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PHOSPHOLIPIDS ARE NEEDED FOR PROPER FORMATION, STABILITY AND FUNCTION OF THE PHOTOACTIVATED RHODOPSIN-TRANSDUCIN COMPLEX

Beata Jastrzebska¹, Anna Goc¹, Marcin Golczak¹, and Krzysztof Palczewski^{1,*}

¹Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA

Abstract

Heterotrimeric G proteins become activated after they form a catalytically active complex with activated G protein-coupled receptors (GPCRs) and GTP replaces GDP on the G protein α subunit. This transient coupling can be stabilized by nucleotide depletion, resulting in an empty-nucleotide G-protein-GPCR complex. Efficient and reproducible formation of conformationally homogenous GPCR-Gt complexes is a prerequisite for structural studies. Herein, we report isolation conditions that enhance the stability, and preserve activity and proper stoichiometry of productive complexes between the purified prototypical GPCR, rhodopsin (Rho), and the rod cell-specific G protein, transducin (Gt). Binding of purified Gt to photoactivated Rho (Rho*) in n-dodecyl- β -maltoside (DDM) examined by gel filtration chromatography was generally modest and purified complexes provided heterogeneous ratios of protein components, most likely because of excess detergent. Rho*-Gt complex stability and activity was greatly increased by addition of phospholipids such as DOPC, DOPE and DOPS, and asolectin to detergent-containing solutions of these proteins. In contrast, native Rho*-Gt complexes purified directly from light-exposed bovine ROS membranes by sucrose gradient centrifugation exhibited improved stability and the expected 2:1 stoichiometry between Rho* and Gt. The above results strongly indicate a lipid requirement for stable complex formation wherein the likely oligomeric structure of Rho provides a superior platform for coupling to Gt, and phospholipids likely form a matrix to which Gt can anchor through its myristoyl and farnesyl groups. Our findings also demonstrate that the choice of detergent and purification method is critical for obtaining highly purified, stable, and active complexes with appropriate stoichiometry between GPCRs and G proteins needed for structural studies.

Keywords

G protein-coupled receptor; heterotrimeric G protein; photoactivated rhodopsin; transducin; phospholipids; gel filtration; sucrose gradient

Hepta-transmembrane helical G protein-coupled receptors (GPCRs) interact with specific heterotrimeric G proteins with appreciable affinity, but it is only after GPCR activation that these transient tight complexes are formed (1,2). Only then do G proteins become activated by the exchange of the nucleotide GDP for GTP on their α -subunits. Ultimately, G proteins in

*Address correspondence to: Krzysztof Palczewski, Ph.D., Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave, Cleveland, Ohio 44106-4965, USA; Phone: 216-368-4631; Fax: 216-368-1300; E-mail: kxp65@case.edu.

Supporting Information Available

Spectral properties of Rho (Figure 1S), and gel filtration of free Rho in DDM without and with designated added phospholipids are presented in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

turn activate their target effector molecules in turn, either through their α -subunits with bound GTP and/or their $\beta\gamma$ -subunits (3).

The most extensively studied GPCR-G protein complex is that between rhodopsin (Rho – apoprotein opsin bound to its chromophore, 11-*cis*-retinal) and its cognate G protein, Gt or transducin (4). Upon exposure to a photon of light, the 11-*cis*-retinal bound covalently to Lys²⁹⁶ on transmembrane helix VII of Rho undergoes isomerization to all-*trans*-retinylidene, converting Rho to its activated state, Rho* (5,6). During its decay from the activated state, a single molecule of Rho binds and activates hundreds of Gt molecules, thereby propagating and markedly amplifying the light signal (7).

Although the above description seems quite straightforward, different GPCR-G protein intermediates can be trapped. For example, complexes are described between Rho and Gt that form upon Rho photoactivation but in the absence of nucleotides, complexes between *cis*-retinoid regenerated Rho* and Gt in the absence of nucleotides, and complexes between opsin and Gt formed in the absence of both retinoids and nucleotides (8,9). Structural differences between these complexes remain to be defined.

Another factor that increases the complexity of GPCR-G protein assembly is the high density of GPCRs in native membranes that can lead to oligomerization (10,11). This can present a problem because the vast majority of experiments have been carried out in detergents that disrupt or compete with hydrophobic interactions between membrane proteins (reviewed in (12,13)). In conventional 2-dimensional crystallization studies of transmembrane proteins, the typical membrane protein is present at a ratio of about 1 protein molecule per 30 phospholipids (13–15). Bacteriorhodopsin crystallizes at a 1:1 (w/w) ratio of protein to lipid (16); however, an increased lipid/protein ratio results in formation of aggregates without crystallization (17).

Specific portion of cone photoreceptor cells called cone outer segments display diffraction patterns suggesting a crystalline form of membrane-bound cone pigment complexes (18,19). Similarly, extraction of free lipids from frog rod outer segments (ROS) yields 2D crystals of Rho (20,21). In mammalian ROS, Rho is packed at a ratio of 1 Rho to ~60 phospholipids or a 1:1.5 (w/w) ratio (22,23), barely more than one layer of phospholipids on average surrounding each Rho, on average. Moreover, Rho at a concentration of 5 mM occupies 50% of the space in ROS membranes (24), and mimics the concentration of Rho in 3D crystals. In fact Rho crystal structures are ~70% hydrated and the protein concentration within these crystals is between 9–14 mM. Thus, it is hardly surprising that Rho forms heterogeneous para-crystalline assemblies in ROS (25–27). Heterogeneity of individual discs within the ROS further complicates this picture (24,28). Some imaging data are consistent with the above geometry and mathematical calculations but are inconsistent with biochemical and biophysical interpretations that assume partial or total fluidity of membranes needed for a highly diffusible receptor and G proteins to amplify light signals (reviewed in (29)). Notably, activation of a single Rho molecule is sufficient to trigger a physiological response and a single Rho molecule also has been shown to effectively catalyze nucleotide exchange in Gt (30–34), even though such an ideal situation is unlikely to hold for *in vivo* complexes. Even if perfect separation of Rho molecules from one another is assumed, the size of the involved proteins would lead to interaction of a single Gt molecule (Gt $_{\alpha}$ ~39 kDa, and the obligate heterodimer Gt $_{\beta}$ (~37 kDa) and Gt $_{\gamma}$ (~8 kDa) (35) with multiple Rho molecules because individual receptors protrude into the cytoplasm with about 10 kDa of protein mass separated by ~4 nm whereas Gt spans ~7.5 nm (see modeling of the complex (36–39)). Reduction of Rho levels in heterozygotes of Rho knockout mice reduces the size of ROS, rather than decreases Rho density (27), whereas increased expression of Rho leads to expansion of the ROS (40). As a consequence of all this potential heterogeneity, it is a challenge to obtain Rho*-Gt complexes for structural studies that are representative of the *in vivo* state.

The phase distribution of GPCRs and G proteins represents yet another complexity. GPCRs are membrane proteins that require detergent solubilization for structural studies (41–45). Selection of a detergent and its concentration may exert a significant influence on protein dispersion/aggregation or delipidation, and the detergent itself may bind to membrane proteins. G proteins are hydrophobic with lipophilic posttranslational modifications that are unlikely to be hydrated (9,13,46,47). Thus, extraction of such proteins from membranes for the purpose of studying complex formation and reconstitution may introduce even more heterogeneity with lipophilic modifications penetrating hydrophobic patches on proteins in an irregular fashion. So, which complexes of heterotrimeric G proteins are physiologically relevant? Towards answering this question, we isolated several such complexes from native ROS membranes in the presence of various detergents and extensively characterized them (48). Here we report how these structures compare with complexes formed from purified components and also document a possible role for phospholipids in these complexes and the effects of different detergents upon them. Phospholipids appear indispensable for their activity, stability and proper stoichiometry of various Rho-Gt complexes.

MATERIALS AND METHODS

Chemicals

Guanosine 5'-3-*O*-(thio)triphosphate (GTP γ S) was purchased from Sigma (St. Louis, MO). *n*-Octyl-glucopyranoside (OG), *n*-nonyl-glucopyranoside (NG), *n*-decyl- β -D-maltoside (DM), *n*-undecyl- β -D-maltoside (UDM), *n*-dodecyl- β -D-maltoside (DDM), and *n*-tridecyl- β -D-maltoside (TDM) were obtained from Anatrace Inc. (Maumee, OH). Bradford ULTRA was purchased from Novexin (Cambridge, UK). Asolectin was procured from Sigma (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine-(DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL).

Purification of Rho

Bovine ROS membranes were prepared from fresh retinas under dim red light according to the Papermaster procedure (49). Rho was solubilized in DDM and purified from ROS by the ZnCl₂-opsin precipitation method that yields Rho with an A_{280 nm}/A_{500 nm} absorbance ratio of 1.63 (42). ZnCl₂ was removed by dialysis in the presence of 0.1% DDM. Rho concentrations were determined with a Cary 50 (Varian, Palo Alto, CA) UV-visible spectrophotometer and quantified by absorption at 500 nm by using the absorption coefficient $\epsilon = 40,600 \text{ M}^{-1}\text{cm}^{-1}$ (50).

Purification of Gt

Bovine ROS membranes were prepared from 100 frozen retinas (W.L. Lawson Co., Lincoln, NE) under dim red light. Gt was purified according to a procedure described elsewhere (9). Briefly, ROS membranes were diluted in 10 ml of isotonic buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM DTT, and 100 mM NaCl) and soluble proteins were removed by gentle homogenization followed by centrifugation at 25,000g at 4°C for 15 min. Gt then was extracted from the pellet in 10 ml of hypotonic buffer (5 mM HEPES, pH 7.5, 0.1 mM EDTA, and 1 mM DTT), membranes were collected by centrifugation at 25,000g at 4°C for 45 min and the supernatant was saved for further purification. This extraction procedure was repeated three times. Combined supernatants were centrifuged at 25,000g for 60 min to remove ROS membrane contaminants and dialyzed overnight at 4°C against the equilibrating buffer (10 mM HEPES, pH 7.5, 2 mM MgCl₂, and 1 mM DTT). This solution was applied at a flow rate of 15 ml/h to a 10 × 100 mm column with 3 ml of pre-equilibrated propyl-agarose resin and the column was washed with 5 column volumes of the equilibrating buffer. Bound proteins were eluted with a 50 ml linear gradient of 0 to 0.5 M NaCl in the equilibrating buffer at a flow rate

of 15 ml/h, and 1 ml fractions were collected. Fractions containing Gt were pooled, dialyzed overnight at 4°C against 10 mM HEPES, pH 7.5, containing 2 mM MgCl₂, 1 mM DTT, and 100 mM NaCl, and then concentrated by 30,000 NMWL Centricon devices (Millipore, Billerica, MA). Gt was purified to homogeneity on a Superdex 200 gel filtration column equilibrated with dialysis buffer at 4°C; flow rate was 0.4 ml/min and 0.4 ml fractions were collected. Fractions containing Gt were combined and concentrated by 30,000 NMWL Centricon devices to ~ 10 mg protein/ml determined by the Bradford assay (51).

Binding of Gt to Rho* examined by gel filtration chromatography

Gt bound to Zn²⁺-extracted Rho under a variety of conditions (different Rho*:Gt molar ratios, in different detergents, and with added phospholipids or NH₂OH) and the resulting complexes were analyzed after size exclusion chromatography on a Superdex 200 10/300 GL column (Amersham Biosciences), with a FPLC instrument (BioRad). The column was run at 4°C with a solution composed of 20 mM BTP buffer, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT and 1 mM DDM for all experiments done in the presence of DDM. For testing of other detergents, the solution contained either 13 mM NG, 3.6 mM DM, 2.3 mM UDM or 0.5 mM TDM. Columns were run at a flow rate of 0.4 ml/min at 4°C and 0.4 ml fractions were collected. Rho (200 µg) was mixed with 100 µg of Gt in 20 mM BTP buffer, pH 6.9 containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT and 1 mM DDM, bleached for 5 min by a Fiber-Light covered with a 480–525 nm bandpass filter at a distance of 10 cm, and incubated on ice for 15 min to form the complex. Proteins then were loaded onto a gel filtration column at 4°C equilibrated with 20 mM BTP, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM DDM. Control gel filtration chromatography experiments were performed with either free Rho and free Gt, a mixture of non-activated Rho and Gt, or a mixture of Rho and Gt in the presence of freshly neutralized 10 mM NH₂OH that was light activated to accomplish Schiff base hydrolysis. Gt dissociation from the complex with light activated Rho* was analyzed by adding 200 µM GTPγS (Roche Diagnostic, Indianapolis, IN) after complex formation. After 30 min incubation on ice, proteins were loaded onto a gel filtration column equilibrated with 5 mM BTP, pH 6.9, containing 10 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 1 mM DDM, and 10 µM GTPγS. Columns were run at a flow rate of 0.4 ml/min at 4°C and 0.4 ml fractions were collected.

The influence of different Rho* and Gt ratios on complex formation was investigated by mixing different amounts of both proteins together to attain the following Rho*:Gt molar ratios: 12.5 µg Rho and 100 µg Gt (1:4); 100 µg Rho and 200 µg Gt (1:1); 200 µg Rho and 200 µg Gt (2:1), and 200 µg Rho and 100 µg Gt (4:1). Proteins were mixed in a buffer composed of 20 mM BTP, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM DDM and then illuminated for 5 min by using a Fiber-Light covered with a 480–525 nm band-pass filter at a distance of 10 cm. After 15 min incubation on ice, protein samples were loaded onto gel filtration columns equilibrated with 20 mM BTP, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM DDM. Columns were run at a flow rate of 0.4 ml/min at 4°C and 0.4 ml fractions were collected.

For testing the influence of lipids (asolectin, DOPC, DOPE and DOPS) on binding of Gt to photoactivated Rho*, samples containing Rho and Gt mixtures were incubated with phospholipids in 20 mM BTP, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM DDM. Asolectin and DOPC, DOPE, DOPS were added to attain a 1:100 Rho:lipid ratio. After 1 h of incubation at room temperature, samples were illuminated and loaded onto gel filtration columns equilibrated with buffer composed of 20 mM BTP, pH 6.9, containing 120 mM NaCl, 2 mM MgCl₂, 1 mM DTT, and 1 mM DDM. Columns were run at a flow rate of 0.4 ml/min at 4°C and 0.4 ml fractions were collected.

Gt activation by Rho*

Activation of Gt was tested as previously described (52). Briefly, purified Gt (250 nM) was added to Rho (25 nM) in 20 mM BTP, pH 7.0, containing 120 mM NaCl, 5 mM MgCl₂, and 1 mM DDM. We used Zn²⁺-extracted Rho with or without added phospholipids such as DOPC, DOPE, DOPS, or those extracted from ROS membranes. Rho was incubated with phospholipids (Rho:lipid molar ratio 1:100) for 2 h at room temperature in the above buffer. Gt was activated by Rho* in ROS membranes for the control experiment. Samples were bleached for 30 s with a Fiber-Light covered with a 480–525 nm band-pass filter (Chroma Technology, Rockingham, VT), followed by a 5 min incubation with continuous low-speed stirring. Then 5 μM GTPγS was added and the intrinsic fluorescence of Gt_α was measured with a LS55 Luminescence Spectrophotometer (Perkin Elmer, Life Science), by using excitation and emission wavelengths of 300 nm and 345 nm, respectively (53,54). The fluorescence change was linear within the range of protein concentrations used. No fluorescence signal was detected in the control experiment without GTPγS.

Isolation of native Rho*-Gt complex by sucrose gradient ultracentrifugation

Native Rho*-Gt complex was isolated from bovine retinas according to the procedure described by Jastrzebska et al. (48). ROS membranes (0.2 mg/ml of protein) were suspended in 20 mM BTP, pH 6.9, containing 120 mM NaCl, 0.1 mM MgCl₂, and 1 mM DTT, bleached for 10 min at a distance of 10 cm under a Fiber-Light covered with a 480–525 nm band-pass filter (Chroma Technology, Rockingham, VT) and incubated for 15 min on ice. To remove soluble proteins present in ROS and endogenous GDP released from the Gt nucleotide-binding site, ROS membranes were washed 5 times with 5 mM BTP, pH 6.9, containing 0.1 mM MgCl₂ and 1 mM DTT followed by a single wash with 20 mM BTP, pH 6.9, containing 120 mM NaCl, 0.1 mM MgCl₂ and 1 mM DTT. Rho*-Gt then was solubilized with 20 mM BTP, pH 6.9, containing 120 mM NaCl, 0.1 mM MgCl₂, 1 mM DTT and 20 mM DDM for 1 h on ice (8). Insoluble material removed by centrifugation at 100,000g for 1 h was discarded. Alternatively, samples were solubilized in different detergents such as 50 mM OG, 50 mM NG, 20 mM DM, 20 mM UDM, or 20 mM TDM. The Rho*-Gt complex was enriched by using a 750–900–900–750 μl step gradient composed of 20–30–40–50% sucrose in 20 mM BTP, pH 6.9, containing 120 mM NaCl, 0.1 mM MgCl₂, 1 mM DTT and 0.3 mM DDM. The density of each fraction was determined from its refractive index. Detergent-solubilized Rho*-Gt complex (300 μl, 2 mg/ml protein) was loaded on top of the gradient, centrifuged at 269,000g for 16 h and fractions (250 μl each) were collected from the top to the bottom of the gradient. Protein content in these fractions was analyzed by SDS-PAGE and immunoblotting. As controls for the above experiments, 50 μg of purified Rho (42) and 50 μg of purified Gt (9) were suspended in 20 mM BTP, pH 6.9, containing 120 mM NaCl, 0.1 mM MgCl₂, and 1 mM DTT, (100 μl total volume) and subjected to sucrose gradient centrifugation after either being kept in the dark or exposed to illumination.

Quantification of total phospholipids

Total phospholipids in Rho*-Gt complexes isolated either by gel filtration chromatography or by sucrose gradient ultracentrifugation were quantified by phosphorus analysis (55). Fractions 28–32 containing no phospholipids or mixture of DOPC and DOPE, or fractions 26–30 containing DOPS obtained after gel filtration chromatography (Figure 1A and Figure 3), and fractions 6–8 obtained after sucrose gradient centrifugation (Figure 6) were collected for phospholipid quantification. Phospholipids were extracted with methanol/CHCl₃ mixed in a 2:5 ratio, dried down in a Speed Vac, and then re-dissolved in CHCl₃. The CHCl₃ solution was separated into three different lipid classes (neutral lipids, glycolipids, and polar lipids) by silica acid column chromatography and subsequently eluted with CHCl₃, acetone, and methanol (56). The methanol fraction containing phospholipids was dried down, redissolved in 10%

H₂O/80% methanol/10% CHCl₃. Sixty percent perchloric acid (650 μ l) was added to 90 μ l of sample and heated 2 hr at 100°C to hydrolyze phospholipids. Then 3.4 ml H₂O, 0.5 ml 2.5 % ammonium molybdate and 0.5 ml 10 % ascorbic acid were added to the sample and incubated for 30 min. Absorption was measured at 797 nm and phosphate content in the sample was calculated from a standard calibration curve.

Determination of molar ratios between Rho* and Gt in isolated Rho*-Gt complexes

Ratios between Gt $_{\alpha}$, Gt $_{\beta}$, and Rho* in fractions containing Rho*-Gt complexes purified by either gel filtration or sucrose gradient ultracentrifugation were calculated from gels stained with Coomassie blue by using the Image J program (<http://rsb.info.nih.gov/ij/> and <http://www.uhnresearch.ca/facilities/wcif/imagej/>).

RESULTS

Formation of the reconstituted Rho*-Gt complex

Purified proteins were used to reconstitute the complex between Rho and Gt. Rho was purified by opsin Zn²⁺-extraction as described in Materials and Methods. Sample purity was determined by its absorption spectra and Coomassie-blue stained gels (Figure S1 A and Figure S1 C (inset)). Typically, this purification procedure yielded Rho with an absorption 280/500 nm ratio of ~ 1.6 (Figure S1 A, black line) that displayed typical behavior under dark and light conditions. Light illumination activated Rho and stimulated formation of Meta II (Figure S1A, grey line), retaining attached chromophore as recognized by protonation of the Schiff base with acid, and a shift in maximum absorption from 380 nm to 440 nm (Figure S1 A, light grey line). However, in the presence of NH₂OH after light illumination, the chromophore was hydrolyzed from its binding pocket, therefore there was no shift of the absorption maximum (Figure S1 B, gray and light grey line). Gt was purified as described in Materials and Methods and its purity was examined by Coomassie-blue stained gels (Figure S1 C (inset)). Formation of the complex between Rho* and Gt was examined by a fluorescence-based assay that involves monitoring guanylyl nucleotide exchange in the α subunit of Gt that occurs upon its activation by Rho* (Figure S1 C). The typical change in the intrinsic fluorescence of Gt $_{\alpha}$ because of nucleotide exchange stimulated by Rho* was observed. The determined rate of activation was similar to rates previously reported under comparable experimental conditions (52, 57).

Gel filtration of reconstituted Rho*-Gt complexes

Binding of purified Gt to Rho* in DDM was examined by gel filtration chromatography (Figure 1). Upon formation of Rho*, Gt binds to the receptor's cytoplasmic surface (58), forming a protein complex with higher molecular mass than its two protein components. Thus, the Rho*-Gt complex should migrate faster in size exclusion chromatography. Although the molecular mass of Gt is 83 kDa (α subunit 39 kDa, β subunit 36 kDa, γ subunit 8 kDa) (58), purified Gt eluted from the gel filtration Superdex 200 column in the presence of DDM spread through fractions 32–42 with higher concentrations in fractions 34 and 40 (Figure 1 F). This might suggest that there are two populations of Gt, i.e. bound and not bound to detergent micelles. Free Rho migrated mainly in fractions 32–34 (Figure 1 E). When the mixture of Rho and Gt was applied to the gel filtration column and eluted in the dark, most of the Gt was present in fractions 36–42. However, some Gt was detected in fractions 32–34, most likely because it bound to the detergent micelles, although pre-coupling to ground state Rho cannot be excluded (Figure 1 C). Complexes of Gt and Rho with higher molecular masses appeared in earlier fractions 28–30 (Figure 1 A). Addition of GTP γ S, a non-hydrolysable analog of GTP, led to release of Gt $_{\alpha}$ and Gt $_{\beta\gamma}$ subunits from Rho*, visible as a shift of Gt to the later 38–40 fractions (Figure 1 B). Addition of the nucleophile NH₂OH, which promotes release of the chromophore from the Rho ligand binding pocket after light activation, did not affect formation of the complex between opsin and Gt that mostly appeared in fraction 30, similar to the same complex

formed in the absence of NH_2OH (compare Figure 1 D and A). Although formation of the $\text{Rho}^*\text{-Gt}$ complex after light activation was detected, its concentration was low, either because of poor complex formation or, more likely, because the complex stability was affected by the presence of detergent.

To determine if the stability of the complex is regulated by the ratio of Rho to Gt, several different ratios of these two purified proteins were mixed together (Rho:Gt 1:4, 1:1, 2:1, 4:1) and subjected to gel filtration after photoactivation (Figure 2). This experiment revealed that an excess of Rho, either 2:1 or 4:1, provided higher yields of the complex and that the complex with a 1:1 $\text{Rho}^*\text{:Gt}$ ratio was present mostly in fraction 30 (Figure 2 A and B, asterisks, two bottom panels). When Rho and Gt were mixed in the presence of an excess or equivalent ratio of Gt to Rho, about a 5 molar excess of Gt compared to Rho^* was detected in fraction 30 (Figure 2 A and B, two upper panels). Some free Gt_β was detected in fractions 38–42 suggesting that photoactivated Rho^* causes partial dissociation of the $\text{Gt}_{\beta\gamma}$ subunit.

The influence of phospholipids on $\text{Rho}^*\text{-Gt}$ complex formation and stability

Many published reports suggesting that a dimer is the structural unit of Rho that forms the interface for Gt binding. Because it is possible that detergent might destabilize the interaction between Rho and Gt, we tested whether exogenous phospholipids could affect this interaction. The $\text{Rho}^*\text{-Gt}$ complex was prepared from purified proteins and phospholipids such as DOPC, DOPE, DOPS, and asolectin were added at a ratio of 100 to 1 Rho (Figure 3). The presence of phospholipids shifted migration of the $\text{Rho}^*\text{-Gt}$ complex to earlier, heavier fractions and enriched these fractions with Rho^* . However, quantification of Rho^* and Gt content in peak fractions containing the $\text{Rho}^*\text{-Gt}$ complex indicated a ratio of about 3:1 for all tested fatty acids except asolectin, suggesting an excess of Rho^* . Thus, it appears that phospholipids stabilize complex formation and/or Rho oligomeric assembly. To explore this question, free Rho was subjected to gel filtration in the presence of phospholipids and its migration was compared with that of Rho without added phospholipids (Figure S2). In this case phospholipids enhanced the migration of Rho, indicating its presence in heavier particles, presumably as oligomers in mixed detergent-lipid micelles. This experiment does not eliminate the possibility of stabilization of the $\text{Rho}^*\text{-Gt}$ complex but the quantified ratios suggest the presence of excess Rho^* contaminating fractions containing the $\text{Rho}^*\text{-Gt}$ complex. Free Gt_β was detected in fractions 40–42 suggesting that photoactivated Rho^* causes partial dissociation of the $\text{Gt}_{\beta\gamma}$ subunit.

Phospholipids then were quantified in fractions containing the reconstituted $\text{Rho}^*\text{-Gt}$ complex obtained after gel filtration of samples either without added phospholipids or containing exogenously added DOPS or a mixture of DOPC/DOPE (Figure 4). About 5 times more phospholipids were found in fractions from samples of reconstituted $\text{Rho}^*\text{-Gt}$ incubated with phospholipids prior to loading the gel filtration column (Figure 4, left) suggesting that Rho naturally incorporates into phospholipids present in detergent-lipid mixed micelles. Significantly, gel filtration of native $\text{Rho}^*\text{-Gt}$ complex without added phospholipids resulted in purified fractions of this complex that contained substantially more lipid (> 2 fold, ~ 16 phospholipids per Rho) than comparable fractions of the reconstituted complex (~ 6 phospholipids per Rho), whereas purification of the native complex by sucrose gradient centrifugation resulted in purified $\text{Rho}^*\text{-Gt}$ complexes that retained lipid in amounts comparable to reconstituted complexes with added phospholipids purified by gel filtration (~ 30 phospholipids per Rho) (Figure 4, right).

Phospholipids also were shown to be critical for optimal Gt activation (Figure 5). Activation rates were faster in the presence of lipids and depended on the type of lipid present. Rho incorporated into DOPS had a far greater effect on Gt activation rate than it did in the presence of either DOPC or DOPE, resulting in 15 times faster activation than purified Rho^* in detergent

micelles; activation in the presence of DOPC and DOPE was 3.4 and 1.8 times faster, as well. Rho incorporated into phospholipids extracted from ROS membranes activated Gt at a rate similar to that of native Rho in ROS membranes and about 7–9 times faster than Rho solubilized in detergent alone. These results strongly indicate the necessity of phospholipids for rapid signal propagation in photoreceptor cells. Phospholipids most likely affect Rho oligomerization (59), providing a better platform for coupling with Gt as well as a matrix for Gt anchorage by its myristyl and farnesyl groups.

Isolation of native Rho*-Gt complex

Because isolation of the reconstituted Rho*-Gt complex by size exclusion chromatography did not yield a homogenous complex with a well defined ratio between Rho* and Gt, we developed a method for purifying the native Rho*-Gt complex. *In vivo*, binding of Gt to Rho* occurs in native ROS membranes where Rho is properly organized in a lipid bilayer. After washing and membrane solubilization, we used a four step gradient of sucrose (20–30–40–50%) to separate the Rho*-Gt complex by ultracentrifugation. Fractions of 250 μ l were collected from the top to the bottom of the gradient and the protein content of each fraction was determined by Coomassie blue-stained SDS-PAGE and immunoblots (Figure 6). After overnight ultracentrifugation, the Rho*-Gt complex was found in fractions 6–8 containing 30–40% sucrose where it was highly enriched but not homogeneous. (Figure 6 A). To determine if the complex was biochemically active, 200 μ M GTP γ S was added to samples before loading them on the sucrose gradient. Most of the complex dissociated and Rho* as well as Gt-GTP γ S were found mainly in fractions 3–5 containing 20–25% sucrose (Figure 6 B), the same fractions that contained free Rho and free Gt in the control experiment (Figure 6 C). The molar ratio between Rho* and Gt quantified in fractions 6, 7, and 8 was 2:1 (Figure 6 D). These results strongly suggest that native phospholipids present in the sample or the method used for Rho*-Gt complex isolation can stabilize the complex in a well defined 2:1 Rho*:Gt molar ratio.

We then compared the effectiveness of sucrose gradient ultracentrifugation and gel filtration chromatography in isolating the native complex (Figure 7). Native Rho*-Gt complex was prepared in ROS membranes as described above but solubilized membranes were loaded onto a gel filtration column instead of a sucrose gradient. Most of the Rho*-Gt complex appeared in fraction 30 whereas excess free photoactivated Rho* eluted in subsequent fractions (Figure 7 A). The isolated complex was functional in that it could be dissociated by GTP γ S that shifted free Gt into later fractions with a peak in fraction 36. The molar ratio between Rho* and Gt in the complex isolated in fraction 30 was ~1:1. This result suggests that during gel filtration more phospholipids surrounding Rho* are sequestered and that their loss most likely causes disruption of the Rho dimeric organization. This result contrasts with that found after sucrose gradient purification where the protein sample is not exposed to a high volume of detergent-containing buffer disrupting the lipid environment protecting Rho. To check this hypothesis, we compared the phospholipid content of native Rho*-Gt complex purified either by sucrose gradient centrifugation or gel filtration (Figure 4). In fact, about 30 phospholipid molecules per one molecule of Rho* were found in fractions of the complex sample purified by sucrose gradient ultracentrifugation whereas only 16 phospholipid molecules per Rho* molecule were found in the fractions purified by gel filtration.

Influence of detergent on Rho*-Gt complex stability

To examine effects of different detergents on properties of the Rho*-Gt complex purified from native ROS, we tested several detergents including OG, NG, DM, UDM and TDM and compared the results with those obtained with DDM (Figure 8). The Rho*-Gt complex in native ROS membranes was prepared as described above but solubilized in one of these detergents and then isolated by sucrose gradient ultracentrifugation. All four alkyl-maltoside detergents seemed to affect complex isolation similarly to DDM such that the Rho*-Gt complexes

appeared mostly in fractions 6–8. In contrast, detergents such as OG and NG that are commonly used to extract membrane proteins had destructive effects on the complex stability. Rho* and Gt were distributed broadly in fractions 6 to 11 suggesting that different heavier complexes were formed and/or aggregated. Some free Rho* and Gt β was also found in fractions 3–5. Possibly alkyl-glucosides are unable to solubilize sufficient phospholipids to maintain a proper lipid to protein ratio, thereby disrupting formed Rho*-Gt complexes.

For comparison, reconstituted Rho*-Gt complexes were extracted in a few different detergents such as NG, DM and TDM and purified by gel filtration in the absence and presence of asolectin (Figure 9). Compared with DDM, Rho in NG and DM migrated in later fractions, most likely because it was present in smaller detergent micelles. In NG, photoactivation clearly shifted migration of some Gt to earlier, heavier fractions (28–30), probably because of aggregation caused by this detergent. Aggregation of non-activated Gt in the presence of NG has been observed on native PAGE gel (not shown). Although Rho* can be found in the same early fractions as Gt (28–30), its concentration is not sufficient to form a stoichiometrically proper complex with Gt. Addition of lipids (asolectin) in the presence of NG failed to stabilize this complex. In DM there was no shift in the migration of Gt and Rho* suggesting complex formation; both Rho* and Gt were present in the same fractions as their control standards. However, prior addition of phospholipids caused them both to shift to earlier fractions, suggesting some stabilizing effect of phospholipids on complex formation. More Rho*-Gt complex was formed in TDM because Gt clearly shifted to earlier fractions (28–30) as compared to control free Gt, and addition of phospholipids caused an even further shift to earlier fractions (24–26).

DISCUSSION

The visual signaling cascade is initiated when Rho - a prototypical G protein-coupled receptor (GPCR) is activated by a photon of light. Conformational changes that occur in the Rho* structure after photoactivation allow proper binding of its cognate heterotrimeric G protein and catalyze rapid GDP/GTP exchange on the Gt α subunit (1). The mechanism of Rho activation and signal transduction is not fully understood. Solving the structure of the complex between Rho* and Gt would greatly improve our knowledge about GPCRs signaling, but before this goal can be achieved biochemical characterization of those complexes is needed.

In this study we examined certain biochemical properties of complexes reconstituted from purified Rho and Gt, and compared them with identical properties of native complexes isolated from ROS membranes. We also tested the effects of adding different phospholipids and using various detergents for Rho*-Gt complex extraction upon the stability of the purified complexes.

Complex formation with purified components: Rho and Gt

The yield of Rho*-Gt complex reconstituted by mixing the two purified proteins in the presence of DDM was poor. One factors that may have affected efficient coupling of Gt to purified Rho is the presence of detergent micelles. Although Rho* solubilized in detergent solution can activate Gt, formation of a stable complex seems to be significantly attenuated. After light illumination we observed that both Rho* and Gt migrated towards earlier, heavier fractions after gel filtration as compared to free Rho and Gt standards. Quantification of proteins in these fractions revealed that they contained excess Gt, suggesting that some oligomers/aggregates of Gt were formed. Although the effect of activated Rho* on Gt oligomerization has yet to be reported, oligomerization of Gt in solution has been observed (60–62). Mixing increasing concentrations of Rho* with Gt resulted in higher complex stability with a ratio of 1:1 in the Rho*-Gt complex. But AFM studies have shown that Rho exists in native ROS disc membranes as highly organized and densely packed dimers (12,25–27,63). Other biochemical and biophysical studies (59,64,65) as well as estimates that account for the sizes of both Rho and

Gt, indicate that two molecules of Rho per one Gt are present in the stable complex. Although one Rho per Gt is sufficient to produce a response in the brain, the second Rho molecule in the dimer most likely serves as a platform for proper Gt docking (37).

Effect of phospholipids on the complex

In ROS membranes Rho is incorporated into the lipid bilayer (about 60 phospholipids per one Rho molecule) (22,23) where phospholipids tightly surrounding Rho most likely stabilize its oligomeric organization (52). Both the headgroups and acyl chains of phospholipids have a significant effect on Meta II formation (66–68) and G protein anchoring (69–71). It has been observed that Phosphatidylethanolamine (PE) and negatively charged phosphatidylserine (PS), especially with polyunsaturated hydrocarbon side chains, favor the formation of Meta II. Enhanced Gt binding affinity to Rho* in the presence of phosphatidylethanolamine (PE) has been reported as well (72,73). But replacement of fatty acids in the lipid matrix of rat ROS membranes not only reduced Rho activation but also Rho*-Gt coupling and PDE activity (74). Therefore, the composition of the lipid matrix is critical not only for Rho function but for the entire process of signal transduction. Here we tested the effect of asolectin, DOPC, DOPE and DOPS on the binding of Rho* to Gt. Rho and Gt were incubated together with each of these phospholipids before light illumination and formation of the complex after photoactivation was examined by gel filtration. Compared to controls (free proteins), migration of both Gt and Rho* was shifted to the earlier, heavier fractions suggesting that coupling had occurred. DOPS had the strongest effect in this regard. This is not surprising because negatively charged PS derivatives reportedly are far more critical for Gt membrane anchoring than uncharged PC (71). The ratio of Rho* to Gt in the presence of phospholipids was changed to over 2:1, indicative of Rho* excess in fractions containing the Rho*-Gt complex. However, when this phospholipid-stabilized complex was subjected again to gel filtration, partial dissociation of the complex occurred unless excess phospholipids were added beforehand (not shown). Stability of the Rho dimer is critically affected by increasing concentrations of detergent, but phospholipids have a stabilizing effect on Rho oligomeric organization (52, 57). Phospholipids may provide a native-like hydrophobic environment for better incorporation of Rho dimer or they may interact with detergent micelles causing a decreased detergent concentration and stimulating Rho self association (59). But if there are not enough phospholipids surrounding Rho, inverted Rho dimers can be formed (41) that affect proper Gt binding and decrease the yield of the Rho*-Gt complex. Formation of inverted dimers is a consequence of non-specific interactions between two monomers, so that adequate binding of phospholipids to protein may hinder formation of these structures and stabilize proper parallel dimer conformations.

Phospholipids are also necessary for rapid Gt activation. Although Gt can be activated by Rho* solubilized in detergent, Gt activation was several times faster in the presence of either synthetic phospholipids such as DOPC, DOPE, and DOPS or a mixture of native lipids isolated from ROS membranes. Both DOPS and native lipids provided Gt activation rates similar to those observed when Gt was activated by Rho* in native disc membranes. Thus, these phospholipids most likely stabilize the quaternary structure of Rho for better Gt docking and membrane anchoring that guarantee rapid responses to light and rapid signal transduction.

Comparison of reconstituted and native membrane complexes between Rho* and Gt

Addition of stabilizing agents such as phospholipids is needed to reconstitute stable complexes of Rho* and Gt from purified components. However, native Rho*-Gt complexes can be isolated directly from ROS membranes wherein phospholipids surrounding Rho have a good stabilizing effect, either on Rho oligomeric structure, Gt binding, or both. We used two methods to isolate the native complex: gel filtration and sucrose gradient centrifugation. Interestingly, Rho*-Gt complexes isolated by these two procedures had different Rho* to Gt ratios. Although the

complex isolated by gel filtration had a 1:1 Rho*:Gt ratio, the complex isolated by sucrose gradient centrifugation displayed a 2:1 Rho*-Gt stoichiometry, similar to that previously reported (48). The Rho*-Gt complex purified by sucrose gradient centrifugation was stable over time and migrated in the same fractions after a second sucrose gradient centrifugation (not shown). As confirmed by lipid analysis of the complex-containing fractions collected by gel filtration, the most likely explanation for this discrepancy is that more phospholipids surrounding Rho were removed by gel filtration resulting in disruption of the Rho* dimeric organization. In contrast, sucrose gradient purification did not expose protein samples to a high volume of detergent-containing buffer, so they retained more phospholipids that protected the Rho oligomeric structure and enhanced Rho*-Gt complex stability. In fact, about 30 phospholipids per Rho* were found in fractions of the complex purified by sucrose gradient ultracentrifugation, whereas only 16 phospholipids per Rho* were quantified in the fractions purified by gel filtration. Thus, a considerably smaller amount of lipid was detected in the reconstituted complex isolated by gel filtration. We also found that the type and concentration of phospholipids and detergent have a strong influence on complex formation.

Effect of detergents on Rho and Gt coupling stability

The detergent used for solubilization of the Rho*-Gt complex had a significant impact on its stability. A few commonly used non-ionic detergents that included alkyl-glucosides and alkyl-maltosides illustrated this effect. All alkyl-maltosides employed (DM, UDM, TDM) seemed to preserve native Rho*-Gt complexes isolated by sucrose gradient centrifugation similar to DDM, whereas alkyl-glucosides destroyed these complexes. Differing results most likely were caused by different amounts of phospholipids extracted by the various detergents. Indeed, it was shown that fewer phospholipids were extracted by OG as compared to other detergents during Rho solubilization from disc membranes (23). In other studies wherein OG was used for solubilization of 5-HT_{1A} receptor, 15% less lipid was co-extracted as compared to DDM (75). Different effects of different detergents on Rho*-Gt complex reconstitution have been noted as well.

CONCLUSIONS

Different complexes of Rho* and Gt were reconstituted with different Rho* to Gt molar ratios. The binding of Gt to Rho* strongly depends on the type of detergent used for Rho solubilization and the amount of co-extracted phospholipids. The presence of phospholipids greatly enhanced the formation and stability of the Rho*-Gt complex. Accordingly, native Rho*-Gt complexes with a Rho*:Gt ratio of 2:1 can be isolated from ROS membranes, where Rho is properly organized and surrounded by native lipids that provide an environment critical for efficient coupling of Gt to Rho*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AFM, atomic force microscopy

Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane
 DDM, n-dodecyl- β -D-maltoside
 DM, n-decyl- β -D-maltoside
 DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine
 DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
 DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoserine
 DTT, dithiothreitol
 HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
 HPLC, high performance liquid chromatography
 GDP, guanosine diphosphate
 GTP, guanosine 5'-triphosphate
 GTP γ S, guanosine 5'-3-O-(thio)triphosphate
 Gt, rod photoreceptor G protein (transducin)
 NG, n-nonyl-glucopyranoside
 OG, n-octyl-glucopyranoside
 PDE, phosphodiesterase
 Rho, rhodopsin
 Rho*, photoactivated rhodopsin or Meta II
 ROS, rod outer segment(s)
 SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 TDM, n-tridecyl- β -D-maltoside
 UDM, n-undecyl- β -D-maltoside

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Figure 1.

Gel filtration of reconstituted Rho*-Gt complexes in DDM under various experimental conditions indicated in the bottom right corner of each panel. **A**, (Light) Gel filtration of Gt and Rho exposed to light. **B**, (GTP γ S) Dissociation of Gt from the Rho*-Gt complex after addition of GTP γ S. **C**, (Dark) Gel filtration of a Rho and Gt mixture in the dark. **D**, (NH₂OH) Gel filtration of Gt and Rho exposed to light in the presence of NH₂OH. **E**, (Rho) Gel filtration of free Rho. **F**, (Gt) Gel filtration of free Gt. Fractions (30 μ l) were analyzed by Coomassie blue-stained SDS-PAGE. * - Indicates fractions containing Rho*-Gt complex. # - Indicates fractions containing free Gt. ◆ - Indicates fractions containing free Rho. Presented gels are representative of three independent experiments.

Figure 2.

Gel filtration of Rho*-Gt complexes obtained by mixing Rho and Gt together at different molar ratios (1:4, 1:1, 2:1, 4:1). **A**, Fractions (30 μ l) were analyzed by Coomassie blue-stained SDS-PAGE. * - Indicates fractions containing the Rho*-Gt complex. **B**, Quantification of band intensities corresponding to Gt α , Gt β and Rho* in fractions collected from the gel filtration column. * - Indicates fractions containing a 1:1 molar ratio of Rho* to Gt. The results suggest that an excess of Rho in the original sample results in formation of a 1:1 Rho*:Gt complex. Presented gels are representative of three independent experiments.

Figure 3.

Gel filtration of Rho*-Gt complexes in the presence of phospholipids at a Rho:lipid molar ratio 1:100. DOPC, DOPE, a 1:1 mixture of DOPC and DOPE, DOPS and asolectin were examined. **A**, Fractions (30 μ l) obtained from gel filtration analyzed by Coomassie blue-stained SDS-PAGE. * Indicates fractions containing the highest concentration of Rho*-Gt complex. **B**, Quantification of band intensities corresponding to Gt α , Gt β and Rho* in peak fractions containing the Rho*-Gt complex (right panels) and calculated Rho*:Gt molar ratios in these fractions (tables, left panels). Results indicate that phospholipids increase the content of Rho in fractions containing the Rho*-Gt complex resulting in molar ratios of Rho* to Gt of greater than three, however asolectin provided a ratio of ~2. Presented gels are representative of three independent experiments.

Figure 4. Phospholipid/Rho* ratios in fractions of reconstituted Rho*-Gt complexes isolated by gel filtration either without (No) or with prior addition of phospholipids (DOPS or DOPC/DOPE) (left). Phospholipid/Rho* ratios in fractions of native Rho*-Gt complexes isolated either by gel filtration (GF) or sucrose gradient ultracentrifugation (SG) are shown for comparison (right). Average numbers of phospholipids molecules per single Rho molecule were calculated from three independent experiments.

Figure 5.

Intrinsic fluorescence increase of the Gt_{α} subunit caused by interaction with photoactivated Rho^{*} without and with addition of synthetic phospholipids or lipids isolated from ROS membranes. Zn^{2+} -extracted Rho (25 nM) was mixed with phospholipids at a 1:100 molar ratio in buffer composed of 20 mM BTP, pH 7.0, containing 120 mM NaCl, 5 mM $MgCl_2$, and 1 mM DDM, and incubated for 2 h at room temperature. Then Gt was added (250 nM) and samples were illuminated for 30 s. After 300 s of recording, 5 μ M $GTP\gamma S$ was added. Reactions were carried out in the above buffer at 20°C in continuously stirred cuvettes. When Gt was activated by ROS membranes, DDM was omitted from the reaction buffer. **A**, Activation of Gt by Rho^{*} without added phospholipids (black line), in the presence of DOPE (blue line), in

the presence of DOPC (purple line), in the presence of DOPS (green line), and in the presence of lipids isolated from ROS membranes (dark cyan line) (upper panel). Activation of Gt by Rho* in ROS membranes (red line) (lower panel). Relative activation rates were calculated from three independent experiments. **B**, Comparison of the relative activation rates. Rates of Gt activation were greater in the presence of phospholipids suggesting an important role of lipids in the Rho*-Gt signaling complex.

Figure 6.

Isolation of native Rho*-Gt complex solubilized in DDM by sucrose gradient ultracentrifugation. Fractions 1–13 (250 μ l each) were collected from the top to the bottom of a sucrose gradient after overnight ultracentrifugation as described in Materials and Methods. Fifteen μ l of each fraction was analyzed by Coomassie blue-stained SDS-PAGE (upper panels) or immunoblots developed with a mixture of anti-Gt α and anti-Gt β antibodies (lower panels). **A**, Separation of the Rho*-Gt complex from excess Rho*. * - Indicates fractions containing the highest concentrations of the Rho*-Gt complex. **B**, Dissociation of the Rho*-Gt complex by 200 μ M GTP γ S. # - Indicates fractions dissociated by Gt-GTP γ S. **C**, Control separation of free Gt (left panel) and detergent solubilized Rho (right panel) by sucrose gradient

ultracentrifugation. **D**, Quantification of band intensities corresponding to Gt_{α} , Gt_{β} and Rho^* (left) and calculations of $Rho^*:Gt$ molar ratios in fractions containing the Rho^*-Gt complex (right). Preparation of the Rho^*-Gt complex in native lipid-containing membranes and its purification by sucrose gradient ultracentrifugation appear important for stability and proper stoichiometry of this complex. Experiments were repeated at least three times.

Figure 7. Purification of native Rho*-Gt complex solubilized in DDM by gel filtration. **A**, Native Rho*-Gt complex, obtained from ROS membranes and isolated as described in Materials and Methods, was subjected to Sephadex gel filtration chromatography. * - Indicates fractions containing the Rho*-Gt complex. **B**, Dissociation of Gt from the Rho*-Gt complex by GTP γ S. # - Indicates fractions containing free Gt. Fractions (30 μ l) obtained from a gel filtration column were analyzed by Coomassie blue-stained SDS-PAGE (upper panels) and by immunoblots developed with a mixture of anti-Gt α and anti-Gt β antibodies (lower panels). **C**, Quantification of band intensities corresponding to Gt α , Gt β and Rho* in fraction 30 containing

the Rho*-Gt complex. The molar ratio of Rho*:Gt was about 1:1. The experiment was repeated three times.

Figure 8.

Purification of native Rho*-Gt complexes solubilized in different detergents by sucrose gradient ultracentrifugation. Native Rho*-Gt complexes were prepared from ROS membranes and isolated as described in Materials and Methods. OG, NG, DM, UDM and TDM in addition to DDM were used for Rho*-Gt complex solubilization. Fractions 1–13 (250 μ l each) were collected from the top to the bottom of sucrose gradients after overnight ultracentrifugation. Fifteen μ l of each fraction was analyzed by Coomassie blue-stained SDS-PAGE. * - Indicates fractions containing the highest concentrations of the Rho*-Gt complex. Alkyl-maltoside detergents (DM, DDM, UDM, and TDM) protected the purified Rho*-Gt complexes and separated them from the excess of illuminated Rho* better than the alkyl-glucoside detergents (OG and NG). Presented gels are representative of three independent experiments.

Figure 9.

Gel filtration of Rho*-Gt complexes, free Rho and free Gt in NG (A), DM (B) or TDM (C) in the absence or presence of asolectin. Fractions (30 μ l) obtained by gel filtration were analyzed by Coomassie blue-stained SDS-PAGE and those containing the highest concentrations of protein are indicated with an asterisk. * - Indicates fractions containing Rho*-Gt complex. # - Indicates fractions containing free Gt. ♦ - Indicates fractions containing free Rho. Presented gels are representative of three independent experiments.