Isolation and structure–function characterization of a signaling-active rhodopsin–G protein complex

The visual photo-transduction cascade is a prototypical G protein–coupled receptor (GPCR) signaling system, in which light-activated rhodopsin (Rho*) is the GPCR catalyzing the exchange of GDP for GTP on the heterotrimeric G protein transducin (GT). This results in the dissociation of GT into its component α, βγ subunit complex. Structural information for the Rho*/GT complex will be essential for understanding the molecular mechanism of visual photo-transduction. Moreover, it will shed light on how GPCRs selectively couple to and activate their G protein signaling partners. Here, we report on the preparation of a stable detergent-solubilized complex between Rho* and a heterotrimer (GT*) comprising a GαT/Gβγ chimera (αT*) and βγ. The complex was formed on native rod outer segment membranes upon light activation, solubilized in lauryl maltose neopentyl glycol, and purified with a combination of affinity and size-exclusion chromatography. We found that the complex is fully functional and that the stoichiometry of Rho* to GαT is 1:1. The molecular weight of the complex was calculated from small-angle X-ray scattering data and was in good agreement with a model consisting of one Rho* and one GαT. The complex was visualized by negative-stain electron microscopy, which revealed an architecture similar to that of the βγ-adrenergic receptor–Gα complex, including a flexible αT helical domain. The stability and high yield of the purified complex should allow for further efforts toward obtaining a high-resolution structure of this important signaling complex.

G protein–coupled receptors (GPCRs), the largest family of transmembrane proteins, are the targets for nearly 50% of all pharmaceutical drugs (1). These receptors modulate cellular responses to a vast array of extracellular signals through the activation of heterotrimeric G proteins, with the active state being defined as the complex that forms between the agonist-bound (or light-stimulated) GPCR and nucleotide-free G protein (2). Attempts to obtain structural information for GPCRs, and especially for signaling-active GPCR–G protein complexes, have garnered a great deal of interest, both as a means to better understand the underlying mechanisms by which this important family of receptors mediates a wide range of biological outcomes and as a critical step in the design of more selective and effective drug treatments. Recent technological advances, including novel protein engineering (3), in meso crystallization (4), and micro-focus beamlines at synchrotron facilities (5), have ushered in significant progress in the determination of high-resolution GPCR structures. However, only a few of those structures are of activated GPCRs, and thus far, only two GPCR–G protein complexes have been solved, namely that of the β2-adrenergic receptor–Gα protein complex (6) and the calcitonin receptor–Gα protein complex (7). Furthermore, virtually all of these structures, with the exception of rhodopsin, have been obtained with GPCRs that were heavily modified so as to facilitate crystallization. Therefore, to help fully understand the mechanisms of GPCR-mediated G protein activation, it will be important to obtain structural information of active complexes formed between native receptors and their different G protein partners.

Rhodopsin, the photoreceptor responsible for dim light vision, is a prototypical member of the GPCR superfamily. Absorption of a single photon by rhodopsin activates many transducin (GT) molecules (the subunits designated as αT, βγ, and γ) in less than a second, generating GTP-bound αT subunits that activate the effector enzyme, cGMP phosphodiesterase (8). The photo-transduction system offers certain advantages for obtaining structural insights into GPCR signaling, as its principal components can be purified from native tissue in large quantities. As a result, rhodopsin represents the first and only GPCR for which X-ray crystal structures (9–13) have been solved in the native form.

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The determination of X-ray crystal structure of an activated rhodopsin–transducin complex not only is essential to providing a comprehensive picture of photo-transduction, but also for obtaining a better understanding of how the specificity
between GPCRs and G proteins is achieved, as well as establishing the stoichiometry of the GPCR–G protein complex in the signaling-active state. To facilitate future crystallographic studies, here we describe a milligram-scale purification of an active rhodopsin–G protein complex formed with native light-activated rhodopsin (Rho*) and a Gα13/Gβ13 chimera (αT*) together with the retinal βγ subunits (Gγ*). The resulting complex is stable and homogeneous, and the stoichiometry between Rho* and Gγ* is 1:1, as determined by both UV-visible spectroscopy and radiolabeled nucleotide binding. Small angle X-ray scattering (SAXS) studies conducted with the purified Rho*–Gγ* complex further confirm that the complex is monodisperse, and molecular weight values calculated based on SAXS data suggest that the complex is composed of a monomeric Rho* bound to one Gγ* molecule. Negative-stain electron microscopy (EM) analyses of the complex confirm its homogeneity and reveal an overall architecture that is reminiscent of both EM (14) and crystallographic (6) results of the β2-adrenergic receptor–Gs protein complex.

**Results**

**Detergent selection for Rho*–Gγ* complex purification**

To purify the Rho*–Gγ* complex, a suitable detergent is needed not only to extract the complex from its native rod outer segment membrane but also to maintain its stability. The first crystal structures of rhodopsin were solved using receptor solubilized in short-chain detergents, such as nonylglucoside (9), C8E4 (10), and octyl glucoside (11–13). Although these detergents can maintain the stability of dark inactivated rhodopsin for several days, the complex formed with light-activated rhodopsin and transducin cannot survive the extraction process and dissociates quickly upon solubilization. Therefore, we set out to select a detergent in which the Rho*–Gγ* complex would remain stably associated.

A fluorescence assay, monitoring the change in the intrinsic tryptophan fluorescence of αT* upon nucleotide exchange (15), was utilized to examine the rhodopsin-catalyzed nucleotide exchange activity in various detergents. When αT* exchanges GDP for GTPyS, a non-hydrolyzable GTP analog, there is an increase in the intrinsic tryptophan fluorescence due to a conformational change in the switch II region, one of three regions of αT* that change conformation upon GTP binding. Therefore, the rate of Rho*-catalyzed nucleotide exchange can be used to assay the activity of Rho* under various detergent conditions. Commercially available detergents from the maltoside and maltose neopentyl glycol (16) families were tested. Most of these detergents were able to maintain the Rho*-catalyzed nucleotide exchange activity at higher concentrations than their corresponding critical micelle concentration (i.e. 2× CMC), with a rate constant of ~3 min⁻¹ (Fig. 1, A and B). There is a clear dependence of Rho* activity on the length of the detergent hydrophobic chain. However, when using detergent concentrations above or below 2× CMC, the Rho*-stimulated nucleotide exchange activity in most of the tested detergents decreased significantly (Fig. 1C, see dodecyl maltoside), suggesting that these detergents are not well-suited for extracting the complex from rod outer segment (ROS) membranes, for which an initial high detergent concentration (typically around 1% w/v) would be required. Of the various detergents tested, only LMNG (lauryl maltose neopentyl glycol) was able to maintain high Rho*-stimulated nucleotide exchange activity at various concentrations (Fig. 1D) and was therefore selected to be used for Rho*–Gγ* complex purification.

**Purification of the Rho*–Gγ* complex**

The purification scheme for the Rho*–Gγ* complex is illustrated in Fig. 2A. The Coomassie Blue-stained SDS-PAGE profiles for the proteins at each step of the purification scheme are shown in Fig. 2B.

The complex was formed by first mixing αT*, a chimeric protein composed of an αT backbone with a stretch of residues from α13, with βγ1γ1 in a 1:1.1 molar ratio. The αT* subunit was expressed in *Escherichia coli* with an N-terminal His6 tag and purified by nickel-nitritoltriacetic acid chromatography followed by ion-exchange and size-exclusion chromatography. The β1γ1 complex was purified from bovine retina. The resulting Gγ* heterotrimer was then mixed with urea-washed bovine ROS membranes containing native Rho in its dark state. Because of the high density of Rho in ROS, a significant amount of Rho may not be accessible to Gγ* upon light activation. Therefore, a substantial excess of Rho was used to assemble the Rho*–Gγ* complex (typically seven Rho per Gγ*). The mixture was subjected to illumination at 4 °C, resulting in the light activation of Rho* and the induction of complex formation on the native ROS membranes. The mixture was then centrifuged, and as a result, the Rho*–Gγ* complex and excess Rho* were in the pellet, whereas the GDP released during Gγ* activation, together with the unbound αT* subunit, remained in the supernatant.

The pellet was solubilized with buffer containing the detergent LMNG and applied to a nickel-Sepharose column. Because of the His6 tag present at the N terminus of αT*, the Rho*–Gγ* complex remained bound to the column and was thus separated from free Rho*. The Rho*–Gγ* complex was eluted from the column with imidazole and further purified by size-exclusion chromatography. The Rho*–Gγ* complex eluted as a single symmetrical peak (Fig. 3A). The yield of a typical purification process was about 80% based on the amount of β1γ1 complex used.

**Stoichiometry, activity, and stability of the Rho*–Gγ* complex**

Bovine Rho has been shown to form arrays of dimers in ROS membranes (17), and there have been questions raised regarding whether the stoichiometry between Rho* and Gγ* in the signaling complex is 1:1 or 2:1. The UV-visible spectrum of the purified complex reveals two peaks at 280 and 380 nm (Fig. 3B), corresponding to the absorbance of the protein moiety, and that of the unprotonated all-trans-retinal in the meta-II Rho* state, respectively. The A280 nm/A380 nm ratio is 3.7, which agrees with a 1:1 stoichiometry based on the reported extinction coefficients for the protein components (18–20) (a 2:1 stoichiometry would result in a A280 nm/A380 nm ratio of 2.58).

Additionally, the stoichiometry between Rho* and Gγ* was determined with a radio-nucleotide filter binding assay, in which the purified Rho*–Gγ* complex was incubated with
[35S]GTPγS and then applied to a nitrocellulose filter. The amount of Gγ* present in the complex was estimated based on its nucleotide binding capacity, i.e. the amount of bound [35S]GTPγS, and was compared with the amount of Rho* calculated from the 380-nm absorbance. The results showed that for 1 mol of Rho*, there was 0.784 ± 0.003 mol of GTPγS present, supporting the idea of a 1:1 Rho*–GT* complex (Fig. 3B).

The purified Rho*–Gγ complex was fully active as it dissociated upon the addition of GTPγS, a non-hydrolysable analog of
GTP (Fig. 3A). The complex was stable at 4 °C in the dark for at least 7 days, as it remained nearly completely intact with less than 5% dissociation (Fig. 3D).

**SAXS analysis of Rho*-G* complex**

To further study the overall conformation of the active state of the Rho*-G* complex, SAXS data were collected over a concentration range of the purified complex, with corrections for the background scattering being obtained by subtracting the scattering due to the SEC buffer from the scattering profiles of the Rho*-G* samples (Fig. 4A). The Guinier regions ($q^2 R_g^2$ < 1.3) of these curves are linear (Fig. 4B), confirming that the Rho*-G* complex is monodisperse. All the parameters calculated from the SAXS scattering profiles are summarized in Fig. 4C. The radius of gyration ($R_g$) values for the Rho*-G* complex calculated from the Guinier plots range from 41.7 to 43.9 Å, which agree well with those calculated from pair distribution functions (P(r)) using GNOM (21) (41.9 – 43.9 Å). These results demonstrate that the value for $R_g$ is concentration-independent. The molecular mass of the Rho*-G* complex was calculated using both the particle mass determination method with ScÅtter software developed by Rambo et al. (22) and SAXS molecular mass developed by Fischer et al. (23). The results from ScÅtter (129.6 – 143.8 kDa) and SAXS molecular mass (127.4 – 128.9 kDa) agree well with each other and confirm that the complex is composed of 1 Rho* and 1 G*, which gives a molecular mass value of 125 kDa. The P(r) curve (Fig. 4D) calculated from GNOM is skewed to the right, indicating an elongated overall particle shape. The cross-sectional $R_c$ values calculated using ScÅtter software range from 32.5 to 34.9 Å, which are about 9 Å smaller than the $R_g$ values, confirming the existence of an elongated shape for the Rho*-G* complex.

**SAXS-based modeling of Rho*-G* complex**

The P(r) curve ($D_{max} = 128$ Å) calculated from the scattering profile for the 1 mg/ml Rho*-G* sample was used for generating ab initio models of the complex. Two different algorithms, DAMMIN (24) and GASBOR (25), were used. The resulting envelopes obtained using each method agree well with each other. Both revealed a small bulge protruding out from the bottom half of the envelopes, indicating the position of the helical domain of $\alpha*$ (Fig. 5, A and B). A structural model of the Rho*-G* complex, using the crystal structure of the $\beta_2$AR–G*
complex (PDB 3SN6) as a template, was generated to fit the envelopes. There is significant discrepancy between the structural model and ab initio envelopes at the Rho* region, which can be attributed to the existence of a detergent micelle around the receptor in solution, and as a result, the model fits poorly to the experimental data with $\chi^2 = 24.2$. To improve the quality of

**Figure 3. Stoichiometry determination and SEC profiles of the purified Rho*–GT* complex.** A, SEC profiles of the purified Rho*–GT* complex (red) and its dissociation upon the addition of GTP$\gamma$S (green). (This is representative of more than 10 similar sets of SEC profiles.) B, UV-visible spectrum of purified complex. (This is representative of more than 20 similar spectra of the purified complex.) C, ratio of Rho* to Gt* in the Rho*–GT* complex was determined by a $[^{35}\text{S}]$GTP$\gamma$S binding assay and the extinction coefficient for the chromophore retinal in Rho*. (This experiment was repeated three times.) D, SEC profiles of the Rho*–GT* complex after 1 day (blue) and 7 days (orange). (This is representative of more than 20 similar sets of SEC profiles.)

**Figure 4. SAXS data and analyses of the Rho*–Gt* complex.** A, SAXS scattering profiles from protein concentrations of 1, 0.5, and 0.25 mg/ml (colored cyan, purple, and red, respectively). (Each curve shown is the average of 10 scattering curves.) B, Guinier plots for q$R_g^2$ region of the scattering curve. C, SAXS parameters and calculation results. D, pair distribution function calculated from 1 mg/ml scattering profile.
the structural model, a detergent corona shaped as an elliptical torus was built around Rho* using the Memprot program developed by Pérez and Koutsioubas (26). The resulting micelle model is composed of 78 LMNG detergent tails and 74 detergent heads, and its dimensions are shown in Fig. 5C. After incorporating the detergent corona into the complex model, the $\chi^2$ value decreased to 3.27, indicating a significant improvement of fitness to the experimental data. In addition, the position of the helical domain was further optimized with the program CORAL (26), and the $\chi^2$ value of the model improved to 1.83 (Fig. 5, D and E). The helical domain position in the resulting model resides between the close and open positions as indicated from X-ray crystal structures (PDB 1GOT and 3SN6, respectively) (Fig. 5E).

**Electron microscopy characterization of negatively stained Rho*–G$_T^*$ complex**

EM visualization of the negatively stained complex samples showed a monodisperse particle population (Fig. 6A). Reference-free alignment and classification of particle projections revealed class averages with an overall density similar to that of the $\beta_2$-adrenergic receptor–G$_\text{S}$ protein complex ($\beta_2$AR-G$_\text{S}$) (Fig. 6B) (14). A central oval density represents Rho* in a detergent micelle with a small protruding density often observed on top corresponding to the N terminus of Rho*. At the bottom of Rho*, two major densities representing the G$_T^*$ heterotrimer are clearly visible. One of the two densities has extensive contact with the receptor density and shows an additional small globular density in various orientations in several class average images. This is similar to what was observed for the $\beta_2$AR–G$_\text{S}$ complex (14) and represents the Ras-like domain of the $\alpha_1^*$ subunit, with a flexible helical domain in the Rho*–G$_T^*$ complex. The other density therefore corresponds to $\beta_1^*\gamma_1$.

**Discussion**

Structural information obtained from a variety of approaches will be invaluable in shedding light on how the photoreceptor Rho engages and activates its signaling partner, the G protein G$_T$. Such analyses will also provide insights with broad rele-
vance toward understanding the underlying mechanisms by which GPCRs demonstrate remarkable specificity for their G protein targets, as well as whether different GPCRs use common or distinct mechanisms in catalyzing the G protein activation event. Recently, there have been several reports of the purification of detergent-solubilized Rho*-G T complexes, in which either native (28, 29) or recombinant Rho (30, 31) was utilized. Our approach toward obtaining an active Rho*-G T complex differs from these previous efforts in two major aspects. First, rather than detergent-solubilizing and purifying Rho prior to allowing it to associate with a purified G protein, the Rho*-G T protein complex was obtained by directly light-activating the urea-washed ROS membranes in the presence of the heterotrimeric and chimeric G protein G T. This allowed the formation of a Rho*-G T complex within a native membrane environment. The subsequent pelleting step readily removed the released GDP, which may otherwise destabilize the complex. Second, instead of using either the glycan linkages or the C terminus of Rho as purification handles, we utilized the N-terminal His tag on the recombinant α1 subunit to achieve a simple and efficient one-step purification of the detergent-solubilized Rho*-G T complex. The resulting complex dissociated with the addition of GTPγS, demonstrating that the complex isolated in this manner is fully active.

Near-infrared light-scattering studies of ROS membranes in the early 1980s by Kuhn et al. (32) suggested a 1:1 stoichiometric association of G T with Rho*. However, atomic force microscopy images of ROS membranes revealed that Rho exists in the form of dimeric arrays in its native environment (17). Recent studies using native rhodopsin solubilized from ROS with detergent for complex formation (28, 29) argued for the existence of a pentameric complex consisting of two Rho* molecules and one G T heterotrimer as the minimal functioning unit. While in these settings, it may appear that Rho* can form dimers, the important question is what stoichiometry of Rho* and G T is required to achieve full activation. Based on our studies of a signaling-active Rho*-G T* complex, as analyzed by UV-visible spectroscopy and radioactivenucleotide binding, as well as by SAXS and EM, we conclude that the minimal unit necessary for full activation is 1 Rho* and 1 G protein. This conclusion is supported by recent studies using rhodopsin-embedded nanodiscs, which have shown that one Rho* is sufficient for coupling to and activating G T (33–34). Moreover, based on nanodisc reconstitution, monomeric rhodopsin has been shown to be sufficient for phosphorylation by G protein–coupled receptor kinase and interaction with arrestin (35–36). The recent X-ray structure of a rhodopsin–arrestin complex (37) further illustrated that rhodopsin binds arrestin in a 1:1 stoichiometry. Similarly, the β2AR has been demonstrated to interact with G s in a monomeric manner (6, 14, 38). Therefore, several lines of evidence now point to class A GPCR monomers being sufficient for promoting their downstream signals.

In addition to the limited stoichiometry necessary to achieve a signaling-active complex, another question of interest is the positioning of the helical domain of the Gα subunit in the receptor–G protein complex. The first high-resolution structure of a G protein α subunit, for GTPγS-bound α1 (39), revealed that the nucleotide is buried inside a cleft formed by the Ras-like domain and helical domain, and it was postulated that an activated receptor must induce an opening of the cleft to allow for nucleotide exchange. The first direct evidence of this inter-domain opening was provided by double electron-electron resonance experiments (40), in which distance increases as large as 20 Å between the Ras-like and helical domains of the Giα subunit were observed upon binding to light-activated ROS. The crystal structure of the β2AR–Gs complex (6) shows a very dramatic 127° rotation of the G s α helical domain, which results in the opening of the nucleotide-binding pocket. Our SAXS model of the Rho*-G T complex shows that the helical domain of α1* adopts a similar open position in solution. However, the opening is not as dramatic as displayed in the β2AR–Gs complex crystal structure. Negative-stain EM studies of the β2AR–Gs complex (14) reveal the flexible positioning of the Gs α helical domain and suggest an ensemble of open helical domain positions that exist under physiological conditions. Because a similarly flexible helical domain is also observed in the negative-stain EM images of the Rho*-G T complex, it is most likely that a displacement of the helical domain is a universal mechanism of receptor-mediated nucleotide exchange for different families of heterotrimeric G proteins.

In conclusion, here we describe procedures for isolating a Rho*-G protein complex with a very high yield that is amenable to a variety of types of biochemical and structural analyses. Given the availability of a number of interesting mutants of α1*, which mimic different stages in the activation event, we should soon be in a position to address fundamentally important questions regarding how different members of the GPCR family engage their G protein signaling partners and induce the necessary structural changes to drive G protein-mediated signal propagation.

Experimental procedures

Materials

Frozen dark-adapted bovine retinai (V. N. O. Cover, 10 minutes) were purchased from the W. L. Lawson Co. (Lincoln, NE). Detergents were from Antrace, and all other chemicals were obtained from Sigma.

Purification of retinal proteins

Urea-washed rod outer segment (UROS) membranes were isolated as described (41), flash-frozen, and stored at −80 °C in HMN buffer (20 mM HEPES, pH 7.5, 2 mM MgCl2, 100 mM NaCl, 1 mM DTT) at a concentration of 300 μM. UROS membranes were used as the source for Rho* in Rho*-G T complex formation. The β1 γ1 subunit complex was purified essentially as described previously (42). Bovine retinai were exposed to light and subjected to sucrose gradient ultracentrifugation to prepare purified ROS membranes. After a series of isotonic and hypotonic washes, 100 μM GTP was added to release G T subunits from the membrane. β1 γ1 was separated from α1 through a 5-ml HiTrap Blue HP (GE Healthcare) column, and the resulting β1 γ1 complex was further purified by anion-exchange chromatography through a 5-ml HiTrap Q HP (GE Healthcare) column, using Buffer A (20 mM HEPES, pH 7.5, 2 mM MgCl2, 1 mM DTT, 10% glycerol) and Buffer B (Buffer A + 1 M NaCl) to form the gradient. The β1 γ1 complex typically elutes at 100 mM NaCl.
and was concentrated to 20 μM, flash-frozen, and stored at −80 °C.

Expression and purification of αT*

An αT*/αT chimera construct designated as pHis,Chi8 was obtained from Dr. Heidi Hamm (Vanderbilt University) (43), in which αT* residues 215–295 were replaced with the corresponding residues from αT, and a His6 tag was introduced at the N terminus. In addition, residues 244 and 247 were changed back to the original amino acids in αT*, resulting in the αT* construct. The αT* subunit can undergo Rho*-catalyzed nucleotide exchange and activate the effector enzyme, phosphodiesterase, in a similar manner as retinal αT*. αT* was expressed in BL21(DE3)-competent cells and purified as described previously (18). The protein was concentrated to 20 μM in HMN buffer with the addition of 10% glycerol, flash-frozen, and stored at −80 °C.

Detergent selection for Rho*–GT* complex purification

Fluorescence measurements were carried out with a Varian eclipse spectrofluorimeter. UROsS light-activated (Rho*) by incubation on ice under ambient light for 5 min. Rho*-catalyzed nucleotide exchange and activate the effector enzyme, phosphodiesterase, in a similar manner as retinal αT*. αT* was expressed in BL21(DE3)-competent cells and purified as described previously (18). The protein was concentrated to 20 μM in HMN buffer with the addition of 10% glycerol, flash-frozen, and stored at −80 °C.

Equation 1

\[ F = F_\infty - (F_\infty - F_0) \cdot e^{-k_{obs} \cdot t} \]

where \( F \) is the fluorescence signal at any time \( t \); \( F_0 \) is the fluorescence signal at time \( t = 0 \); \( F_\infty \) is the fluorescence signal at time \( t = \infty \); and \( k_{obs} \) is the observed rate constant.

Rhodopsin–Gαt* complex formation and purification

The G protein heterotrimer Gαt* was formed by mixing 22 nmol of αT* with 20 nmol of βTt γt and then incubated on ice for 5 min. The Rho*–Gαt* complex was formed on ROS membranes by mixing Gαt* with UROsSs containing 140 nmol of Rho and illuminating the mixture under a halogen lamp covered with a UV-absorbing glass and a 495-nm long-pass filter at 4 °C for 30 min. The suspension was aliquoted into 1.5-ml Eppendorf tubes and centrifuged at 14,000 × g for 30 min. From here on, all subsequent steps were carried out in the dark under dim red light. The supernatant was discarded, and the pellets containing the Rho*–Gαt* complex and excess Rho* were resuspended in 3 ml of HMN buffer + 1% (w/v) LMNG. The mixture was incubated at 4 °C with rocking for 1 h to allow for complete solubilization. HMN buffer (12 ml) was then added to lower the LMNG concentration to 0.2%, and the sample was further incubated at 4 °C with rocking for 1 h. Solubilized complex was loaded onto a 1-ml HisTrap FF column (GE Healthcare) pre-equilibrated with HMN buffer + 0.003% LMNG. The Rho*–Gαt* complex was eluted from the column with an imidazole gradient in HMN buffer + 0.003% LMNG, as a single peak at ~100 nm imidazole. Peak fractions were pooled and concentrated with an Amicon Ultra-0.5 100-kDa MWCO concentrator (EMD Millipore) to 500 μl and injected onto a Superdex 200 10/300 GL column (GE Healthcare), pre-equilibrated with HMN buffer + 0.003% LMNG. Peak fractions were pooled and concentrated with an Amicon Ultra-0.5 100-kDa MWCO concentrator (EMD Millipore) to 50 μl, resulting in a complex concentration of ~40 mg/ml.

Determination of the stoichiometry of the Rho*-Gαt* complex

The stoichiometry of Rho* and Gαt* within the complex was determined using two methods, UV-visible absorption spectroscopy and a [35S]GTPγS binding assay. For stoichiometry determinations using UV-visible spectroscopy, a 100-μl sample of the purified Rho*–Gαt* complex was examined in a quartz cuvette with a 1 cm path length, using a Beckman DU600 UV-visible spectrophotometer scanning from 240 to 700 nm at 240 nm/min. The ratio for Rho* to Gαt* within the complex was determined from the \( A_{280\text{ nm}}/A_{380\text{ nm}} \) ratio, using the following extinction coefficients: Rho* (\( \epsilon_{280\text{ nm}} = 61,800\ M^{-1} \cdot cm^{-1} \)); \( \epsilon_{380\text{ nm}} = 42,000\ M^{-1} \cdot cm^{-1} \)). The extinction coefficients for Gαt* and βTt γt were calculated based on protein sequence using the ExPASy ProParam tool (44). Therefore, a 1:1 Rho*–Gαt* stoichiometry would result in an \( A_{280\text{ nm}}/A_{380\text{ nm}} \) value of 3.69, whereas a 2:1 Rho*–Gαt* stoichiometry would yield a \( A_{280\text{ nm}}/A_{380\text{ nm}} \) value of 2.58.

For stoichiometry determinations using a [35S]GTPγS binding assay, purified Rho*–Gαt* complex was incubated with 50 μM [35S]GTPγS for 10 min on ice in 20 μl of HMN buffer + 0.003% LMNG. The solution was then applied to prewet nitrocellulose filters (Schleicher & Schuell, pore size 0.45 μm) on a suction manifold. The filters were washed twice with HMN buffer, added to scintillation liquid (30% LSC Scintisafe Mixture), and counted in a scintillation counter (LS6500 multipurpose scintillation counter). The calculated amount of bound [35S]GTPγS was used as an estimate for the amount of Gαt* in the complex and was compared with the amount of Rho* calculated from \( A_{380\text{ nm}} \) using the extinction coefficient of Meta II rhodopsin, \( \epsilon_{380\text{ nm}} = 42,000\ M^{-1} \cdot cm^{-1} \).

SAXS data collection and processing

SAXS data were collected at the G1 station of the Cornell High Energy Synchrotron Source (CHESS). G1 operated with an energy of 9.86 keV and provided a flux of \( 3 \times 10^{11} \) photons/s for a 250 × 250-μm beam. Purified Rho*–Gαt* complex was eluted through a Superdex 200 5/150 GL column (GE Healthcare), pre-equilibrated with HMN buffer without detergent, immediately prior to the collection of SAXS data. The center peak fraction was collected, and a concentration series, 0.25, 0.5, and 1 mg/ml, was prepared in HMN buffer and kept on ice. Samples were centrifuged at 14,000 × g for 10 min before being loaded into the SAXS sample exposure window. Samples were exposed for 30 s with oscillation. Ten datasets were collected for each sample for possible radiation damage detection, and undamaged exposures were averaged. Buffer measurements with HMN Buffer were conducted in-between each sample measurement. Data reduction and background subtraction were
SAXS-based modeling of the Rho*–G* complex

Ab initio models were calculated using two different programs, DAMMIN (24) and GASBOR (25). In DAMMIN calculations, 17 envelopes were generated (average $\chi^2 = 0.90$), and the results were averaged using DAMAVER (46) to produce the averaged and filtered envelopes. None of the 17 DAMMIN envelopes was rejected during the DAMAVER calculation, and the mean normalized special discrepancy value was $0.482 \pm 0.053$, indicating the model is of good quality. In addition to DAMMIN, GASBOR calculations were performed to fit the intensity in reciprocal space. The resulting envelope fits well to the experimental curve with $\chi^2 = 0.895$. A structural model was built using the X-ray crystal structure of the $\beta_2$AR–G* complex (PDB code 3SN6) as a template. In this model, the X-ray crystal structures of meta-rhodopsin II (PDB code 3PXO), and the $\beta_1\gamma_1$ complex from the $G_\text{T}$ heterotrimer (PDB code 1GOT), were used for Rho* and $\beta_1\gamma_1$, respectively. A homology model of the $\alpha_1^*$ subunit was built based on the $\alpha_2$ subunit from the X-ray crystal structure of the $\beta_2$AR–G* complex (PDB code 3SN6) using the SWISS-MODEL server (44). The program Memprot (26) was used to model the LMNG detergent micelle around Rho*, in which a coarse-grained fitting algorithm was used to add detergent molecules by assuming an elliptical model for the detergent corona. Electron density values of 0.28 and 0.52 $\text{e}^{-}\text{Å}^{-3}$ were used for the hydrophobic and hydrophilic portions of the LMNG detergent molecule, respectively. The position of the helical domain (residues 57–176 in $\alpha_1^*$) was optimized using the program CORAL (27), in which the linker regions (residues 51–56 and 177–181) connecting the helical domain to the GTPase domain were set to be random flexible loops. The resulting structural model was aligned with the envelopes using the SUPCOMB program (47).

Specimen preparation and EM imaging of negative-stained samples

Purified Rho*–G* complex was prepared for electron microscopy using the conventional negative-staining protocol (48) and imaged at room temperature with a Tecnai T12 electron microscope operated at 120 kV, using low-dose procedures. Images were recorded at a magnification of $\times 71,138$ and a defocus value of $\sim 1.5 \mu\text{m}$ on a Gatan US4000 CCD camera.

Two-dimensional classifications of the Rho*–G* complex

All images were binned (2 × 2 pixels) to obtain a pixel size of 4.16 Å on the specimen level. Particles were manually excised using e2boxer (49) (part of the EMAN2 software suite). Two-dimensional reference-free alignment and classification of particle projections were performed using ISAC (50). A total of 9303 projections of Rho*–G* were subjected to ISAC, producing 134 classes consistent over two-way matching and accounting for 6774 particle projections.

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Isolation and structure–function characterization of a signaling-active rhodopsin–G protein complex
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