz Biotechnology) for cTα. The fluorescence signals from chi8 was quantified, plotted and fitted with the best polynomial curve, and served as the standard to calculate the quantity of Tα in each lane.

FIGURE 5. Rod and cone Tα undergo similar light-induced translocation. A, Tα (green) is primarily localized to the outer segment (os) of WT and cTα++ rods in the dark adapted state. Endogenous cTα, here visualized in a dark-adapted rTα (GNAT1) knockout retina, is also localized to the outer segment. The bottom rTα-/--panels are also co-labeled with rhodamine-conjugated peanut agglutinin (red), a known cone marker. Adjacent panels show the differential interference contrast image of the same field to highlight the boundary of the outer nuclear layer. Exposure to 2,000 lux light causes endogenous Tα in both rods and cones to move towards the inner segment compartments (is, onl). This pattern was also demonstrated by ectopically expressed cTα in transgenic rods. The sections were counterstained with DAPI to visualize cell nuclei (blue). White scale bar = 25 µm, black scale bar = 10 µm.

FIGURE 6. Light threshold for cTα translocation does not correlate with Tα concentration. Both rTα and cTα were detected using the TF15 antibody (green). The left strip of each panel shows rhodopsin immunofluorescence (red) highlighting the outer- and inner segment boundary, whereas the remaining panel is the merged image of rhodopsin (red) and Tα (green). The nuclei were stained with DAPI (blue). A-C, C57 retinas (WT); D-F, cTα++ retinas; G-I, cTα+ retinas. Translocation of rTα in WT retina was detected at 9.7 x 10$^3$ R*/rod·sec from using a 2.8 OD neutral density filter (B). At this light intensity translocation for cTα++ can be detected (E), but was much more robust at 1.7 x 10$^4$ R*/rod·sec (F, 2.6 OD). For cTα+ translocation is evident at 1.7 x 10$^4$ R*/rod·sec (H, 2.6 OD). The green arrow marks the light intensities where translocation is evident. Scale bar = 25 µm.
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Figure 1

A

4.4 kb opsin promoter cTa cDNA pA

B

WT rTα+/− rTα/− cTα+ cTα++

52 kD
38 kD
52 kD
38 kD

actin cTα rTα

C

Relative levels of Tα

WT rTα+/− rTα/− cTα+ cTα++

D

WT rTα/− cTα+

Gα1
Gα63
Gβα1L
RG9S-1

E

WT cTα

AIr2−
rTα− cTα
Concentration dependence of $G_\alpha$ on rod sensitivity

Figure 2
Concentration dependence of $G_{\alpha_t}$ on rod sensitivity

Figure 3

A

B

C

WT

cT$\alpha+$

D

E

$R/R_{\text{max}}$

$R_{\text{wave}}/R_{\text{max}}$

$R/R_{\text{max}}$

Phosphons/um$^2$
Concentration dependence of $G_\alpha$ on rod sensitivity

Figure 4

A

B

C

D

Fluorescent unit

Fluorescent unit

0 0.1 0.2 0.3 0.4

0 0.05 0.1 0.15 0.2

0.5 1.0 1.5 2.0 2.5

chi8 (pmol)

chi8 (pmol)
Concentration dependence of $G_{\alpha t}$ on rod sensitivity

Figure 6
Functional Comparison of Rod and Cone Gαt on the Regulation of Light Sensitivity
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