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RHODOPSIN-TRANSDUCIN HETEROPENTAMER: THREE-DIMENSIONAL STRUCTURE AND BIOCHEMICAL CHARACTERIZATION

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Abstract

The process of vision is initiated when the G protein-coupled receptor, rhodopsin (Rho), absorbs a photon and transitions to its activated Rho* form. Rho* binds the heterotrimeric G protein, transducin (G_t) inducing GDP to GTP exchange and G_t dissociation. Using nucleotide depletion and affinity chromatography, we trapped and purified the resulting nucleotide-free Rho*•G_t complex. Quantitative SDS-PAGE suggested a 2:1 molar ratio of Rho* to G_t in the complex and its mass determined by scanning transmission electron microscopy was 221±12 kDa. A 21.6Å structure then was calculated from projections of negatively stained Rho*•G_t complexes. The molecular envelope thus determined accommodated two Rho molecules together with one G_t heterotrimer, corroborating the heteropentameric structure of the Rho*•G_t complex.

Keywords

G protein-coupled receptor; heterotrimeric G protein; photoactivated rhodopsin; scanning transmission electron microscopy; transducin

Introduction

A wealth of structural, biophysical and biochemical data about its function makes rhodopsin (Rho) a paradigm for understanding G protein-coupled receptors (GPCRs) and their activation. GPCRs, essential proteins for signal transduction across cellular membranes, are involved in most physiological processes. Rho, the primary molecule in the rod visual signaling cascade, is activated by a single photon that isomerizes its bound inverse agonist, 11-*cis*-retinal, to its all-*trans*, full agonist state. This leads to formation of the Rho* activated state, which catalyzes nucleotide exchange on multiple transducin (G_t) heterotrimers,

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providing an initial signal amplification (Hofmann et al., 2009; Palczewski, 2006; Sakmar et al., 2002). The first crystal structure determined for a GPCR was that of Rho (Palczewski et al., 2000), and Rho crystals have yielded the highest resolution and only native structure of any GPCR determined to date (Okada et al., 2004). The structure of Rho* has been solved (Salom et al., 2006), the interaction site between Rho and the G_t C-terminus structurally defined (Choe et al., 2011; Scheerer et al., 2008; Standfuss et al., 2011), and the G_{tα} activation by Rho* characterized by site-directed spin labeling and spectroscopy (Van Eps et al., 2011). However, the most complete insight into the interactions between a GPCR and its cognate G protein is provided by the recent structure of the human β₂ adrenergic receptor•G_s protein complex (Rasmussen et al., 2011). Despite this progress, the molecular mechanisms by which receptor-G protein activation occurs are still not fully understood (Han et al., 2009; Jastrzebska et al., 2011).

A long-standing question concerns the stoichiometry of the fully active receptor•G protein complex. Near-infrared light scattering experiments initially indicated a stoichiometry of 1:1 for an active Rho*•G_t complex (Kuhn et al., 1981), but subsequent studies on the light-dependent binding of G_t to Rho* suggested that two or even four receptors were needed for G_t coupling (Wessling-Resnick and Johnson, 1987). Extensive biophysical and biochemical evidence suggests the existence of GPCR dimers in living cells (Angers et al., 2002). Recent surface plasmon resonance measurements and a holistic systems modeling approach indicate that prevalently dimeric Rho and Gt form transient complexes (Dell'orco and Koch, 2011). These authors conclude that Rho molecules and Rho-Gt complexes can both absorb photons and trigger the visual cascade. Imaging of native rod outer segment (ROS) membranes by atomic force microscopy (AFM) revealed Rho to be organized in rows of parallel dimers (Fotiadis et al., 2003). Considering the native dimeric state of Rho, the surfaces of Rho and G_t, and the regions of both proteins postulated to be involved in complex formation, a Rho*-Rho dimer was proposed to be the functional unit required for G_t activation (Liang et al., 2003). Although activation of a single Rho suffices to trigger a physiological response (Bayburt et al., 2007; Baylor et al., 1979; Whorton, et al. 2008), these *in vitro* findings do not provide information about the actual stoichiometry of the Rho*•G_t complex in its native membrane.

The most compelling evidence for a dimer in GPCR-mediated signaling comes from *in vivo* studies of the luteinizing hormone receptor (LHR), which demonstrated intermolecular cooperation between ligand-binding deficient and G protein activation deficient mutants (Rivero-Muller et al., 2010). Functional complementation assays showed that the minimal signaling unit consists of two class A and a single G protein (Han et al., 2009), and dimerization of the β₂-adrenergic receptor was found to be obligatory for cell surface targeting (Salahpour et al., 2004). Furthermore, studies probing the lateral diffusion of Rho in photoreceptor membranes (Govardovskii et al., 2009), as well as Monte Carlo simulations of Rho* and G_t in disc membranes support the binding of one G_t to at least two GPCRs and suggest that the high density packing of Rho into dimers and higher order oligomers provides a kinetic advantage that enables a rapid response to light (Dell'Orco and Schmidt, 2008).

To provide new information about the most native system available, we have purified the Rho*•G_t complex in the absence of GTP, and determined its stoichiometry by biochemical methods as well as its mass by scanning transmission electron microscopy (STEM). This complex has a mass of ~221 kDa, which is consistent with the biochemically measured stoichiometry of 2 Rho molecules (39 kDa) and one G_t heterotrimer (86 kDa) per complex, taking 1-2 detergent micelles into account. Model building demonstrates that the three-dimensional (3D) molecular envelope of negatively stained complexes accommodates one

Rho dimer and one G_t heterotrimer, corroborating biochemical analyses and STEM mass measurements.

Results and Discussion

Purification and characterization of $Rho^*\bullet G_t$

Solubilized Rho was purified as described in Supplementary Information, bound to succinylated Concanavalin (sConA) affinity resin, photoactivated, and saturated with G_t . The reconstituted $Rho^*\bullet G_t$ complex then was eluted with α -methyl-mannoside (Fig. 1A, middle panel). No complex formation was observed without illumination (Fig. 1A, upper panel) indicating that activated Rho was required for G_t binding. Moreover, the $Rho^*\bullet G_t$ complex formed on the sConA affinity column was biochemically active, because G_t dissociated from Rho^* when 200 μ M GTP γ S was applied to the column (Fig. 1A, bottom panel). Reverse phase HPLC confirmed that the purified complex was in the nucleotide-free state (Fig. S1A), and its UV-visible spectrum exhibited an absorbance maximum at 380 nm, characteristic of the Meta II activated state (Fig. S1B). Under acidic conditions the deprotonated Schiff base of the Meta II photoproduct became protonated, shifting its absorption peak to 440 nm (Fig. S1B), as would be expected for Meta II Rho. Retinoid analysis showed that the major isomerization state of chromophore in the complex was all-*trans*-retinal, with a minimal amount of dark state (11-*cis*-retinal) chromophore (Fig. S1C).

As previously noted, the type of detergent and quantity used to reconstitute the $Rho^*\bullet G_t$ complex have a significant influence on complex stability (Jastrzebska et al., 2009a). Only alkyl maltosides, including n-tetradecyl- β -D-maltoside and n-dodecyl- β -D-maltoside (DDM), allowed purification of the isolated complex; non-maltoside detergents either dissociated the complex or inhibited its formation. sConA affinity chromatography allowed lipids to be removed without compromising reconstitution of the active complex on the column. Lipid removal was advantageous, as excess lipids characteristic for the sucrose density gradient purification protocol described previously increased sample heterogeneity (Jastrzebska et al., 2009b). Moreover, sConA affinity chromatography allowed the DDM concentration to be lowered to 0.5 mM, which sufficed to prevent Rho precipitation while maintaining the native Rho dimer conformation (Suda et al., 2004).

Stoichiometry and mass of the $Rho^*\bullet G_t$ complex

Two different protein in-gel staining techniques allowed the ratio between Rho^* and G_t in the purified $Rho^*\bullet G_t$ complex to be measured (see Fig. S2 for the calibration procedure). Densitometric analysis of Sypro-Ruby stained SDS-gels from three independent purifications is displayed in Fig. 1B and yielded a ratio of 2:1 $Rho^*:G_t$, consistent with the stoichiometry of sucrose gradient purified $Rho^*\bullet G_t$ complexes reported previously (Jastrzebska et al., 2009b).

Unstained, freeze-dried $Rho^*\bullet G_t$ complexes were imaged by low-dose dark-field STEM (Fig. 2A, and mass values calculated from their electron scattering power were assembled in a histogram (Fig. 2B). The major particle population had a mass of 221 ± 12 kDa, whereas secondary peaks represented multiples of this value (Table 1). This result agrees best with a 2:1 Rho (39 kDa) : G_t (86 kDa) absolute stoichiometry because the expected heteropentamer mass is ~210-240 kDa, depending on the amount of bound detergent. To obtain particle dimensions, average projections were calculated for particles from the major population having a mass of ~220 kDa; class averages revealed elongated particles that are about 130 Å long and 90 Å wide (inset Fig. 2A).

Size exclusion chromatography revealed two major peaks: a ~220 kDa peak, corresponding to the purified $Rho^*\bullet G_t$ complex (peak #1), and an ~80 kDa peak (peak #2) corresponding to

free G_t subunits (Fig. S3A). Purified DDM-solubilized Rho as well as the DDM-G_t complex eluted at ~140 kDa in control experiments (Fig. S3B). Size exclusion chromatography not only supports the 2:1 Rho:G_t stoichiometry but also demonstrates the fragility of this complex.

Visualization of Rho*•G_t complexes by TEM

Negatively stained preparations of crosslinked Rho*•G_t exhibited two major particle populations (Fig. 3). To obtain a statistically relevant estimate of these populations, the auto-boxing feature of EMAN2 was employed to select and visualize a large number of particles. The larger, elongated particles accounted for 32% of all particles on the grid with lengths of 130 ± 6 Å ($n=60$) (Table 2) and variable widths. Such particles most likely represent the Rho*•G_t complex. The smaller particles had diameters of 82 ± 6 Å ($n=60$), sizes characteristic of either Rho dimers solubilized in DDM (Suda et al., 2004) or heterotrimeric G_t (Jastrzebska et al., 2009a).

Because gel filtration chromatography (Fig. S3A) suggested that the complex dissociates during purification, crosslinked samples were separated by size exclusion chromatography just prior to grid preparation. Again, two size populations were observed, but in this case the elongated particles were the major species (72%, Table 2).

Negatively stained preparations of uncrosslinked Rho*•G_t revealed the same elongated complexes (length= 131 ± 6 Å; Table 2) found predominately in the crosslinked preparation after size exclusion chromatography. The percentage of larger particles was similar to that calculated for crosslinked Rho*•G_t preparations before sizing chromatography (25%; Table 2). These results suggest that neither adsorption to the carbon film nor staining with uranyl acetate markedly promoted complex dissociation, but that cross-linking is required to augment the fraction of intact Rho*•G_t complexes.

3D structure of the Rho*•G_t complex

Structural variability and possible dissociation of the Rho*•G_t complex not only constituted a challenge for particle selection but also required a large particle pool to obtain class averages suitable for calculating an unbiased initial model. We therefore established an initial 3D map from projections of crosslinked Rho*•G_t complexes enriched by sizing chromatography. Because these elongated complexes adsorbed to the carbon film in preferential orientations, images were recorded at 0°, 45° and 60° tilt to improve the coverage of projection angles. Elongated particles were selected manually for 3D reconstruction of the negatively stained complex, namely 5,156 projections recorded with a Philips CM10 and 5,663 with an FEI TF20. CTF-corrected projections of samples prepared by the sandwich method (Cheng et al., 2006) were used to initiate calculation of the 3D map. In all, 160 class averages were calculated to sample the projection angle range at sufficient density (Figs. 4A and S4C). The highest scoring initial model determined by EMAN2 (Tang et al., 2007) was then refined against the TF20 data (5,663 projections), the CM10 data (5,156 projections) and the total data set (10,819 projections). Refinement results were similar for all data sets, and converged within the first few refinement cycles (Fig. S4A). Fig. 4B (top row) shows different views of the 3D map contoured to comprise a mass of 220 kDa. According to the EMAN2 even-odd test, the resolution of the 3D map obtained from the final refinement run that employed 10,819 projections was 21.6 Å (Fig. S4B) with extensive angular coverage (Fig. S4C).

As a control, a data set of negatively stained uncrosslinked Rho*•G_t complexes comprising 634 projections from images recorded at 0° and 499 particles recorded at 45° tilt was used to calculate 31 class averages (Fig. S5A). The initial model obtained was then refined against

this data set, yielding the 3D map displayed in Fig. 4B, lower row. This map exhibits a resolution of 28.4 Å (Fig. S5B) and is markedly similar to the 3D map obtained from crosslinked preparations.

Our results contrast the recent structure of the human β_2 adrenergic receptor• G_s protein complex (Rasmussen et al., 2011), which reveals a single GPCR molecule interacting with the G_s heterotrimer. Nevertheless, the consistency of biochemical analyses and our STEM mass determination (Figs. 1 and 2) together with the two independent 3D maps obtained (Fig. 4B) provide solid evidence for the heteropentameric nature of the $Rho^*\bullet G_t$ complex.

Modeling of Rho dimer and G_t into the molecular envelope

The calculated 3D map shows the $Rho^*\bullet G_t$ complex to be ~ 130 Å long and 30 Å thick, with a narrower 70 Å wide end, and a wider end with two distinct domains (Fig. 4B). To explore possible arrangements of the components, available structures of Rho and G_t were fitted into the map by hand (supplementary information). One of the two domains of the wider end corresponded best to the $G_{\beta\gamma}$ subunits, indicating the orientation of the intact transducin heterotrimer (PDB ID: 1GOT; (Lambright et al., 1996)). The narrower end was much too large to contain only one Rho monomer, and could accommodate a Rho dimer. Further information was obtained by analyzing both X-ray structures of GPCRs that contain crystallographic or non-crystallographic dimers and computed models of Rho packing in ROS discs. All these dimers were explored for the best fit, both geometrically and biochemically. Squid rhodopsin, CXCR4 and the 1N3M model are quite similar and all fit well into the map obtained by EM. Because the extreme C terminus of $G_{t\alpha}$ is not observed in the 1GOT structure, we spliced a corresponding α -helical segment into this region based on the structures of the $Rho^*\bullet G_{t\alpha}$ -peptide complex (PDB ID: 2X72, 3DQB).

The molecular envelope of the $Rho^*\bullet G_t$ complex accommodates one Rho dimer and one nucleotide-bound G_t heterotrimer (Figs. 4C and 5). According to this model, the C terminal helix of $G_{t\alpha}$ is close to the position observed in the opsin + G_t peptide structures (Choe et al., 2011; Standfuss et al., 2011). Furthermore, the extreme N-terminus (not observed in the 1GOT structure) would also be in close proximity to helix 8 on the receptor, best satisfying receptor - N-terminal interactions upon activation (Downs et al., 2006; Kisselev and Downs, 2006). In this orientation the G_γ and the G_α subunits could insert their respective farnesylation and myristoylation moieties into the plasma membrane (Fig. 5).

Given the recent results that demonstrate a marked flexibility of the G_α subunit in the nucleotide empty state (Rasmussen et al., 2011; Van Eps et al., 2011), we treated the alpha helical and ras-like domains as independent rigid bodies connected by residues 50-58 and 172-178 forming a flexible hinge. By opening the alpha subunit at the nucleotide binding cleft by about 30°, the fit markedly improved (Figs. 4C and 5B) compared to that obtained with the nucleotide bound form of $G_{t\alpha}$ (Fig. 5A). This rotation is much smaller in magnitude than the 130° opening of the alpha subunit seen in the β_2 adrenergic receptor• G_s protein T4L- β_2 AR- G_s -nb structure (PDB ID: 3SN6) (Rasmussen et al., 2011) and is in line with the magnitude of changes observed in recent EPR studies (Van Eps, 2011 #35).

When we superpose the T4L- β_2 AR- G_s -nb structure with our model, it reveals a very similar interaction between the C-terminal helix and the cleft formed by the outward movement of Helix 5 and 6 upon activation. The T4L- β_2 AR- G_s -nb fits quite well into our density with the exception of the extremely open conformation of the alpha-helical domain; however, there is a significant unoccupied density, which corresponds to the position for the second Rho comprising the dimer observed within our preparations (Fig. 5C).

In short, this work presents the first direct observation of the native, physiologically active complex formed between Rho and G_t and indicates that the minimal functional signaling unit is a dimer of Rho complexed to a single G_t heterotrimer. This is firmly supported by biochemical analyses and STEM mass measurements. In addition, X-ray structures determined of the individual components were used together with extant biochemical data about interactions present in the complex to interpret the molecular envelope obtained by single particle 3D reconstruction methodology. The low-resolution structure of the heteropentameric Rho*•G_t complex is compatible with a wealth of results from biochemical, biophysical and *in-vivo* analyses, and provides a working model of the native Rho*•G_t complex as well as GPCR-G protein signaling in general.

Methods

Purification of Rho*•G_t complex by sConA A affinity chromatography

sConA affinity resin was prepared by coupling sConA (Vector Laboratories, CA) to CNBr-activated agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a density of 8 mg sConA/ml of resin. A column containing sConA resin was equilibrated with 20 mM BTP, pH 6.9 containing 120 mM NaCl, 1 mM MnCl₂, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM DTT and 0.5 mM DDM (buffer A). Purified Rho was diluted to ~0.2 mg/ml and loaded onto the column at 0.5 ml/min. The resin was washed with 5 column volumes of buffer A and then illuminated for 10 min with a 150 Watt fiber light (Dolan Jenner Industries Inc.) delivered through a 480-520 nm band pass filter. Purified native-expressed G_t, diluted to ~0.2 mg/ml with buffer A, was applied to the column immediately after light exposure at 0.5 ml/min and washed with 10 column volumes of buffer A. Complex was eluted at 0.2 ml/min with buffer A containing 200 mM α -methyl-D-mannoside. Protein elution profile was examined by SDS-PAGE and fractions containing Rho*•G_t complex were used for TEM and STEM analysis. Protein concentration was determined by Bradford ULTRA (Novexin, UK) using bovine serum albumin as a standard.

STEM measurements of the Rho*•G_t complex

Purified complex was diluted 30x in buffer A and immediately adsorbed for 1 min to glow-discharged, thin carbon films spanning a thick fenestrated carbon film supported by a gold-coated 200 mesh copper grid. Grids were washed on 8 droplets of quartz double-distilled water, blotting after each drop, quick frozen in liquid nitrogen and freeze-dried at -80°C and 5·10⁻⁸ Torr overnight in a Vacuum Generator HB-5 STEM interfaced to a modular computer system (Tietz Video and Image Processing Systems). Dark-field images comprising 512×512 pixels of 9.2 Å diameter were recorded at an acceleration voltage of 80 kV, depositing recording doses of 5 ± 0.3 electrons/Å². Beam-induced mass-loss was experimentally determined and corrected for as described (Müller et al., 1992). Tobacco Mosaic Virus (TMV; kindly supplied by R. Diaz-Avalos) and the hexagonally packed intermediate layer of *Deinococcus radiodurans* (HPI-layer; kindly supplied by H. Engelhardt) served for absolute mass calibration.

Mass analysis was carried out with the MASDET program package (Krzyzanek et al., 2009). Particles having a size between ~100-200 Å were selected in optimized circular boxes, total mass was calculated and background subtracted. Final mass values were binned into histograms and fitted by a series of Gaussian curves. The overall experimental uncertainty of the results (±12 kDa for the main population) was estimated from the corresponding SE (SE = SD/√*n*) and the ~5% uncertainty in the calibration of the instrument by calculating the square root of the sum of the squares (Table 1).

TEM of negatively stained Rho*•G_t complex and image analysis

Purified Rho*•G_t complex was prepared with or without cross-linking with 5% glutaraldehyde for 15 min on ice. The cross-linking reaction was quenched with 100 mM Tris-HCl pH 8.0 for 10 min. Samples were diluted to ~20 µg/ml in buffer A and adsorbed for 1 min to glow-discharged, 400 mesh, carbon-coated grids. Grids were washed on 6-8 drops of distilled H₂O and negatively stained with 2% (w/v) uranyl acetate. Both single film and double film sandwich negative staining methods were used (Cheng et al., 2006). Micrographs were either taken with a Philips CM 10 (Philips Research, Hamburg, Germany) operated at 80 kV or an FEI TF20 operated at 200 kV (FEI, Eindhoven, Netherlands). Electron micrographs were recorded at 0°, 45° or 60° tilt, either at a nominal magnification of 136,000 × on a Veleta – 2k × 2k side-mounted TEM CCD Camera (Olympus) (CM10) or 69,000 × on a Gatan Ultrascan 4k CCD (Gatan Inc, Pleasanton, CA 94588, USA) (TF20). Images were scaled according to the magnification determined by measuring the meridional reflection of co-prepared TMV.

Image processing was performed with the EMAN and EMAN2 software packages (Ludtke et al., 1999; Tang et al., 2007). Particles were selected manually by using BOXER or automatically using the auto-boxing feature of EMAN2. The latter method was used only for picking all recognizable particles to determine the particle size distribution. For CTF correction, a Wiener filter approach was implemented in the SEMPER package (Saxton, 1996), and applied to all images prior to particle selection. For images of tilted samples the defocus was determined for 8x8 regions containing 512² pixel to define tilt angle and axis. Stripes with a defocus range $\Delta f < 50\text{nm}$ were selected, CTF-corrected and merged in the CTF-corrected image. 160 class averages were calculated from the Rho*•G_t complexes prepared by the sandwich method and recorded with TF20 at a 0°, 45° and 60° tilt (5,663 projections). The EMAN2 module e2initialmodel.py calculated initial models from this class average set, and the best initial model was refined against the TF20, the CM10, and the combined data sets.

Modeling procedure

Available structures of Rho and G_t were fitted into the map by hand. The crystal structures containing dimers analyzed included photoactivated rhodopsin PDB ID: 2I37, opsin PDB ID: 3CAP, squid rhodopsin PDB ID: 2Z73 and CXCR4 PDB ID: 3ODU, and a model of Rho derived from AFM constraints PDB ID: 1N3M. All of the putative dimers were explored for the best fit both geometrically and biochemically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Purification of the Rho*•G_t complex by sConA affinity chromatography and stoichiometry

A) Formation of the complex between solubilized Rho coupled to sConA resin and G_t has been explored in the dark (top panel, diamond) or after illumination (middle panel). The functionality of Rho*•G_t complex is evidenced by its dissociation in the presence of 200 μ M GTP γ S (bottom panel). No complex formation was observed without photoactivation, indicating the necessity of activated Rho for G_t binding to occur. Rho*•G_t can be dissociated with GTP γ S, indicating that G_t within the complex is functional. UB – unbound fraction; W – last wash; GTP – fractions eluted after incubation with GTP γ S; 1-9 – fractions eluted with buffer containing α -methyl mannoside (see Material and Methods). 20 μ l of each fraction

were analyzed by Coomassie blue- and Sypro-Ruby-stained SDS-PAGE. Intensities of bands marked by * have been quantitatively assessed. # - marks fractions containing dissociated free G_t (bottom panel). x - marks fractions containing residual $Rho^* \bullet G_t$ complex and free Rho^* . B) Sypro-Ruby stained SDS gel containing $Rho^* \bullet G_t$ complexes from three independent purifications with the respective relative band intensities displayed in the bottom panel. Protein bands were quantified by Molecular Imager FX (BioRad). The calibration of relative band intensities is documented in Supplementary Information.

Fig. 2. STEM mass determination

A) Low-dose STEM dark-field image of freeze-dried non-crosslinked Rho*•G_t complex. White circles mark particles selected for mass analysis and grey circles the areas selected for background determination. Selection was based on size, shape and brightness of the particles. The large fraction of particles in this field with either low intensities or sizes smaller than 100 Å resulted from dissociated Rho*•G_t complexes and are either smaller than 100 Å or have low intensities. Scale bar: 500 Å. Inset: A total of 136 particle projections from the major mass-histogram population (average mass 211 ± 17 kDa; B) were analyzed with EMAN software (Ludtke et al., 1999). In all, 116 particles were separated into the 3 classes shown. The class averages indicate that the projections of the selected unstained,

freeze-dried particles are elongated and have a length of ~ 130 Å. The precision of the measurement is limited by the pixel size of 9.2 Å required to record digital, low-dose, dark-field STEM images for mass measurement. Scale bar: 150 Å. B) Histogram of particle masses measured for a freeze-dried, unstained Rho*•G_t sample by STEM. The major peak is at 221 kDa suggesting that the Rho*•G_t complex is composed of 2 Rho molecules (monomer mass: 39 kDa) and 1 G_t molecule (heterotrimer mass: 86 kDa) with associated DDM. The fitted Gaussian peaks have variable widths (standard deviations); the position of the major peak has an uncertainty of ± 12 kDa (Table 1). Secondary peaks are Rho*•G_t aggregates comprising 2, 3 or 4 complexes.

Fig. 3. TEM of negatively stained Rho*•G_t complexes

A TEM image of negatively stained purified Rho*•G_t complexes is shown at the top. Regions marked by broken squares indicate elongated particles selected for structural analysis of the complex. Regions marked with broken circles indicate smaller particles, presumably Rho dimers or G_t. A set of 44 elongated particles is displayed at the bottom. Scale bar, 500 Å. Box dimensions, 250 Å × 250 Å. TMV was used as a standard for particle size calibration.

Fig. 4. 3D map of the Rho*•G_t complex at 21.6 Å resolution

A) Class averages of the Rho*•G_t complex obtained by manual selection of particles prepared by the sandwich negative staining method. This set was used to calculate an initial model. Asterisks mark class averages, which document the bipartite morphology of the complex. (Box dimensions, 250 Å × 250 Å. B) Top row: The 3D map calculated from projections of crosslinked Rho*•G_t complexes has a resolution of 21.6 Å according to the FSC function, 50% criterion (see Fig. S4B). The views were generated by Chimera (Pettersen et al., 2004), rotating the Rho*•G_t complex around its long axis in 45° increments. Bottom row: Independent 3D map having a resolution of 28.4 Å calculated from projections of native Rho*•G_t complexes. Scale bar, 100 Å. C) Semi-empirical model of the Rho*•G_t complex fitted into a 3D envelope established by single particle analysis. Because a single photon can activate the complex, the Rho molecule, which directly interacts with the G_t C-terminus is depicted in yellow to denote that it is the activated subunit. The second Rho is denoted in red. G_{tα}, G_{tβ}, G_{tγ} are colored dark blue, green and magenta, respectively.

Fig. 5. Proposed orientation of the Rho*-G_t complex in the plasma membrane and comparison with the T4L-β₂AR-G_s-nb structure

Activation of rhodopsin by a single photon of light results in a Rho*-Rho heterodimer; the Rho* within the dimer binds to G_t through an induced fit mechanism, allowing the C-terminus of G_{tα} and other contact points in G_t to interact productively to initiate expulsion of GDP from the subunit. Once G_t attains the nucleotide-free state, a GTP becomes bound from the bulk solution, which promotes dissociation of G_t from Rho and G_t into its component G_{tα} and G_{tβγ} subunits thereby triggering downstream signaling events. A) Model with the 1GOT (GDP bound alpha) structure leaves a large portion of unoccupied density above the second rhodopsin molecule. B) A 30° hinge-like motion of the alpha helical domain allows this domain to fill the unoccupied density noted with the “un-bent” structure. EPR experiments (Van Eps et al., 2011) and the T4L-β₂AR-G_s-nb structure (Rasmussen et al., 2011) indicate that this change in structure is feasible. C) Fitting the T4L-β₂AR-G_s-nb into our EM density reveals that the conformation of the G_α helical domain is inconsistent with our observed density. Given the 130° rotation observed in that structure, it is likely that in the nucleotide free state the G_α helical domain is free to “flop” about. The Rho* molecule (or β₂-adrenergic receptor in the case of the T4L-β₂AR-G_s-nb structure), which directly interacts with the G_t C-terminus is depicted in yellow to denote that it is the activated subunit. The second Rho is colored red. G_α, G_β, G_γ are colored dark blue, green and magenta, respectively.

Table 1

Overall experimental error of STEM mass analysis

The mass calibration was performed by using tobacco mosaic virus (TMV) and the hexagonally packed intermediate (HPI) layer of *Deinococcus radiodurans* as standards. The calibration error is a conservative estimate accounting for all experimental errors related to sample preparation (background uncertainty), instrumentation, and mass-loss correction (Müller et al., 1992). SE is the standard error of the mean, and the overall uncertainty is estimated assuming statistical error propagation.

Gauss peak position ± SD (kDa)	% of 790 particles under peak	Number of particles in Gauss peak	SE (kDa)	5% calibration error (kDa)	Overall uncertainty (kDa)
221 ± 84	61.3%	484	± 3.8	± 11.1	± 12
429 ± 88	26.7%	211	± 6.1	± 21.5	± 22
636 ± 95	7.7%	61	± 12.2	± 31.8	± 34
975 ± 137	4.3%	34	± 23.5	± 48.8	± 54

Table 2
Data collection and statistical analysis of particle size distribution

Images have been collected with a Tf20 and a CM10 transmission electron microscope. Particles were either selected using the auto-boxing feature of EMAN2 (Tang et al., 2007) for particle size statistics and manually for 3D reconstruction.

Raw data collection									
Rho*•Gt purification method	sConA affinity chromatography with cross-linking		sConA affinity chromatography with cross-linking and sizing chromatography			sConA affinity chromatography without cross-linking			
# of preparations	4		1			1			
# of images collected per preparation ^a	50-100 at 0°		100 at 0°			26 at 0°			
	50-100 at 45°		200 at 45°			24 at 45°			
	20-30 at 60°		20 at 60°						
Particles selected by auto-boxing for size statistics									
Total # of particles	5627		9782			5069			
Type of particles ^b	small	elongated	aggregated	small	elongated	aggregated	small	elongated	aggregated
(% of total)	66	32	2	26	72	2	75	25	<1
Particle size (Å) ^c	82±6	130±6	152±8	82±6	133±8	177±13	81±7	131±6	165±18
Particles selected manually for 3D reconstruction									
Total number of elongated particles ^d	5156		5663			1133			

^aThe number of images collected for each preparation varied between values given.
^bAuto-boxed particles were selected by eye based on their size to determine the size distribution.
^c60 small or elongated particles and 30 aggregates were measured
^dParticles were selected by eye using BOXER.