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Diffusion and light-dependent compartmentalization of transducin

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Abstract

Diffusion and light-dependent compartmentalization of transducin are essential for phototransduction and light adaptation of rod photoreceptors. Here, transgenic *Xenopus laevis* models were designed to probe the roles of transducin/rhodopsin interactions and lipid modifications in transducin compartmentalization, membrane mobility, and light-induced translocation. Localization and diffusion of EGFP-fused rod transducin- α subunit ($G_{\alpha t1}$), mutant $G_{\alpha t1}$ that is predicted to be N-acylated and S-palmitoylated ($G_{\alpha t1}A3C$), and mutant $G_{\alpha t1}$ uncoupled from light-activated rhodopsin ($G_{\alpha t1}-Ct\alpha_s$), were examined by EGFP-fluorescence imaging and Fluorescence Recovery After Photobleaching (FRAP). Similarly to $G_{\alpha t1}$, $G_{\alpha t1}A3C$ and $G_{\alpha t1}-Ct\alpha_s$ were correctly targeted to the rod outer segments in the dark, however the light-dependent translocation of both mutants was markedly impaired. Our analysis revealed a moderate acceleration of the lateral diffusion for the activated $G_{\alpha t1}$ consistent with the diffusion of the separated $G_{\alpha t1}GTP$ and $G\beta_1\gamma_1$ on the membrane surface. Unexpectedly, the kinetics of longitudinal diffusion were comparable for $G_{\alpha t1}GTP$ with a single lipid anchor and heterotrimeric $G_{\alpha t1}\beta_1\gamma_1$ or $G_{\alpha t1}-Ct\alpha_s\beta_1\gamma_1$ with two lipid modifications. This contrasted the lack of the longitudinal diffusion of the $G_{\alpha t1}A3C$ mutant apparently caused by its stable two-lipid attachment to the membrane and suggests the existence of a mechanism that facilitates axial diffusion of $G_{\alpha t1}\beta_1\gamma_1$.

1. Introduction

The principal components of the vertebrate phototransduction cascade, rhodopsin, transducin (G_{t1}), and cGMP phosphodiesterase-6 (PDE6), reside on the disc membranes within a specialized outer segment (OS) compartment of rod photoreceptor cells. Photoexcited rhodopsin (R^*) stimulates exchange of GTP for GDP on the α -subunit of heterotrimeric G_{t1} ($G_{\alpha t1}\beta_1\gamma_1$) causing $G_{\alpha t1}GTP$ to dissociate from $G\beta_1\gamma_1$ and R^* and activate PDE6 (Burns and Arshavsky, 2005; Fu and Yau, 2007; Lamb and Pugh, 2006). The diffusion controlled encounter between the phototransduction proteins at the membrane surface is thought to control the kinetics of photoresponse (Calvert et al., 2001; Pugh and Lamb, 1993). In addition to lateral diffusion along the plane of the disc membranes, $G_{\alpha t1}GTP$, $G\beta_1\gamma_1$, and heterotrimeric G_{t1} can diffuse longitudinally (along the long axis of the OS). The axial diffusion or inter-disc protein transfer apparently underlies light-induced translocation of $G_{\alpha t1}GTP$ and $G\beta_1\gamma_1$ from the OS to the inner segment (IS) (Artemyev,

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2008; Calvert et al., 2006; Nair et al., 2005; Rosenzweig et al., 2007; Slepak and Hurley, 2008; Sokolov et al., 2002; Wang et al., 2008). Furthermore, longitudinal diffusion may serve as a mechanism for the IS→OS transport of translocated or newly synthesized G_{t1} in the dark. The N-terminal acylation of $G_{\alpha t1}$ and farnesylation of $G_{\gamma 1}$ provide for a relatively weak interaction of the individual subunits with the disc membrane, whereas the dual lipidation of $G_{\alpha t1}\beta_1\gamma_1$ promotes a comparatively stable membrane association (Bigay et al., 1994; Kosloff et al., 2008). According to the diffusion model of light-dependent translocation of transducin, the activation of G_{t1} by R^* facilitates dissociation of $G_{\alpha t1}GTP$ and $G\beta_1\gamma_1$ subunits from the membrane allowing them to diffuse to the IS (Artemyev, 2008; Burns and Arshavsky, 2005; Calvert et al., 2006; Slepak and Hurley, 2008). For the protein translocation to occur, the G_{t1} activation rate must exceed the capacity of the GTPase activating protein (GAP) complex to inactivate $G_{\alpha t1}GTP$ (Kerov et al., 2005; Lobanova et al., 2007). Inactivation of $G_{\alpha t1}GTP$ with subsequent formation of the heterotrimer leads to re-association of G_{t1} with the membrane and prevents diffusion. The simple diffusion model predicts that the longitudinal diffusion of activated transducin is faster than that of the heterotrimeric G_{t1} . However, a recent investigation of transducin diffusion in rods of transgenic *Xenopus laevis* yielded surprising findings. The activated $G_{\alpha t1}GTP$ was found to diffuse laterally and longitudinally significantly slower than $G_{\alpha t1}\beta_1\gamma_1$ (Wang et al., 2008). It is not unexpected that $G_{\alpha t1}$ would diffuse slower as a part of the activated $G_{\alpha t1}/PDE6$ complex, particularly since the diffusion of PDE6 in ROS is remarkably slow (Muradov et al., 2009). Yet, the slower mobility was exhibited by the entire pool of activated $G_{\alpha t1}$ well in excess to rod PDE6 (Wang et al., 2008). The proposed underlying mechanism is sequestration of $G_{\alpha t1}GTP$ by lipid microdomains (Wang et al., 2008). Although the reported slow rate of longitudinal diffusion of activated $G_{\alpha t1}$ is sufficient to explain the light-dependent redistribution of transducin, the study underscores the lack of complete understanding of the translocation phenomenon (Wang et al., 2008).

To probe transducin targeting and translocation mechanisms, we have generated transgenic *Xenopus laevis* expressing human EGFP- $G_{\alpha t1}$ and its mutants in rod photoreceptors. Localization and diffusion of mutant transducins were examined by EGFP-fluorescence imaging and Fluorescence Recovery After Photobleaching (FRAP). The A3C mutation was introduced into EGFP- $G_{\alpha t1}$ to create an artificial palmitoylation site. By analogy with $G_{\alpha i}$, the A3C mutant is expected to be both N-acylated at Gly2 and palmitoylated at Cys3, thereby enhancing its affinity for the membrane (Chen and Manning, 2001; Wedegaertner et al., 1995). A $G_{\alpha t1}$ mutant with the replacement of the C-terminal 11 residues by the corresponding residues of $G_{\alpha s}$ ($G_{\alpha t1}$ -C α_s) was produced to investigate the effects of rhodopsin/transducin interactions on the light-dependent compartmentalization and diffusion of transducin. The C-terminus of $G_{\alpha t1}$ is a major rhodopsin recognition site (Bourne, 1997; Oldham et al., 2006; Scheerer et al., 2008) that could potentially serve as a signal motif for targeting of transducin to the OS. Biochemical studies indicated that the $G_{\alpha t1}$ -C α_s mutant is not activated by R^* (Natochin et al., 2001). Our analysis revealed an increase rather than decrease in the lateral D value of the activated $G_{\alpha t}$ consistent with the diffusion of the separated $G_{\alpha t1}GTP$ and $G\beta_1\gamma_1$ on the membrane surface. Interestingly, the axial diffusion kinetics were equivalent for $G_{\alpha t1}GTP$ with a single lipid anchor and heterotrimeric G_{t1} with two lipid modifications. In contrast, the $G_{\alpha t1}$ A3C mutant failed to diffuse axially, apparently due to its strong two-lipid attachment to the membrane.

2. Materials and methods

2.1. Generation of transgenic tadpoles

The transgene construct was prepared using a modified pXOP(−508/+41)EGFP vector (Mani et al., 2001; Muradov et al., 2009). The EGFP sequence was inserted into the helical domain of $G_{\alpha t1}$ between Met¹¹⁵ and Pro¹¹⁶ via a 6-residue linker sequence (SGGGGS) (Fig.

1) (Wang et al., 2008). The cDNA sequence of human $G\alpha_t$ was obtained from the Missouri cDNA Resource Center. The $G\alpha_t$ 1-115 sequence was PCR-amplified using a 5'-primer with an NcoI site and a 3'-primer with the N-terminal sequence of EGFP added to $G\alpha_t$ specific sequence. The $G\alpha_t$ 116-350 sequence was PCR-amplified using a 5'-primer with the C-terminal sequence of EGFP added to $G\alpha_t$ specific sequence, and a 3'-primer containing an XbaI site. The two resulting PCR products were used as primers in the PCR reaction with pXOP(-508/+41)EGFP as a template. The $G\alpha_t$ 1 construct with the internally-fused EGFP sequence was subcloned into the pXOP vector using NcoI/XbaI sites to produce pXOP(-508/+41)EGFP- $G\alpha_t$ 1. The SalI site was introduced in front of the promoter region for linearization of the plasmid and transgenesis by restriction enzyme mediated integration (REMI) (Kroll and Amaya, 1996). The pXOP(-508/+41)EGFP- $G\alpha_t$ 1 template was amplified with primers coding the A3C mutation or the 11 C-terminal amino acids of $G\alpha_s$ to generate transgenic $G\alpha_t$ 1A3C and $G\alpha_t$ 1-C α_s tadpoles. The sequences of the final constructs were confirmed by direct sequencing. Preparation of sperm nuclei and egg extract, and REMI were performed as previously described (Kroll and Amaya, 1996). Transgenic tadpoles were identified 7–8 days post-injection by visual examination for EGFP fluorescence in the eye using a dissecting microscope equipped with a GFP filter.

2.2. Live cell imaging

X. laevis tadpoles at about stage 50 were dark-adapted overnight and anesthetized with 0.1% tricaine methylsulphonate. Eyes were enucleated, retinas extracted and put into 60 μ l oxygenated Ringer's solution on a microscope glass under dim red light. Retinas were dissected into $\sim 0.2 \times 0.2$ mm pieces using two 30G needles. For light adaptation, tadpoles were exposed to light in Petri dishes (~ 800 lux, 40 min) prior to anesthesia, and the retina pieces were prepared under room light as described above. EGFP-fluorescence was examined using a Zeiss LSM 510 confocal microscope. Mean fluorescence intensities in the OS and IS of light-adapted EGFP- $G\alpha_t$ 1 and EGFP- $G\alpha_t$ 1A3C rods were quantified using Z-stack projection images and ImageJ. The Z-stack projection images were obtained from 1 μ m Z-sections covering an entire diameter of a single rod (~ 7 – 8 μ m). $G\alpha_t$ 1 and EGFP- $G\alpha_t$ 1A3C rods with average and similar fluorescence were selected and analyzed with the same microscope settings.

2.3 Immunofluorescence

X. laevis tadpoles were dark-adapted for at least 12 hrs, anesthetized in 0.1% tricaine methylsulphonate, and dissected under dim red light. The heads were fixed in 4% paraformaldehyde in PBS at 22°C for 4 hours in full darkness. For light adaptation, tadpoles in Petri dishes were placed under white fluorescent bulbs (~ 800 lux, 40 min), followed by anesthesia and dissection. After fixation, the eyeballs were submersed in a 30% sucrose solution in PBS for 12 hrs at 4°C and then embedded in tissue freezing medium (TBS) and frozen on dry ice. Radial sectioning (10 μ m) of the retina was performed using a cryomicrotome Microm HM 505E. Retinal cryosections were air-dried and kept at -80°C until use. Before staining, sections were incubated in 0.1% Triton/PBS for 30 min followed by incubation with 5% BSA in PBS for another 30 min. Labeling with rabbit anti-rod $G\alpha_t$ antibody K-20 (1:1000)(Santa Cruz Biotech, Santa Cruz, CA) was performed in 0.1% Triton/PBS containing 5% BSA for 3 hours at 25°C. Following 2-hr incubation with goat anti-rabbit AlexaFluor 555 secondary antibodies (Invitrogen) (1:1000), the sections were visualized using a Zeiss LSM 510 confocal microscope.

2.4. Transducin extraction

After overnight dark-adaptation of transgenic *X. laevis*, tadpole eyeballs (~ 20) were isolated under room light (~ 1000 lux), homogenized with pestle and sonicated in 250 μ l of 20 mM Tris (pH=7.0) buffer containing 150 mM NaCl, 2 mM MgSO₄, complete protease inhibitor

mixture (Roche) and 10 μ M GTP γ S (buffer A). The homogenate was exposed to 2000 lux light for 5 minutes, spun (2500g, 1 min) to remove cell debris and nuclei, and centrifuged for 1 hour at 100000g. The supernatant was collected as soluble fraction S. The pellet was rinsed with 200 μ l of buffer A, resuspended in 120 μ l of buffer A, and centrifuged (1 hour, 100000g). The procedure was repeated using buffer A without GTP γ S, and the pellet was dissolved in 100 μ l 1% SDS (membrane fraction M). All procedures were performed on ice or at +4°C.

2.5. Analysis of transducin diffusion

The lateral and longitudinal (axial) diffusion of transducin were assessed by measuring Fluorescence Recovery After Photobleaching (FRAP) in living *Xenopus* rods as previously described (Wang et al., 2008) with minor modifications (Muradov et al., 2009). A typical recording involved imaging of a transgenic rod cell attached to a small piece of retina at 25°C in Ringer's buffer (5 mM HEPES, pH 7.7, 110 mM NaCl, 2 mM CaCl₂, 2.5 mM KCl, 1.2 mM MgCl₂). To study diffusion of G $\alpha_{t1}\beta_1\gamma_1$, rhodopsin in transgenic rods was converted to opsin by pre-incubation with 10 mM hydroxylamine under room light conditions. Diffusion of G α_{t1} GTP was monitored using Ringer's buffer oxygenated with 95% O₂/5% CO₂ and containing 10 mM glucose. The membrane permeabilization with α -toxin, used previously to study diffusion of G α_{t1} GTP γ S (Wang et al., 2008), caused an apparent loss of native rod morphology and was excluded from our analysis. Nucleotide depletion with 6 mM 2-deoxyglucose and 10 mM sodium azide was used to accumulate the Gt-R* complex (Wang et al., 2008). Diffusion coefficients were determined according to Wang et al. (2008) using GraphPad Prism 4. Groups of measurements were compared with two-tailed unpaired t test.

2.6. Western Blotting

Tadpole retinas were homogenized in 5% SDS and boiled for 5 min. After centrifuging at 16 000 g for 5 minutes at 22°C supernatant was collected. The protein concentration was determined by a detergent-compatible method (DC Protein Assay, Bio-Rad, 500–0114). Samples of retinal homogenates after centrifugation (16 000 g, 5 min) were subjected to SDS-PAGE in 10% gels, electrotransferred onto nitrocellulose membranes, and probed with antibodies against EGFP (A-11122, Invitrogen) and rod transducin (K-20, Santa Cruz Biotech) (both at 1:1000 dilution). The antibody-antigen complexes were detected using anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) and ECL reagent (Amersham Pharmacia Biotech.).

3. Results

3.1. Expression, localization, and translocation of EGFP-G α_{t1} and mutants in transgenic rods

The EGFP-fusion protein of human G α_{t1} , and the A3C and G α_{t1} -Cta ς mutants were expressed in rods of transgenic *X. laevis* under control of the *Xenopus* opsin promoter XOP (Mani et al., 2001) (Fig. 1A). The site for insertion of EGFP in the helical domain of G α_{t1} between Met¹¹⁵ and Pro¹¹⁶ was selected based on an earlier report that showed complete functionality of the similarly fused G α_q (Hughes et al., 2001). The intact properties of the EGFP-fusion protein of bovine G α_{t1} with the analogous insertion site have been confirmed (Wang et al., 2008). As it has been shown for other XOP-directed transgenes, G α_{t1} transgene expression varied between retinas from different tadpoles and also between rods within the same retina (Moritz et al., 2001; Wang et al., 2008). Western blot analysis indicated that on average the levels of EGFP-G α_{t1} , A3C, and G α_{t1} -Cta ς were less than 10% of the endogenous frog G α_{t1} (Fig. 1B). Thus, levels of EGFP-G α_{t1} and mutants in the majority of rods were significantly lower than the endogenous G α_{t1} level. EGFP-fluorescence imaging

of the retina of transgenic tadpoles showed that all three EGFP-fusion proteins, $G\alpha_{t1}$, A3C, and $G\alpha_{t1}$ -Cta_s, were properly targeted to the OS in the dark-adapted tadpoles, suggesting intact protein transport (Fig. 2). Following light adaptation of transgenic EGFP- $G\alpha_{t1}$ tadpoles (800 lux, 40 min), a fraction of EGFP- $G\alpha_{t1}$ translocated from the OS to the IS (Fig. 2). A similar degree of translocation ($I_{OS}/I_{IS}=2.3\pm0.2$, $n=7$) of native frog $G\alpha_{t1}$ under these illumination conditions was observed in WT tadpoles using immunofluorescence analysis with $G\alpha_{t1}$ specific antibodies K-20 (not shown), suggesting that the EGFP insert did not alter transducin translocation. In comparison to the frog $G\alpha_{t1}$, light-induced translocation of $G\alpha_{t1}$ in mouse rods is more extensive (Kerov et al., 2005; Lobanova et al., 2007; Rosenzweig et al., 2007). The quantitative difference of $G\alpha_{t1}$ translocation in frog and mouse rods appears to be species-specific.

EGFP- $G\alpha_{t1}$ -Cta_s (hereafter EGFP is omitted) did not translocate from the OS to the IS in response to light (Fig. 2A). This observation is in agreement with the lack of $G\alpha_{t1}$ -Cta_s activation by R^* and the requirement of G_t activation for its translocation (Artemyev, 2008; Calvert et al., 2006; Slepak and Hurley, 2008). In comparison to $G\alpha_{t1}$, the translocation of the $G\alpha_{t1}$ A3C mutant was clearly impaired. Only a very weak EGFP signal was detectable in the IS of the majority of rods after light adaptation (Fig. 2AB). Control immunofluorescence staining of the $G\alpha_{t1}$ A3C transgenic retina demonstrated that light-dependent translocation of endogenous $G\alpha_{t1}$ in rods expressing $G\alpha_{t1}$ A3C was unaffected (Fig. 3). The $G\alpha_{t1}$ A3C phenotype is consistent with palmitoylation of the majority of $G\alpha_{t1}$ A3C molecules. To further examine the extent of palmitoylation of $G\alpha_{t1}$ A3C, we performed analysis of transducin partitioning between the soluble GTP γ S extract and membrane-bound fraction. In control *X. laevis*, the bulk of endogenous frog $G\alpha_{t1}$ and transgenic $G\alpha_{t1}$ was found in the GTP γ S extract with only ~15% of transducin remaining in the membrane fraction (Fig. 4). In contrast, about 50% of $G\alpha_{t1}$ A3C remained in the membrane fraction following extraction with GTP γ S, whereas the membrane-bound fraction of frog $G\alpha_{t1}$ was similar to that in control *X. laevis* (Fig. 4). Since palmitoylated $G\alpha_{t1}$ A3C may partially be extracted from the membrane, the results of the extraction experiments indicate that ~50% or more of the mutant $G\alpha_{t1}$ is palmitoylated.

3.2. Lateral diffusion of $G\alpha_{t1}$ and mutants in transgenic rods

The mobilities of $G\alpha_{t1}$, $G\alpha_{t1}$ A3C, and $G\alpha_t$ - $G\alpha_s$ Ct, in various states of activation were assessed by FRAP (Fig. 5, Table 1). To validate the FRAP protocol, we first measured lateral diffusion of rhodopsin using the transgenic rhodopsin-EGFP *X. laevis* model described previously (Jin et al., 2003). The D value for rhodopsin-EGFP was $0.10 \mu\text{m}^2/\text{s}$ (or $0.28 \mu\text{m}^2/\text{s}$ after the correction for the slowing effect of incisures) (not shown) (Govardovskii et al., 2009; Poo and Cone, 1974). This value is comparable to D values obtained previously for rhodopsin-EGFP with a similar FRAP analysis (Wang et al., 2008) and for native rhodopsin with alternative biophysical techniques (Govardovskii et al., 2009; Poo and Cone, 1974; Wey et al., 1981).

To study diffusion of $G\alpha_{t1}\beta_1\gamma_1$, rhodopsin in transgenic rods was converted to opsin by pre-incubation with 10 mM hydroxylamine similarly as described (Wang et al., 2008). The lateral diffusion coefficient for $G\alpha_{t1}\beta_1\gamma_1$ was $0.105\pm0.007 \mu\text{m}^2/\text{s}$. Diffusion of $G\alpha_{t1}$ GTP monitored using an oxygenated Ringer's buffer containing 10 mM glucose (Wang et al., 2008) was moderately accelerated compared to $G\alpha_{t1}\beta_1\gamma_1$ ($D=0.145\pm0.013 \mu\text{m}^2/\text{s}$) ($p=0.013$). A significant decrease in the mobility of $G\alpha_{t1}\beta_1\gamma_1$ was observed upon its sequestration by R^* under nucleotide-depleting conditions ($0.065\pm0.008 \mu\text{m}^2/\text{s}$) ($p=0.0023$). In comparison to $G\alpha_{t1}$ GTP, the lateral diffusion of the activated $G\alpha_{t1}$ A3C mutant was modestly slower ($p=0.048$) (Table I). The diffusion of the heterotrimeric A3C was not investigated since hydroxylamine promotes hydrolysis of palmitoyl moieties. Interestingly, the kinetics of the lateral diffusion of $G\alpha_{t1}$ -Cta_s were similar under all tested conditions suggesting that $G\alpha_{t1}$ -

Ct α_s was not sequestered or activated by R* (Table I). The calculated immobile fractions of transducin and mutants for lateral diffusion in all of the examined states (G $\alpha_{t1}\beta_1\gamma_1$, G α_{t1} GTP, and G $\alpha_{t1}\beta_1\gamma_1$ /R*) were negligible (<1%).

3.3. Longitudinal diffusion of G α_{t1} and mutants in transgenic rods

The longitudinal diffusion of G α_{t1} , G α_{t1} A3C, and G α_{t1} -G α_s Ct was probed by bleaching a stripe perpendicular to the long axis of the transgenic ROS (Fig. 6). The longitudinal diffusion of G $\alpha_{t1}\beta_1\gamma_1$ was determined to be markedly slower than its lateral diffusion (Table I). Surprisingly, the axial D value for the activated G α_{t1} GTP was analogous to that for the heterotrimeric protein. Sequestration of G $\alpha_{t1}\beta_1\gamma_1$ by R* decreased D for longitudinal diffusion by more than 2-fold ($p=0.0027$). The A3C mutation essentially blocked the longitudinal diffusion of the activated state or the R*-bound transducin (Table I). The interdisc transfer rates of G α_{t1} -Ct α_s were highly variable and not significantly different across the three experimental conditions. The axial diffusion rates of G α_{t1} -Ct α_s were generally comparable to those of G α_{t1} GTP and G $\alpha_{t1}\beta_1\gamma_1$ (Table I).

4. Discussion

Biochemical studies have shown that the activation of G α_{t1} by R* loosens its interaction with the disc membranes and causes solubilization of G α_{t1} GTP *in vitro* (Bigay et al., 1994; Kosloff et al., 2008). Considering the high density of disc membranes in the OS, it remains unclear whether G α_{t1} GTP partitions to the cytosolic fraction *in vivo*. Because the viscosity of the membrane is much greater than the viscosity of cytoplasm, diffusion of a protein with a lipid anchor on a surface of a membrane is dictated by the embedded anchor (Saffman and Delbruck, 1975). If G α_{t1} GTP dissociates from the membrane, its lateral diffusion coefficient would be increased by more than an order of magnitude as suggested by comparison of the diffusion coefficients for transducin and EGFP in the ROS (Wang et al., 2008). If, however, G α_{t1} GTP dissociates from G $\beta_1\gamma_1$ but stays on the membrane, the embedded radius would be reduced by ~2 fold and only a ~10–15% increase in the diffusion coefficient is then predicted from the theoretical calculations of Saffman and Delbruck (1975). Our data show a moderate ~40% increase in the lateral diffusion rate of G α_{t1} GTP in comparison to G $\alpha_{t1}\beta_1\gamma_1$. We interpret these results as an indication that G α_{t1} GTP remains largely associated with the membrane, but its lifetime on the membrane is reduced compared to G $\alpha_{t1}\beta_1\gamma_1$.

The lateral diffusion coefficients of G α_{t1} GTP and the R*-G $\alpha_{t1}\beta_1\gamma_1$ complex obtained in this study are in general agreement with the previous results (Wang et al., 2008). However, the lateral and longitudinal D values of G $\alpha_{t1}\beta_1\gamma_1$ reported by Wang et al. (2008) are ~3.5 and ~10-fold greater, respectively, than the values obtained here. One potentially significant difference between the experimental protocols is that we analyzed only rods that are still attached to small pieces of retina. To test this possibility, we also analyzed diffusion of G $\alpha_{t1}\beta_1\gamma_1$ in dissociated rods. We found that G $\alpha_{t1}\beta_1\gamma_1$ diffuses laterally ~1.5 times faster ($D=0.16\pm0.02\ \mu\text{m}^2/\text{s}$) and longitudinally ~2.5 times faster ($D=0.0095\pm0.0025\ \mu\text{m}^2/\text{s}$) in comparison to the diffusion in attached rods. Apparently, dissociated rods have reduced viability, especially after the treatment with hydroxylamine, and this in part accounts for the discrepancy. The diffusion analysis of Wang et al. (2010) presumes bleaching through the full width of the ROS. In this regard, Calvert et al. (2010) pointed out that with the bleaching at a single focus level, Wang et al. (2010) might have overestimated the diffusion rates due to radial diffusion of unbleached molecules from out-of-focus regions into the scan plane. We imaged the 3-dimensional bleached volume under our experimental settings using EGFP immobilized in 40% polyacrylamide gel (Suppl. Fig. 1). This analysis indicated a relatively uniform bleaching in the axial direction within at least $\pm 3\ \mu\text{m}$ from the focal plane. Thus, the bleached volume spanned the entire width of the rods used in this study.

Generally, the observed diffusion coefficients for $G\alpha_{t1}GTP$ and G_t , corrected for the effect of incisures in frog discs membrane, are lower than previously assumed (Pugh and Lamb, 1993) or theoretically predicted (Saffman and Delbruck, 1975; Wang et al., 2008). This may indicate that transducin diffusion is retarded by protein-protein interactions, macromolecular crowding, and/or association with more viscous lipid microdomains. A recent study indicated that the mobility of a soluble photoactivatable GFP in the frog ROS is much slower than predicted (Calvert et al., 2010). According to the analysis of Pugh and Lamb (1993) (equation 3), the D values for G_t ($0.105 \mu m^2/s$) (Table 1) and R^* ($0.14 \mu m^2/s$) (Wang et al., 2008) would result in the encounter rate of $\sim 900 s^{-1}$ between a single R^* and G_t in frog ROS. Even without the correction for incisures, this rate is well above the actual activation rate of $120\text{--}150 G_t s^{-1}$ per R^* estimated in amphibian rods (Leskov et al., 2000). Thus, the apparently slow diffusion of transducin is sufficient to account for its known R^* -dependent activation rate.

The D values for longitudinal diffusion of G_t and $G\alpha_{t1}GTP$ were 27 and 37 fold smaller than corresponding D for the lateral diffusion. Unexpectedly, the interdisc transfer kinetics of G_t and $G\alpha_{t1}GTP$ were equivalent despite a lower membrane affinity of $G\alpha_{t1}GTP$. It appears that the longitudinal diffusion of G_t and $G\alpha_{t1}GTP$ is limited by tortuosity rather than by the protein dissociation from the discs. The tortuosity factor of the frog OS due to obstruction of axial diffusion by stacked membrane discs is ~ 20 or even ~ 40 if the incisures are inaccessible for diffusion (Calvert et al., 2010). Although the longitudinal diffusion of $G\alpha_{t1}GTP$ is rather slow, it might be adequate to explain the light-dependent translocation of transducin in frogs rods, which appears to be less efficient than in mouse rods. Transducin transport in the dark in the IS $\rightarrow$ OS direction is significantly slower than its light-dependent translocation. In terms of kinetics, this process may well be accounted for by the axial diffusion.

The equivalence of the rates of longitudinal diffusion of G_t and $G\alpha_{t1}GTP$ raises important questions. What determines compartmentalization of G_t to the OS and prevents G_t from leaking out to the IS in the dark? Ciliary proteins such as centrins can potentially block diffusion of G_t in the OS $\rightarrow$ IS direction (Giesl et al., 2006). Another possibility is that G_t is targeted and retained in the OS in the dark due to its affinity for the membranes and/or its interactions with rhodopsin. The $G\alpha_{t1}\text{-}Ct\alpha_s$ mutant transgenic *Xenopus* model was generated to probe the effects of rhodopsin/transducin interactions on the light-dependent compartmentalization and diffusion of transducin. The C-termini of $G\alpha$ subunits are the major interaction sites of heterotrimeric G-proteins with activated GPCRs. The C-terminus of $G\alpha_{t1}$ is a major rhodopsin recognition site that is required for the G_t activation by R^* (Burns and Arshavsky, 2005; Lamb and Pugh, 2006). It could potentially serve as a signal motif for targeting transducin to the OS. The C-terminus of $G\alpha_{t1}$ also weakly interacts with opsin (Scheerer et al., 2008) and may even interact with the ground “dark” state of rhodopsin as suggested by studies using plasmon-waveguide resonance spectroscopy (Alves et al., 2005) and computational analyses (Fanelli and Dell’Orco, 2005). Two main observations have been made using the $G\alpha_{t1}\text{-}Ct\alpha_s$ model. Correct localization of $G\alpha_{t1}\text{-}Ct\alpha_s$ to the OS demonstrates the $G\alpha_{t1}$ C-terminus is not involved in the targeting of G_t (Fig. 2). The failure of $G\alpha_{t1}\text{-}Ct\alpha_s$ to translocate to IS during light adaptation indicates that $G\alpha_{t1}\text{-}Ct\alpha_s\text{-}\beta_1\gamma_1$ is not activated by R^* and confirms that this activation is necessary for G_t translocation to the IS. The analysis of $G\alpha_{t1}\text{-}Ct\alpha_s$ diffusion is consistent with the impaired interaction with R^* . The kinetics of lateral diffusion of $G\alpha_{t1}\text{-}Ct\alpha_s$ were similar under conditions favoring formation of $G\alpha_{t1}\beta_1\gamma_1$, $G\alpha_{t1}GTP$ or the $R^*\text{-}G\alpha_{t1}\beta_1\gamma_1$ complex. Furthermore, the $G\alpha_{t1}\text{-}Ct\alpha_s$ model presents an important control for the analysis of longitudinal diffusion of G_t . It allowed exclusion of transducin activation by opsin as a source of the axial diffusion of G_t . G_t is activated by opsin with 10^{-3} R^* efficiency (Cornwall and Fain, 1994; Melia et al., 1997), but this still may potentially influence the

slow longitudinal FRAP kinetics. Indeed, the D value for the axial diffusion of $G\alpha_{t1}$ - $Ct\alpha_s\beta_1\gamma_1$ was comparable to that of $G\alpha_{t1}\beta_1\gamma_1$, demonstrating the interdisc transfer for the heterotrimer uncoupled from R^* activation.

If longitudinal diffusion of transducin underlies its light-dependent translocation to the IS, a correlation must exist between the two phenomena. Blocking or slowing of the axial diffusion of $G\alpha_{t1}$ would then lead to the block or attenuation of the protein translocation. We sought to manipulate the membrane affinity of $G\alpha_{t1}$ by introducing an artificial palmitoylation site at the N-terminus with the A3C mutation. Several lines of evidence suggest that a dominant fraction of the $G\alpha_{t1}$ A3C mutant in frog rods was palmitoylated. The $G\alpha_{t1}$ A3C mutant was properly targeted to the OS in the dark, but its light-dependent translocation to the IS was markedly impaired (Fig. 2). A large fraction of $G\alpha_{t1}$ A3C remained membrane-bound following extraction of retinal membranes with $GTP\gamma S$. The kinetics of the lateral diffusion of $G\alpha_{t1}$ A3C were slower than those of $G\alpha_{t1}$ with one lipid anchor, and similar to the kinetics of $G\alpha_{t1}\beta_1\gamma_1$ with two lipid modifications. Lastly, the A3C mutation halted the longitudinal diffusion of transducin with immobile fraction exceeding 80%. Apparently, two lipids, N-acyl and S-palmitoyl, cause stable attachment of $G\alpha_{t1}$ A3C to the membrane. The lack of axial diffusion of $G\alpha_{t1}$ A3C differs from the ability of $G\alpha_{t1}\beta_1\gamma_1$ to diffuse longitudinally. We hypothesize that the longitudinal diffusion of $G\alpha_{t1}\beta_1\gamma_1$ in the OS is facilitated by sequestration of one of the two lipid anchors via interactions with phosducin, PrBP/d, UNC119, or another unknown binding partner (Norton et al., 2004; Sokolov et al., 2004; Zhang et al., 2010). Our analysis supports the essential role of the lipid modifications of transducin in the control of the protein mobility and compartmentalization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

G_{t1}	rod G protein transducin
$G\alpha_{t1}$	rod transducin α -subunit
PDE6	photoreceptor phosphodiesterase-6
R^*	photoexcited rhodopsin
FRAP	Fluorescence Recovery After Photobleaching
ROS (OS)	rod outer segment(s)
RIS (IS)	rod inner segment(s)
$GTP\gamma S$	guanosine 5'-O-(3-thiotriphosphate)

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Fig. 1. Expression of EGFP- $G\alpha_{t1}$ and mutants in retinas of transgenic *X. laevis* (A). Map of the transgene. L – linker. The Sal I site was used to linearize the transgene plasmid for transgenesis. (B) A piece of retina of a transgenic tadpole expressing EGFP- $G\alpha_{t1}$. EGFP-fluorescence/DIC overlay. Bar – 20 μ m. (C) Samples of retinal extracts from tadpoles expressing EGFP-fused $G\alpha_{t1}$ (1), $G\alpha_{t1}$ A3C (2), and $G\alpha_{t1}$ -Ct α_s (3) (equivalent to 2 retinas at about stage 50 *X. laevis* tadpole with an average fluorescence intensity in the eye) were immunoblotted with rabbit anti-GFP antibodies (Invitrogen) (left) or with anti- $G\alpha_{t1}$ antibodies K-20 (Santa Cruz Biotech) (right). Arrow indicates frog $G\alpha_{t1}$.

Fig. 2. Localization of $G\alpha_t$, $G\alpha_{t1}A3C$, and $G\alpha_{t1}-Ct\alpha_s$ in dark- and light-adapted rods
(A) EGFP-fluorescence in living rods expressing EGFP-fusion proteins of $G\alpha_t$, $G\alpha_{t1}A3C$, and $G\alpha_t-G\alpha_sCt$. Transgenic tadpoles were dark adapted overnight (D). After dark adaptation, some tadpoles were exposed to light (800 lux/40 min) in a Petri dish (L). Bar -10 μ m. **(B)** Mean fluorescence intensities in the OS (red line) and IS (yellow line) of light-adapted rods were quantified using Z-stack projection images and ImageJ. The ratio of the intensities (I_{OS}/I_{IS}) (mean $\pm$ SE) was determined for control $G\alpha_{t1}$ rods (2 tadpoles, 8 rods) and $G\alpha_{t1}A3C$ rods (2 tadpoles, 8 rods) ($p=0.003$).

Fig. 3. Light-dependent translocation of total transducin in EGFP- $G\alpha_t$ A3C rods
Localization of total $G\alpha_t$ (red, anti- $G\alpha_t$ K-20 immunofluorescence) and EGFP- $G\alpha_t$ A3C (green, EGFP-fluorescence) in the dark (D) and after light exposure (800 lux, 40 min) (L). OS – outer segment, IS – inner segment. Bar -5 μ m.

Fig. 4. Membrane association of Gα_{t1}A3C

Soluble GTPγS-extracted (**S**) and membrane (**M**) fractions were obtained from control EGFP-Gα_{t1} and EGFP-Gα_{t1}A3C tadpoles as described in *Materials and Methods*. The fractions were analyzed by Western blotting with anti-Gα_{t1} antibodies K-20 (Santa Cruz Biotech) (*right*). Open and filled arrows indicate EGFP-fused and frog Gα_{t1}, respectively.

Fig. 5. Representative measurement of lateral diffusion of EGFP-G $\alpha_{t1}\beta_1\gamma_1$

(A). Pre- and post-bleach images of a transgenic ROS at different time points. The retina was pre-incubated in Ringer's buffer containing 10 mM hydroxylamine under ambient light conditions to monitor diffusion of G $\alpha_{t1}\beta_1\gamma_1$. 150 images (256×256 pixels) were collected at 196 msec/frame with no intervals between the scans. (B) Intensity integrated along y-axis for each x-axis pixel of the selected box region from the first post-bleach image. The plot is used to determine the width of a bleach region with a Gaussian profile and the depth of bleach according to the protocol of Wang et al. (2008). (C) Intensities of the bleached region during the experimental time course corrected for background and fading (black) and the fitting curve to a one-dimensional diffusion equation 3 in Wang et al. (2008).

Fig. 6. Representative measurement of longitudinal diffusion of EGFP- $G\alpha_{t1}\beta_1\gamma_1$
(A). Pre- and post-bleach images of a transgenic ROS at different time points. The retina was pre-incubated in Ringer's buffer containing 10 mM hydroxylamine to monitor diffusion of $G\alpha_{t1}\beta_1\gamma_1$. 150 images were collected with a 2-sec interval between the scans. **(B)** Intensities of the bleach region during the experimental time course corrected for background and fading (black), and the fitting curve to one-dimensional diffusion equation 3 in Wang et al. (2008).

Table 1Lateral and longitudinal diffusion coefficients of EGFP- $G\alpha_{t1}$ and its mutants in different activation states

Lateral:			
State	$G\alpha_{t1}$	$G\alpha_{t1}A3C$	$G\alpha_{t1}-Cta_s$
$G\alpha_{t1}GTP$	0.145 ± 0.013 (6) *	0.114 ± 0.008 (11)	0.120 ± 0.017 (8)
$G\alpha_{t1}\beta_1\gamma_1/R$ *	0.065 ± 0.008 (7)	0.076 ± 0.004 (5)	0.125 ± 0.007 (6)
$G\alpha_{t1}\beta_1\gamma_1$	0.105 ± 0.007 (8)	-	0.119 ± 0.018 (6)

Longitudinal:			
State	$G\alpha_{t1}$	$G\alpha_{t1}A3C$	$G\alpha_{t1}-Cta_s$
$G\alpha_{t1}GTP$	0.0039 ± 0.0004 (6)	$<0.001, F>0.8(6)$ **	0.0051 ± 0.0013 (7)
$G\alpha_{t1}\beta_1\gamma_1/R$ *	0.0018 ± 0.0004 (4)	$<0.001, F>0.8$ (5)	0.0039 ± 0.0011 (9)
$G\alpha_{t1}\beta_1\gamma_1$	0.0039 ± 0.0003 (6)	-	0.0046 ± 0.0025 (6)

* mean $\pm$ standard error (N). Data are not adjusted for the effect of incisures

** F - immobile fraction