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REVIEW ARTICLE

Complexes between photoactivated rhodopsin and transducin: progress and questions

Beata JASTRZEBSKA[1], Yaroslav TSYBOVSKY and Krzysztof PALCZEWSKI[1]

Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965, U.S.A.

Activation of GPCRs (G-protein-coupled receptors) leads to conformational changes that ultimately initiate signal transduction. Activated GPCRs transiently combine with and activate heterotrimeric G-proteins resulting in GTP replacement of GDP on the G-protein α subunit. Both the detailed structural changes essential for productive GDP/GTP exchange on the G-protein α subunit and the structure of the GPCR–G-protein complex itself have yet to be elucidated. Nevertheless, transient GPCR–G-protein complexes can be trapped by nucleotide depletion, yielding an empty-nucleotide G-protein–GPCR complex that can be isolated. Whereas early biochemical studies indicated formation of a complex between G-protein and activated receptor only, more recent results suggest that G-protein can bind to pre-activated states of receptor or even couple transiently to non-activated receptor to facilitate rapid responses to stimuli. Efficient and reproducible formation of physiologically relevant, conformationally homogenous GPCR–G-protein complexes is a prerequisite for structural studies designed to address these possibilities.

Key words: G-protein-coupled receptor (GPCR), heterotrimeric G-protein, photoactivated rhodopsin, transducin (G_t).

INTRODUCTION

The discovery of molecular events involved in visual transduction, especially in rod photoreceptor cells, has led to an increased understanding of GPCR (G-protein-coupled receptor) signalling in general. The critical first step in this process is the interaction between the G-protein and its cognate receptor. Despite many studies of GPCR signalling effects, highly purified native GPCR–G-protein complexes have not been available for detailed structural characterization. Recently this situation has changed such that various forms of the rhodopsin–transducin (G_t) complex can be used for this purpose.

THE Rho*–G_t COMPLEX FORMATION MECHANISM

Rho (rhodopsin) is a prototypical GPCR that is activated by light and, through its cognate heterotrimeric G-protein, G_t, initiates the visual signalling cascade. A photon of light captured by Rho causes isomerization of the Rho chromophore, 11-*cis*-retinal, to all-*trans*-retinal. As a result of this photoreaction, Rho changes its conformation to form activated Rho* (Meta II), which binds to and activates G_t [1–4]. Structural changes in the Rho molecule stimulates dissociation of GDP from its nucleotide-binding pocket in the G_t α subunit followed by formation of a transient complex between activated Rho* and G_t with an empty nucleotide-binding pocket. If no GTP is available, this isolated 'high-affinity' transient complex is stable for hours. However, it never accumulates within the cell because GTP binding is rapid and irreversible, causing Rho*–G_t complex disruption, as well as dissociation of the G_t heterotrimer to its α and $\beta\gamma$ subunits which then activate different downstream pathways (Figure 1). Although the structures of both Rho and a chimaeric variant G_t have been solved, the molecular mechanism of this receptor-mediated G-protein activation is not fully understood.

COMPLEXES BETWEEN Rho* INTERMEDIATES AND G_t

A number of different Rho intermediates that occur after light illumination have been identified spectroscopically [1–4], and several of these were also crystallized [5–10]. Among them Meta II, which exists in equilibrium with its precursor Meta I, is crucial for G_t activation [11]. Because G_t preferentially binds Meta II, this equilibrium shifts towards Meta II formation in the presence of G_t, indicating coupling of G_t to Rho*. Excess Meta II formed in the presence of G_t is called 'extra' Meta II. Extra-Meta II does not accumulate in the presence of GTP, which causes complex dissociation. GDP has a negative effect on the formation of extra-Meta II as well, suggesting that G_t associated with extra-Meta II must be free of bound nucleotide [4].

The existence of two forms of Meta II was proposed by Arnis and Hofmann [12]. These are Meta IIa (also called Meta II) formed within milliseconds after deprotonation of the Schiff base, and Meta IIb (also called Meta II H^+) formed more slowly by proton uptake from the aqueous solution that is dependent on pH, temperature and ionic strength. In fact, experiments with a pH-sensitive dye showed that after Rho photolysis, the aqueous environment initially is more acidic because of proton release and then it becomes more basic due to proton uptake. As shown recently, both Meta IIa and Meta IIb can bind a G_t-derived peptide [13]. In fact, G_t activation is pH-dependent, with higher activation rates observed at a lower pH, indicating the importance of proton uptake for tight binding between Meta II and G_t, and rapid signal transduction. Thus the existence of two species of Meta II, namely Meta IIa and Meta IIb,

Abbreviations used: AFM, atomic force microscopy; BRET, bioluminescence resonance energy transfer; DDM, dodecyl-β-D-maltoside; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; G_t, transducin; GTP[S], guanosine 5′-[γ-thio]triphosphate; HDL, high-density lipoprotein; LHR, luteinizing hormone receptor; NSOM, near-field scanning optical microscopy; PE, phosphatidylethanolamine; Rho, rhodopsin; Rho*, activated Rho*; SPR, surface plasmon resonance.

[1] Correspondence may be addressed to either of these authors (email kxp65@case.edu or bxj27@case.edu).

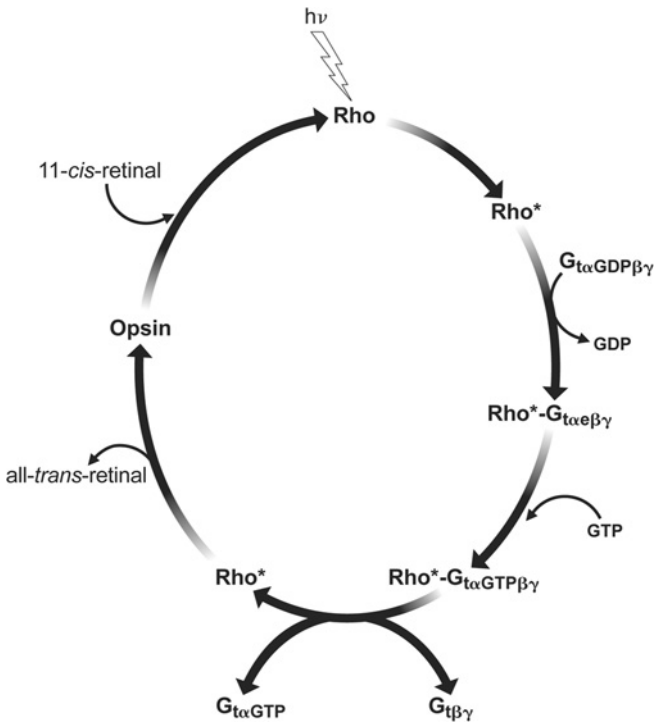

Figure 1 Schematic diagram of the reaction pathway for G_t activation

Light causes transition of dark state Rho to activated Rho* that binds heterotrimeric $G_{t\alpha GDP\beta\gamma}$. This binding induces GDP release from the G_t nucleotide-binding pocket and formation of the transient nucleotide-free Rho*–$G_{t\alpha e\beta\gamma}$ complex. Uptake of GTP then leads to formation of the Rho*–$G_{t\alpha GTP\beta\gamma}$ complex with subsequent release and dissociation of $G_{t\alpha GTP}$ and $G_{t\beta\gamma}$ from Rho*. Free Rho* then decays to opsin and all-*trans*-retinal. Subsequent uptake of 11-*cis*-retinal completes the cycle by regenerating Rho.

is thought to be required for full catalytic activity involving nucleotide exchange in G_t [14]. It should be kept in mind, however, that these experimental results were obtained by substituting typically low-affinity G-protein peptide fragment(s) for the whole heterotrimeric G-protein complex, so their extrapolation to the naturally occurring complex could be questioned. Moreover, the quality of the protein preparations is also critical for such studies. The vast majority of work in this field was performed with the G-protein α subunit reconstituted with its $\beta\gamma$ subunits based on the assumption that the resulting trimeric complex would be identical with the one existing *in vivo*. However, recent studies by Goc et al. [15] demonstrated differences in conformation and heterotrimer complex formation between reconstituted and native G_t preparations along with altered stability of the reconstituted G_t, which assembled differently from the native protein [15]. This work suggested that G_t extracted and purified without subunit dissociation appears to be more appropriate for future studies. These observed differences presumably arose from post-translational modifications of G-proteins, such as palmitoylation, myristoylation and isoprenylation of the α and γ subunits. Once a G-protein is extracted from membranes and dissociated into subunits, these hydrophobic groups are not likely to be hydrated and may instead bind preferentially to the protein core of its cognate polypeptide chain rather than protrude into the aqueous medium. Thus it is highly improbable that these groups will properly reorient themselves in the reconstituted complex in precisely the same way as they exist in native membranes. Moreover, G_t subunits expressed in insect cells or bacteria have no post-translational modifications at all so their biochemical and biophysical properties might differ from those of native vertebrate proteins.

For many years, Meta II was considered to be the only Rho intermediate that binds G_t after photoactivation and it was postulated that it undergoes large structural changes to permit this coupling and stimulate nucleotide dissociation (reviewed in [2]). However, subsequent studies by the Shichida group [16–19] identified an intermediate other than Meta II which interacted with G_t in its GDP-bound state without stimulating nucleotide dissociation from the G_t α subunit. This Meta II precursor, named Meta Ib, had an absorption maximum that was shifted $\sim$20 nm towards shorter wavelengths of the spectrum as compared with that of previously identified Meta I (presently called Meta Ia) [16–19]. Transient stabilization of this complex was observed in the presence of excess GDP that simultaneously inhibited excess Meta II accumulation. Stabilization of the Meta Ib–G_t–GDP complex was sustained even in the presence of GTP, whereas stabilization of the Meta II–G_t complex was abolished. The binding affinity of Meta Ib and G_t–GDP was approx. 2-fold lower than that of Meta II and nucleotide-free G_t. According to these results, Meta Ib clearly binds to G_t in a different manner from that of Meta II.

Different forms of Rho were identified because 11-*cis*-retinylidene, the chromophore bound to opsin, as well as its isomerization product, all-*trans*-retinylidene, are both optically active in the visible spectrum of light. However, directly correlating changes in the absorption spectrum with changes in protein conformation can be misleading, as was well illustrated in the case of structures of Rho, lumirhodopsin (the deprotonated form of Rho*) and the Meta I form of Rho (reviewed in [20,21]). Whereas the absorption spectra of these proteins differ dramatically in the visible range, they do not reflect significant structural changes. Moreover, if any structural changes do occur, they are within the 1–3 Å (1 Å = 0.1 nm) fluctuation of a typical protein polypeptide at physiological temperature [22–24]. Indeed, only an indirect relationship exists between measured spectroscopic changes observed for a protein and the actual alterations in its structure. This generalization applies spectroscopic techniques such as UV–visible absorption spectroscopy, fluorescent spectroscopy and EPR (electron paramagnetic resonance).

More recently, FTIR (Fourier-transform infrared resonance) spectroscopic studies involving binding of the G_t C-terminal peptide to Rho* demonstrated clear differences between the active site conformation of Meta II stabilized by this peptide and the active-site conformation stabilized instead by the agonist, all-*trans*-retinal [25]. These results strongly indicate that allosteric changes in Rho, as well as in G_t, are important in the regulation of photoactivation and mediation of the light signal. However, a realistic picture of the Rho*–G_t complex assembly with detailed structural arrangements of both components can only be obtained after the fine structure of the complex is solved.

COMPLEXES BETWEEN DARK Rho AND G_t

How do membrane-associated signalling proteins transfer stimuli affecting one side of a membrane to effector proteins located on the opposite side? One hypothesis is that interactions between receptors, mediators and effectors occur through ‘collisional coupling’ and free diffusion [26]. This idea stemmed from early classical work on phototransduction in mammalian rod cells. However, in many G-protein-mediated signalling pathways activation is rapid, with responses occuring within milliseconds to seconds. Thus a ‘physical scaffold’ hypothesis suggesting direct or indirect interactions of specific protein components has also been proposed [27].

The simple observation that G_t associates with membranes under ionic conditions closely reflecting the physiological state

indicates that G_t proteins in the cell must be in close proximity to membranes, and therefore to Rho as well. Such an arrangement would permit rapid G_t binding and signal transmission to specific effectors. However, this binding cannot be too stable because amplification of the signal requires that a single activated receptor catalyses the exchange of nucleotide on 10–100 G-protein molecules [28].

Historically, Rho was the first GPCR purified to homogeneity in the 1970s, and its functional coupling to G_t, the first isolated heterotrimeric G-protein, was demonstrated in the early 1980s [29,30]. G_t was identified by first testing enzymatic activities in a mixture of proteins extracted from rod outer segment membranes and then by recombining these soluble proteins with washed disc membranes. These experiments showed that the interaction of G_t with disc membranes depended on ionic strength, light, pH and temperature. At high ionic strength, G_t remained bound to membranes under dark conditions and could be extracted in low-ionic-strength buffer. Light illumination of rod outer segment membranes induced G_t binding independently of ionic strength, either at low or high (1 M) salt concentrations [31,32]. Magnesium ions enhanced binding between rod outer segment membranes and G_t [31], and binding was achieved in a pH range of 5.8–8.4 [33]. After binding occured, G_t became soluble in a spontaneous slow reaction that was highly dependent on temperature, such that it did not occur at 0 °C and required approx 1 h at 20 °C [29,31]. This probably relates to conformational changes in Rho* structure that led to its relaxation and chromophore release. A fraction of G_t bound to membranes after light activation could be extracted by GTP or its analogues in low-ionic-strength buffer [29].

The affinity of G_t for Rho in rod outer segment in the dark is low, with a calculated dissociation constant of approx. 10 μM [34]. Light illumination of Rho and its activation causes structural changes that open the nucleotide-binding pocket in G_t, enabling the release of GDP and increasing the affinity of Rho* for nucleotide-free G_t to 0.9 nM, as compared with 200 nM for G_t–GDP. This indicates that the association of Rho* with G_t in the absence of nucleotide is extremely tight [35]. A structural comparison of deprotonated Rho* with dark Rho provided evidence that light receptors in both states share a common recognition docking mode for heterotrimeric G_t [36]. Other evidence derived from plasmon waveguide resonance spectroscopy showed a G_t binding affinity constant of $\sim$60–64 nM to dark state Rho and 0.7 nM to Rho* reconstituted into an egg PC (phosphatidylcholine) bilayer. This might suggest that the existence of bound complexes between Rho and G_t under dark conditions assures an immediate specific response to a light stimulus and possibly increases the effectiveness of signal transduction [37,38].

Real-time studies conducted on living cells using FRET (fluorescence resonance energy transfer) or BRET (bioluminescence resonance energy transfer) techniques to examine interactions between different forms of GPCRs with specific heterotrimeric G-proteins confirmed the possible existence of complexes formed before receptor activation [39,40]. This in turn may suggest that functional interactions of receptor–G-proteins are regulated by conformational changes rather than an association–dissociation cycle. Moreover, NMR studies corroborate that the same structural changes occur in G_t during heterotrimer reconstitution as those observed upon formation of the G_t 'transition/activation' state $\{GDP \cdot AlF^{4-}/Mg^{2+}$ and GTP[S] (guanosine 5′-[γ-thio]triphosphate)/$Mg^{2+}\}$, suggesting that the G_t $\beta\gamma$ subunit is required for activation of the G_t α subunit prior to the interaction with Rho* [41]. This finding provides further evidence that signalling proteins are prepared for fast action before the activating signal arrives.

Based on the above findings, one can also speculate that a massive rearrangement of helices in Rho* is not required for coupling with G_t as proposed previously [42,43], but rather that subtle structural changes are essential for GDP/GTP exchange. These changes may involve protonation/deprotonation of key residues, reactions catalysed by internal water molecules [44–46].

COMPLEXES BETWEEN Rho* AND $G_{t\alpha}$ AND $G_{t\beta\gamma}$

Early studies on interactions between Rho* and G_t conducted by Kühn and Chabre [47] revealed that only heterotrimeric G_t binds to rod outer segment membranes with high affinity in a light-dependent manner. $G_{t\alpha}$ in the absence of $G_{t\beta\gamma}$ remained soluble, and only approx. 15 % of the total added $G_{t\beta\gamma}$ associated with rod outer segment membranes at high ionic strength. However, mixing of $G_{t\alpha}$ and $G_{t\beta\gamma}$ in a 1:1 ratio stimulated subunit association and significantly increased their binding affinity to light-activated Rho* [47]. This observation was confirmed by subsequent studies of ^{125}I-labelled $G_{t\alpha}$ binding to urea-stripped rod outer segment membranes. $G_{t\alpha}$ exhibited 1000-fold weaker binding than heterotrimeric G_t under either dark or light conditions. Addition of $G_{t\beta\gamma}$ to a mixture of $G_{t\alpha}$ and rod outer segment membranes significantly increased association of $G_{t\alpha}$ to the membranes, indicating that G_t binds to Rho* in a co-operative manner [48].

Kinetic analysis of G_t coupling to Rho* measured by SPR (surface plasmon resonance) showed independent binding of 0.65 μM $G_{t\beta1\gamma1}$ with a specific binding signal of 41 resonance units, whereas almost no binding was seen with 0.48 μM $G_{t\alpha}$ (the specific binding signal was only 2 resonance units). However, a mixture of G_t subunits produced more than an additive signal of 583 resonance units. Thus the binding affinity of $G_{t\alpha}$ is significantly increased by the $\beta\gamma$ subunit [49], which agrees with previous observations [47,48].

Rho*–G_t BINDING STOICHIOMETRY

Studies of the composition of rod outer segment proteins, as well as their interactions in the light-triggered activation cascade, revealed that rod outer segment membranes contain approx. ten Rho molecules per one G_t molecule [32,47]. Considering that Rho occupies approx. 50 % of the surface of rod outer segment membranes and that the footprint of a G_t molecule is approx. 4-fold that of a Rho molecule, the surface occupied by G_t constitutes approx. 20 % of all the available membrane surface in this segment of the cell (Figure 2). Such a high density of G_t in a physiological setting would probably promote organization of G_t into larger clusters, as has been proposed for G-proteins in general [50,51] and G_t specifically [52,53].

The stoichiometry and kinetics of Rho and G_t association was studied by Kühn et al. [54] who recorded near-IR light scattering of native rod outer segment membrane suspensions. In the absence of GTP, light caused an increase of scattering that became saturated when $\sim$10 % of Rho was bleached, which is approximately equal to the amount of G_t present in rod outer segment membranes. This suggested that the binding stoichiometry between illuminated Rho and G_t is 1:1. The observation was confirmed in a reconstituted system wherein purified G_t was mixed with washed rod outer segment membranes. G_t was added to achieve from 0.25- to 4-fold the native G_t/Rho ratio. Binding saturation was reached when the molar amount of bleached Rho was equal to that of G_t, again suggesting a 1:1 binding stoichiometry between these components [54].

Conversely, research conducted by two independent groups on light-dependent interactions between G_t and either Rho in

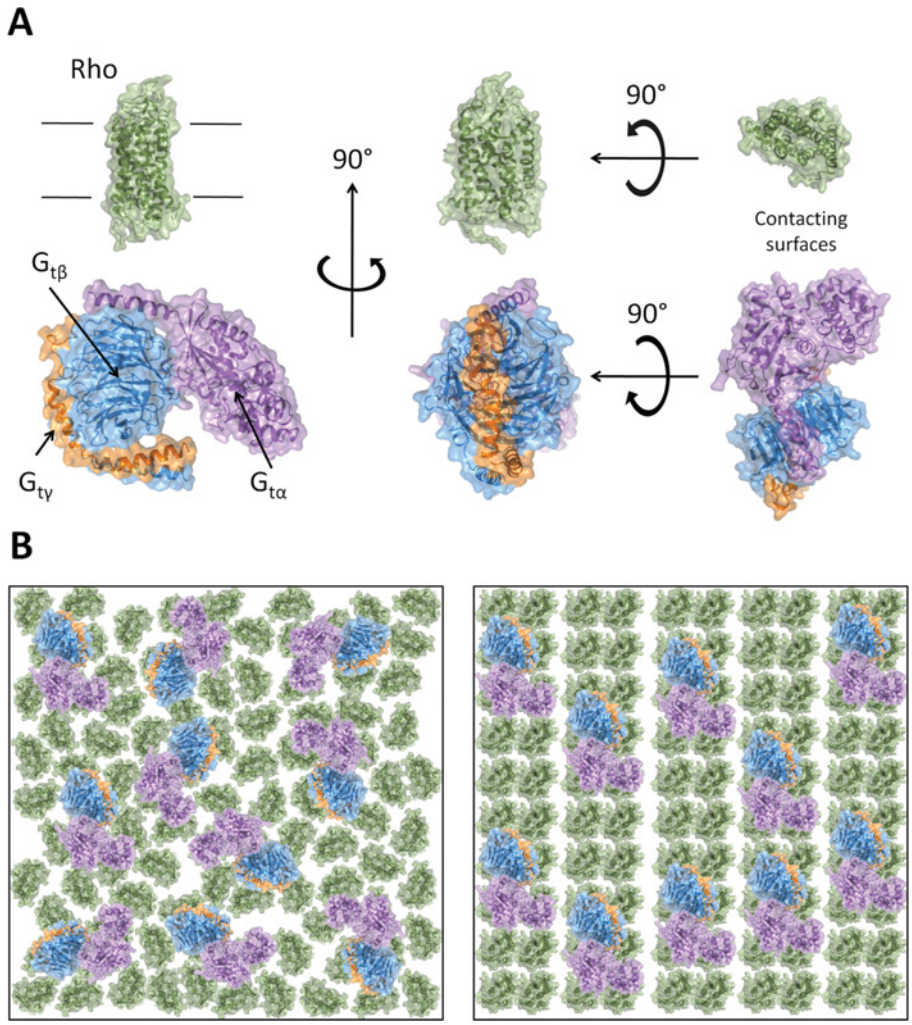

Figure 2 Possible organization of the Rho*–G_t complex in rod outer segment membranes

(**A**) Size comparison of a Rho monomer (PDB ID: 1F88) and a G_t molecule (PDB ID: 1GOT). The approximate position of the lipid bilayer is shown with black lines. Location of the contacting surfaces is based on modelling studies [52]. The area of projection of G_t on the plane of the membrane is approximately four times that of Rho. A three-dimensional interactive version of this Figure is available at http://www.BiochemJ.org/bj/428/0001/bj4280001add.htm. (**B**) Rho*–G_t complexes on the surface of rod outer segment membranes composed of randomly distributed Rho monomers (left-hand panel) and Rho dimers organized in rows (right-hand panel), as suggested by AFM studies [68]. Rho occupies approx. 50 % of the surface of the membrane. The molar ratio of Rho monomer to G_t is ~10:1 [32,47]. Tight packing of Rho molecules favours their tight organization to reduce the total occupied area and avoid steric clashes. A high abundance of G_t molecules on the surface of the membrane may promote formation of G_t clusters.

urea-washed membranes or purified Rho reconstituted into lipid vesicles suggests that oligomeric forms of Rho may be involved in interactions with G_t, wherein at least two or even four receptors provide surfaces for G_t binding [55,56]. Oligomeric association of G_t with Rho* was also a conclusion derived from studies that employed an indirect light-scattering technique [35]. In other studies, binding of G_t to Rho* incorporated in a thin membrane film measured by SPR spectroscopy revealed a molar ratio of G_t bound to Rho* of ~0.6 at saturating concentrations of both proteins, implying a coupling stoichiometry of approx. one G_t per two Rho molecules [37].

Mapping the interacting surfaces between Rho* and G_t that could shed light on the nature and stoichiometry of the activated complex was further pursued by other approaches. First, the Rho*–G_t complex interface was studied in a competition assay involving different peptides from Rho [57,58]. However, it is highly probable that small fragments of Rho may not properly mimic the physiological conformation of Rho loops. In a different approach, Khorana's group [59–61] pioneered the use of site-directed cysteine mutagenesis and cross-linking methods to identify residues essential for the interaction between Rho* and G_t. Their studies showed that the second and third loops, as well as cytoplasmic helix 8 on the Rho* surface, are important for G_t binding. G_t was docked to Rho* by the C-terminus and residues within the $\alpha 4$–$\beta 6$ loop of $G_{t\alpha}$ [59–61]. Other studies using competition assays and cross-linking of the C-terminal peptide of $G_{t\alpha}$ (residues 340–350) confirmed that the C-terminal region of $G_{t\alpha}$ is involved in binding to the Rho* receptor, thereby stabilizing the Meta II conformation [62,63]. Biochemical experiments have shown that the $G_{t\beta\gamma}$ subunit directly associates with different parts of Rho cytoplasmic helix 8 [64–67]. Notably, the interface engaged in the complex probably encompasses the entire cytoplasmic surface of Rho. Thus focusing on specific short regions of G_t or Rho may lead to oversimplification of this interaction.

Several different observations such as (i) spectacular images of Rho oligomers in native photoreceptor disc membranes visualized by AFM (atomic force microscopy), (ii) the presence

of Rho dimers in cell plasma membranes wherein Rho was heterologously expressed [68–74], (iii) the relative areas of the Rho and G_t surfaces, and (iv) the fact that Meta II is stabilized by C-terminal fragments of $G_{t\alpha}$ and $G_{t\gamma}$ subunits, all suggest that a Rho*–Rho dimer might be the functional unit required for G_t binding and its activation [52,75,76]. Although Rho monomers in DDM (dodecyl-β-D-maltoside) did activate G_t, activation rates were much greater when G_t was activated by rows of Rho dimers in HDM (hexadecyl-β-D-maltoside), similar to its activation by Rho in rod outer segment membranes [77]. Recent work on lateral diffusion of Rho in photoreceptor membranes [78] supports the model of binding one G_t to at least two receptors and explains mistakes made in an earlier work on this topic (referenced in [78]). Even as one Rho is photoactivated, the other serves as a platform for proper accommodation of the G_t molecule [52]. More recent studies based on mesoscopic Monte Carlo simulations of stochastic encounters between Rho* and G_t in disc membranes suggest that the high density of Rho and its highly ordered packing would provide a kinetic advantage for rapid photoresponses as compared with freely diffusing randomly distributed monomers [79].

Apparently contradicting the above results, subsequent experiments wherein either Rho monomers or Rho dimers were incorporated into HDL (high-density lipoprotein) particles proved that Rho monomers can efficiently activate G_t, suggesting an important role of lipids for G_t anchoring. Indeed, Rho dimer incorporated into HDL activated G_t at lower rates than the monomer [80,81]. But an alternative explanation for the above result could be formation of antiparallel dimers during reconstitution into a lipid bilayer, similar to what was observed in the first crystal structure of Rho [82]. Moreover, it was recognized that the structural arrangement of protein and lipids in nascent HDL is an antiparallel double superhelix wrapped around an ellipsoidal lipid phase [83], instead of idealized 'discs'. This could perturb G_t activation by affecting the dimer-binding surface. The crystal structure of the receptor–G_t complex must be solved in order to obtain a clear picture of Rho and G_t surfaces involved in the complex.

On a separate note, the question of whether GPCR monomers can activate G-proteins may be of secondary importance. In particular, if monomers rarely occur in the native state, the above model studies do not differ significantly from earlier studies demonstrating that a short peptide, mastoparan, activated G-proteins (for example, see [84]). The problem of extremely slow G_t kinetics *in vitro* (1000–10000 lower kinetic rates as compared with physiological responses) also complicates data interpretation. Thus differences found in the rates of activation between different preparations (e.g. mutant GPCRs, mutant G-proteins, nucleotide and metal ion conditions) noted with *in vitro* assays are likely to be misinterpreted.

ISOLATION OF Rho*–G_t COMPLEXES

Isolation of the Rho*–G_t complex in its natural state is challenging because of its dynamic characteristics. One method to achieve this objective is separation of the complex by gel-filtration chromatography from a mixture of the two purified proteins after light illumination and appropriate incubation. Gel-filtration chromatography facilitates the separation of proteins and their complexes according to differences in molecular mass. Although this technique is quite simple for soluble proteins, membrane proteins need first to be solubilized and processed in detergent solutions that may cause disruption of fragile complexes and influence the resolution of separation. A number of detergents have been tested for their effects on the stability of the Rho*–G_t complex and its isolation by gel-filtration chromatography [85]. Only maltosides such as decyl-β-D-maltoside, DDM and tetradecyl-β-D-maltoside were found to be appropriate for keeping Rho* and G_t tightly bound together, whereas glucosides (octyl-β-D-glucoside and nonyl-β-D-glucoside) destroyed this complex. Stability of the Rho dimer was also adversely affected by increasing concentrations of detergent. However, addition of phospholipids helped to stabilize both Rho*–G_t complexes and Rho oligomeric organization [77,86]. Phospholipids may provide a native-like hydrophobic environment for optimal incorporation of Rho dimer or they may interact with detergent micelles to decrease detergent concentrations and stimulate Rho self-association [73]. The headgroups, as well as the acyl chains, of particular phospholipids had significant effects on both Meta II formation [87–89] and G-protein anchoring [90–92]. PE (phosphatidylethanolamine) and negatively charged PS (phosphatidylserine) with polyunsaturated hydrocarbon side chains favoured the formation of Meta II. Enhanced G_t binding affinity to Rho* in the presence of PE has also been observed [38,93]. Therefore phospholipids most probably stabilize the quaternary organization of Rho for better G_t docking and membrane anchoring that together guarantee rapid responses to light and rapid signal transduction.

To overcome problems concerning reconstitution of Rho*–G_t from purified components, the complex can be formed by light activation in native rod outer segment membranes, where Rho is properly organized in rows of dimers. Then the complex must be separated from excess Rho* and other contaminating proteins. Indeed, photoactivation of Rho leads to retention of G_t on rod outer segment membranes and Rho*–G_t complex formation. Moreover, GDP released from the G_t nucleotide-binding pocket can be washed out and the captured transitory nucleotide-free complex is stable for hours [94]. Recently Jastrzebska et al. [95] developed a protocol for solubilization of the Rho*–G_{te} complex (where the subscript 'e' denotes an empty nucleotide-binding pocket). This was accomplished by extraction with a maltoside detergent followed by partial purification from excess

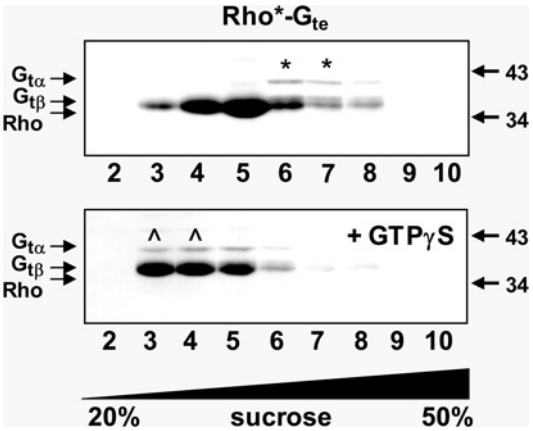

Figure 3 Isolation of Rho*–G_{te} complex by sucrose-gradient centrifugation

Fractions 1–13 (250 μl each) were collected from the top to the bottom of a sucrose gradient after overnight ultracentrifugation. A 20 μl aliquot of each fraction was analysed by SDS/PAGE. Upper panel: separation of the Rho*–G_{te} complex from excess Rho*. Fractions containing the highest concentrations of the Rho*–G_{te} complex are indicated with * . Lower panel: dissociation of the Rho*–G_{te} complex by 200 μM GTP[S]. Fractions containing $G_{tGTP[S]}$ are indicated with ^ . Figure adapted, with permission, from [95], © 2009 The Federation of American Societies for Experimental Biology.

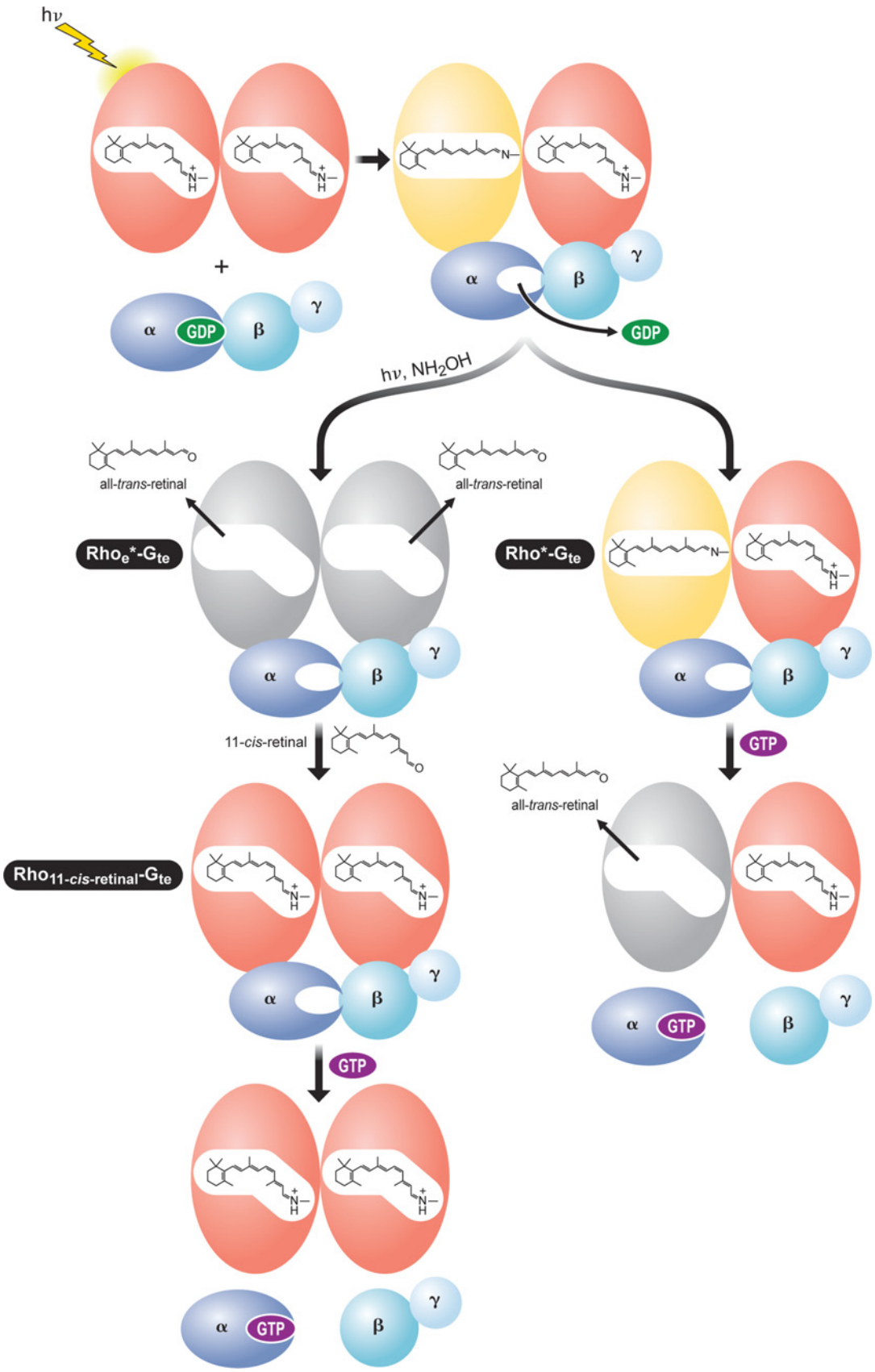

Figure 4 **For legend see facing page.**

Rho*/Rho by sucrose-gradient ultracentrifugation. The Rho*–G_{te} complex in mixed detergent–endogenous lipid micelles had a higher density than Rho itself and migrated further in the sucrose gradient than Rho*/Rho. Because Rho constitutes >90 % of the protein in disc membranes [1], the purity of the isolated complex was typically greater than 95 % (Figure 3). Notably, the protein stoichiometry in this isolated native complex was two Rho* molecules per one G_t molecule, confirming predictions of other studies [52]. Binding of G_t to Rho packed in native membranes, where its conformation was unaffected by detergent, ensured proper complex formation. Moreover, sufficient retention of endogenous phospholipids during the purification procedure helped to stabilize this complex. Therefore this isolated native Rho*–G_{te} complex constitutes high-quality material for future crystallization and other structural studies.

VARIOUS Rho–G_t COMPLEXES AND THEIR STABILITY

Although structures of variously activated individual components of the Rho*–G_t complex have been solved at different resolutions, not much is known about the mechanism by which Rho* binds to its cognate G-protein and catalyses nucleotide exchange. However, there are two events of critical importance for formation of the stable complex between Rho and G_t, namely the activation of Rho by light and the release of GDP from the nucleotide-binding pocket in G_t. Keeping the nucleotide-binding pocket free assures tight binding of these two signalling proteins. In fact, even though G_t is in the nucleotide-free state in the isolated native Rho*–G_{te} complex, the complex still retains the ability to interact with GTP. Rho* in this complex spectrally remains in an equilibrium between the Meta II and Meta I states (deprotonated and protonated photoproducts respectively). The all-*trans*-retinylidene chromophore is stable and bound to the apo-protein via a Schiff base bond. Although light activation causes rapid dissociation of Rho* to opsin and free all-*trans*-retinal, the decay of Rho* in the isolated Rho*–G_{te} complex is completely blocked, and the complex is stable for weeks. This observation strongly suggests a protective role for G_t coupled to Rho* that stabilizes the Rho* conformation and inhibits chromophore hydrolysis and release from the chromophore-binding pocket [95]. However, addition of GTP[S] to a Rho*–G_{te} sample stimulates its dissociation and decay of Rho* from the Meta II to the Meta III photoproduct and eventually to inactive opsin (B. Jastrzebska, unpublished work).

Surprisingly, the Schiff base between apo-protein and all-*trans*-retinylidene in the Rho*–G_{te} complex is accessible to hydroxylamine which promotes hydrolysis of the chromophore. Nonetheless, removal of the all-*trans*-retinylidene from its binding pocket has no effect on either the formation of the Rho_e*–G_{te} complex or its activity and stability. This again indicates that opsin in this complex is conformationally stabilized by the presence of G_t, a situation that differs from free opsin which is unstable in detergent solutions [96–98]. Moreover, opsin (Rho_e) in the complex with empty nucleotide- and retinoid-binding pockets (Rho_e*–G_{te}) could be up to 75 % regenerated with exogenous 11-*cis*-retinal, and the resulting regenerated $Rho_{11\text{-}cis\text{-}retinal}$–$G_{te}$ (Rho–G_{te}) complex was stable and active, even in the presence of 11-*cis*-retinylidene (Figure 4) [95]. This observation strongly suggests that once the complex is formed, Rho is stabilized by the presence of G_t and a variety of complexes between these two proteins can be formed. However, structural differences between these complexes remain to be defined. One might expect even more heterogeneity in complex formation in view of Rho dimerization/oligomerization. As previously mentioned, G_t can pre-couple to Rho in its ground conformation to form a Rho/Rho–G_t complex, but two different complexes could be created after light stimulation (Rho/Rho*–G_t or Rho*/Rho*–G_t) because either one Rho or two Rho molecules could be activated in the Rho dimer. Considering that one G_t molecule has a footprint as large as four Rho molecules, even more variety might be expected (Figure 2) [52]. Many experimental results support the functional role of GPCR asymmetry. Even as activation of only one Rho molecule is needed for the neuronal response in the brain, it is hard to believe that one G_t interacts with just one Rho molecule, because in native disc membranes Rho exists as dimers tightly packed in higher-order oligomers (Figure 2). The concept of GPCR asymmetry and its functional role in G-protein activation is supported by studies on other receptors such as BLT1 (leukotriene B_4 receptor), $GABA_B$ (γ-aminobutyric acid B), taste receptor T1R and the metabotropic glutamate receptor mGluR (for a review see [99]).

Over the last several years, the idea that GPCRs exist and function as dimers or higher-order oligomers has been favoured. Many biochemical and biophysical experimental approaches such as co-immunoprecipitation, blue native electrophoresis, analytical centrifugation, FRET, BRET and AFM apparently support this concept. Most of these experiments have been conducted *in vitro* on isolated proteins or *in situ* with cell lines expressing high non-physiological amounts of receptor that may possibly force its oligomerization in the plasma membrane, so the findings may not apply to native systems. Thus *in vivo* studies in living animals must be designed very carefully to demonstrate adequately the functional importance of GPCR dimers. Very recently an elegant *in vivo* study by Rivero-Müller et al. [100] on LHR (luteinizing hormone receptor), which belongs to the rhodopsin-like family, demonstrated intermolecular co-operation in signalling between the two receptors comprising a dimer [100]. The authors generated mice with a LHR-knockout background that co-expressed two LHR mutants, one deficient in hormone binding and the other deficient in signalling. In contrast with the original LHR-knockout mice, which displayed hypogonadism and infertility, the newly generated mice exhibited normal sexual development, as well as normal gametogenesis and reproductive behaviour. This observed complementation between the two disabled types of receptors stresses the functional importance of GPCR dimerization. This elegent *in vivo* strategy can be further employed to demonstrate the physiological relevance of oligomeriation for other GPCRs.

Along with *in vivo* studies, various high-resolution imaging methodologies should be explored to show the existence of GPCR

Figure 4 Schematic representation of differing complexes formed between Rho* and G_t

Light illumination triggers structural changes in Rho and allows the binding of G_t to the Rho*–Rho dimer. In the pathway on the right-hand side, activation of one Rho subunit in the dimer suffices to induce G_t association, which stimulates GDP release from the G_t nucleotide-binding pocket. This results in formation of the Rho*–G_{te} complex with a free nucleotide-binding pocket. However, loading of GTP causes complex dissociation. In the pathway on left-hand side, Rho*–G_{te} is accessible to hydroxylamine that promotes all-*trans*-retinylidene chromophore hydrolysis and dissociation from the chromophore-binding pocket. This results in formation of the complex with the empty chromophore-binding pocket and an empty nucleotide-binding pocket in Rho_e*–G_{te}. The Rho_e*–G_{te} complex can be regenerated with 11-*cis*-retinylidene resulting in a Rho–G_{te} complex that can be dissociated in the presence of GTP. Red ovals represent Rho in the dark state, yellow ovals represent photoactivated Rho* (Meta II), grey ovals represent opsin (Rho_e) and blue ovals represent heterotrimeric $G_{t\alpha\beta\gamma}$. An animated version of this Figure is available at http://www.BiochemJ.org/bj/428/0001/bj4280001add.htm.

di-/oligo-mers *in vivo*. The most powerful techniques so far are AFM and NSOM (near-field scanning optical microscopy), which allow the visualization of single proteins or protein clusters in their native environment. For example, AFM was successfully used to show bacteriorhodopsin in membranes of *Halobacterium salinum* [101], functionally related conformational changes for the channel protein OmpF porin from *Escherichia coli* [102] and rhodopsin dimers organized in oligomers in native photoreceptor disc membranes [68]. In its turn, NSOM has been applied to visualize the cell surface of neonatal and embryonic cardiac myocytes isolated from mice. This study demonstrated that functional β-adrenergic receptors are organized into small clusters of at most five molecules, suggesting the importance of oligomerization for this receptor [103]. Increasing the resolution of the above techniques could be instrumental in visualizing other receptors from the GPCR family.

GPCRs are targets for various extracellular stimuli including light, calcium, nucleotides, amino acids, odorants, pheromones and neurotransmitters. Abnormal GPCR function is associated with many diseases, making GPCRs the most common targets for drug discovery and development. Thus understanding the detailed structure and precise function of these receptors and their partner G-proteins is critical to designing more selective medicines and improving patient therapy.

CONCLUSIONS AND PERSPECTIVES

Complexes between Rho and G_t have been investigated for about 30 years. These studies include discovering the stimuli for their formation, determining the requirements for their stabilization, revealing their stoichiometry and binding affinities, establishing structural changes in both proteins required for active complex formation and defining the roles of these complexes in downstream signalling. Several critical observations have been made.

G_t binds to Rho* with high affinity (<1 nM) but it can also bind to dark-adapted Rho with an affinity of ~ 60 nM to 1 μM. After light illumination, Rho undergoes a series of transitions before reaching its activated state, and some of the intermediates can also form complexes with G_t.

Activation of a single Rho molecule is sufficient to trigger a physiological response, and a single Rho molecule has also been shown to effectively catalyse GDP/GTP exchange in G_t [80,81,104,105]. However, as spectacularly revealed by AFM, the latter is unlikely to happen in rod outer segment membranes *in vivo* where Rho is highly organized and tightly packed in rows of dimers surrounded by lipids [53,68–70,72].

Despite the considerable progress to date, we have yet to define the conformation of activated Rho (Meta II), the conformation of activated G_t, the precise structural changes in Rho that allow G_t binding and nucleotide exchange after light stimulation, the interacting surfaces involved in binding, and the *in vivo* Rho functional unit. Thus elucidation of the atomic structure of the Rho*–G_t complex is of primary importance. To achieve this goal, two-dimensional crystallization of Rho* bound to the cognitive G-protein heterotrimer and studies by electron crystallography could be an option. However, a three-dimensional crystal structure of such a complex determined by X-ray crystallography at high resolution would yield the most information.

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