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Structural basis of function in heterotrimeric G proteins

William M. Oldham and Heidi E. Hamm*
Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA

Abstract. Heterotrimeric guanine-nucleotide-binding proteins (G proteins) act as molecular switches in signaling pathways by coupling the activation of heptahelical receptors at the cell surface to intracellular responses. In the resting state, the G-protein α subunit (Gα) binds GDP and G$\beta\gamma$. Receptors activate G proteins by catalyzing GTP for GDP exchange on Gα, leading to a structural change in the Gα(GTP) and G$\beta\gamma$ subunits that allows the activation of a variety of downstream effector proteins. The G protein returns to the resting conformation following GTP hydrolysis and subunit re-association. As the G-protein cycle progresses, the Gα subunit traverses through a series of conformational changes. Crystallographic studies of G proteins in many of these conformations have provided substantial insight into the structures of these proteins, the GTP-induced structural changes in Gα, how these changes may lead to subunit dissociation and allow Gα and G$\beta\gamma$ to activate effector proteins, as well as the mechanism of GTP hydrolysis. However, relatively little is known about the receptor–G protein complex and how this interaction leads to GDP release from Gα. This article reviews the structural determinants of the function of heterotrimeric G proteins in mammalian systems at each point in the G-protein cycle with special emphasis on the mechanism of receptor-mediated G-protein activation. The receptor–G protein complex has proven to be a difficult target for crystallography, and several biophysical and computational approaches are discussed that complement the currently available structural information to improve models of this interaction. Additionally, these approaches enable the study of G-protein dynamics in solution, which is becoming an increasingly appreciated component of all aspects of G-protein signaling.

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* Author for corresponding: Dr H. E. Hamm, Earl W. Sutherland Jr. Professor and Chair, Department of Pharmacology, Vanderbilt University School of Medicine, 2222 Pierce Avenue, 442 Robinson Research Building, Nashville, Tennessee 37232-6600, USA.
Tel.: (615) 343-3533; Fax: (615) 343-1084; E-mail: heidi.hamm@vanderbilt.edu

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1. Introduction

Heterotrimeric guanine-nucleotide-binding proteins (G proteins; subunits α, β, γ) act as molecular switches in signaling pathways by coupling the activation of heptahelical receptors at the cell surface to intracellular responses. This role depends on the ability of G-protein α subunits (Gα) to cycle between a resting conformation primed for interaction with an activated receptor (R*) and a signaling conformation capable of modulating the activity of downstream effector proteins, including a variety of enzymes and ion channels. As the primary intermediary between receptors and effectors, G proteins play an important role in determining the specificity and temporal characteristics of the cellular responses to a diverse array of extracellular stimuli.

G proteins are inactive in the heterotrimeric conformation where Gα binds GDP and the constitutive G$\beta\gamma$ dimer. Extracellular stimuli, such as hormones, neurotransmitters, chemokines, light and odorants, activate receptors by inducing a conformational change that permits G-protein binding and catalyzes GDP release from Gα, thereby resulting in the formation of a stable, high-affinity complex between the activated receptor and G protein. Binding of GTP to Gα destabilizes this complex, leading to a structural rearrangement of Gα(GTP), G$\beta\gamma$ and the receptor. Both subunits, Gα(GTP) and G$\beta\gamma$, go on to interact with downstream effector proteins. The cellular response is terminated when Gα hydrolyzes GTP to GDP and re-associates with G$\beta\gamma$, thus completing the cycle (Fig. 1). After an introduction to the structure of heterotrimeric G proteins, we will use this series of conformational steps to guide our review of the structural determinants of the function, specificity and regulation of G-protein signaling, with

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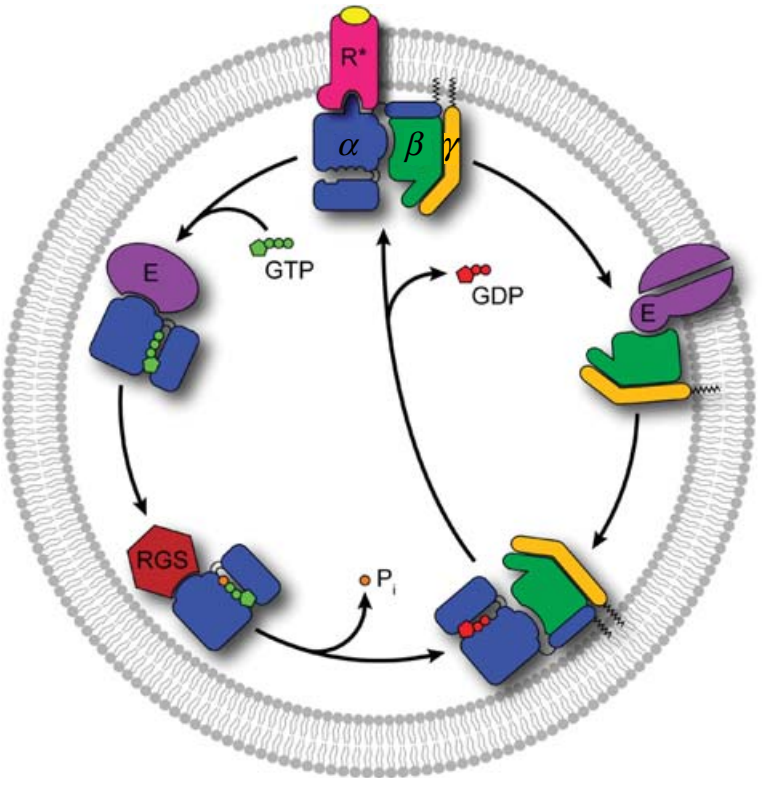


Fig. 1. Overview of the G-protein cycle. In the resting state, G proteins are heterotrimers of GDP-bound α (blue), β (green) and γ (gold) subunits. Agonist binding to heptahelical receptors (pink) in the cell membrane results in a conformational change leading to G-protein binding and, subsequently, GDP release. The high-affinity, nucleotide-free complex is stable until GTP binding causes dissociation of R*, Gα(GTP) and G$\beta\gamma$. The G-protein subunits go on to activate a variety of downstream effector proteins (E, purple). The signal is terminated upon the hydrolysis of GTP to GDP by Gα, which may be catalyzed by RGS proteins (dark red).

particular emphasis on the receptor–G protein complex and the mechanism of G-protein activation. In this review, we focus on G-protein signaling in mammalian systems, but readers are directed to excellent reviews on these signaling proteins in plants and yeast and the references therein (Dohlman & Thorner, 2001; Dohlman, 2002; Assmann, 2005).

2. Heterotrimeric G-protein structure

Although nearly 900 different heptahelical receptors are known (Fredriksson & Schiöth, 2005), they interact with a relatively small number of G proteins composed of α, β and γ subunits. At the last count, there were 21 Gα subunits encoded by 16 genes, 6 Gβ subunits encoded by 5 genes and 12 Gγ subunits in humans (Downes & Gautam, 1999).

2.1 G-protein α subunit

Heterotrimeric G proteins are typically divided into four main classes based on the primary sequence similarity of the Gα subunits: Gα_s, Gα_i, Gα_q and Gα_{12} (Simon *et al.* 1991). Members

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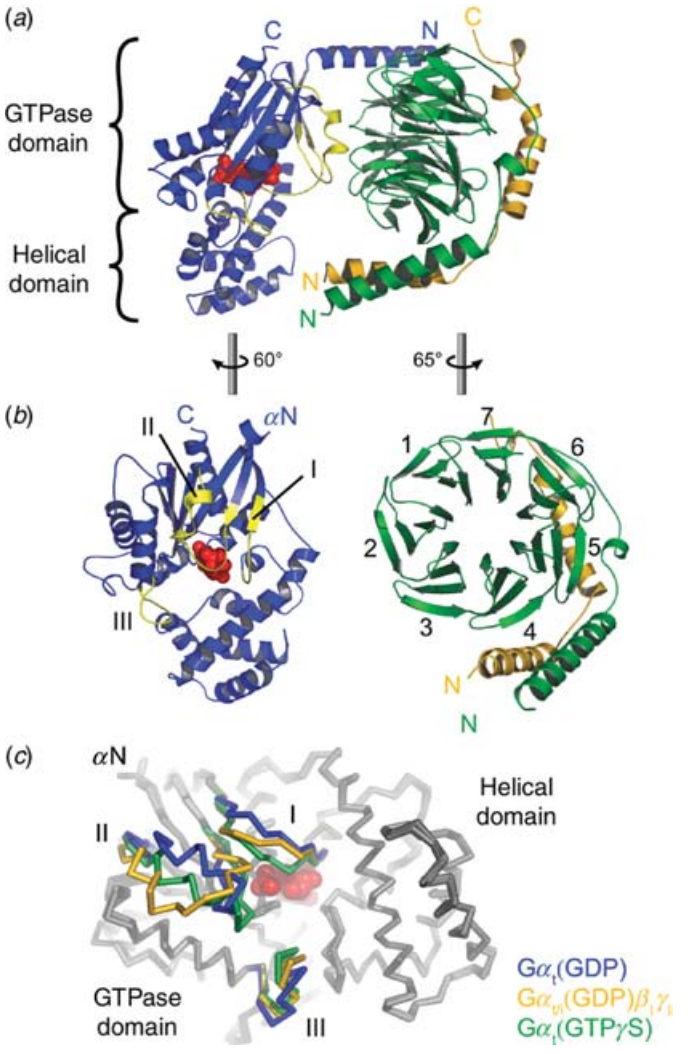


Fig. 2. Structural features of heterotrimeric G proteins. (*a*) Ribbon model of G$\alpha_{t/i}$(GDP)$\beta_1\gamma_1$ heterotrimer (1GOT), where α is blue, β is green and γ is gold. The three Switch regions in Gα are highlighted in yellow. GDP (red) is buried between the GTPase and helical domains of Gα. (*b*) The subunits have been rotated to show the intersubunit interface, which is primarily composed of the αN-helix (truncated in this view) and Switch II on Gα and blades 1–3 on Gβ. (*c*) When the GDP-bound (1TAG), GTPγS-bound (1TND) and heterotrimeric (1GOT) structures of Gα are aligned, significant conformational differences are found in the three Switch regions (compare gray and colored backbone). The crystal structures indicate that, upon GTP binding (green backbone), the three Switch regions collapse toward the γ-phosphate of GTP. The Gα(GDP) conformation is likely a short-lived transition state *in vivo* due to the large hydrophobic surface presented by this conformation of the Switch regions (blue backbone).

of this family range in size from 39–52 kDa and share between 35% and 95% sequence identity (Downes & Gautam, 1999). Many crystal structures of these proteins in a variety of conformations have been resolved, and these structures provide the framework for our understanding of the biomechanics of G-protein signaling (Table 1).

All of the Gα structures reveal a conserved protein fold composed of two domains, a GTPase domain and a helical domain (Fig. 2). The GTPase domain is structurally homologous to the

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family of monomeric G proteins, and is composed of a six-stranded β-sheet surrounded by five α-helices. The most highly conserved sequences in this domain are the five loops that contain the consensus sequences for guanine nucleotide binding, the diphosphate-binding (P-) loop (GXGESGKS), the Mg^{2+}-binding domain (RXXTXGI and DXXG) and the guanine ring-binding motifs (NKXD and TCAT). This domain also contains three flexible loops near the γ-phosphate binding site, named Switches I, II and III, where significant structural differences between the GDP-bound (Lambright *et al.* 1994; Mixon *et al.* 1995) and GTPγS-bound (Noel *et al.* 1993; Coleman *et al.* 1994a) conformations of Gα are found (Fig. 2*c*). The GTPase domain hydrolyzes GTP and contains sites for binding to the G$\beta\gamma$ dimer, heptahelical receptors and downstream effector proteins. The helical domain is composed of six α-helices that form a lid over the nucleotide-binding site, burying bound nucleotides in the core of the protein. Functions of the helical domain include increasing the affinity of Gα for guanine nucleotides (Warner *et al.* 1998; Remmers *et al.* 1999a) and increasing the GTP hydrolysis activity of the protein (Markby *et al.* 1993). As this domain is the most divergent among Gα subunits, it may also play a significant role in coupling specific G proteins to specific effectors (Liu *et al.* 1998).

Less is known about the structure of two other important regions of Gα, the amino and carboxyl termini, because they were either removed from the protein or disordered in the crystals. In the two structures of G-protein heterotrimers, the N-terminus forms an α-helix that is ordered by its interaction with Gβ (Fig. 2*a*) (Wall *et al.* 1995; Lambright *et al.* 1996), however, its structure and location in other conformations of the protein remain unknown. Both termini are key determinants of receptor-binding specificity and play a critical role in G-protein activation.

The N-terminus is also the site of the fatty-acid modifications on Gα subunits that regulate G-protein localization and protein–protein interactions. All of these proteins, except $G\alpha_t$, are modified post-translationally with the saturated 16-carbon fatty acid palmitate, and some ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$) are palmitoylated at multiple sites. Palmitate is attached by a reversible thioester bond to cysteines near the N-terminus of Gα, which is subject to regulation by protein acyltransferases and thioesterases (reviewed in Chen & Manning, 2001; Smotrys & Linder, 2004). Members of the $G\alpha_i$ family are also myristoylated, which results from the co-translational addition of the saturated 14-carbon fatty acid myristate to an N-terminal glycine following removal of the initiating methionine residue. Since a stable amide bond links the myristate to the protein, myristoylation is an irreversible modification (reviewed in Chen & Manning, 2001).

Recently, we have shown that myristoylation also plays an important role in the structure of the N-terminal helix of Gα. In the non-myristoylated protein, this region is dynamically disordered, and upon binding to G$\beta\gamma$, the N-terminus becomes ordered (Medkova *et al.* 2002), consistent with its conformation as an ordered α-helix in the crystal structures of G-protein heterotrimers (Wall *et al.* 1995; Lambright *et al.* 1996). However, the myristoylated N-terminus is highly ordered in Gα(GDP), and no further conformational changes were observed upon binding to G$\beta\gamma$ (Preininger *et al.* 2003). Thus, myristoylation plays a structural role in ordering the αN-helix, even in the absence of G$\beta\gamma$. Furthermore, steady-state fluorescence and fluorescence anisotropy indicate that the myristoylated N-terminus is less solvent-exposed and less mobile than the non-myristoylated N-terminus, pointing to a putative intramolecular binding site in Gα(GDP) for the myristoylated N-terminus (Fig. 3) (Preininger *et al.* 2003). Myristoylation also affects the interactions between Gα and effectors (Gallego *et al.* 1992; Taussig *et al.* 1993; Dessauer *et al.* 1998) and the soluble guanine nucleotide exchange factor (GEF) resistance to inhibitors of cholinesterase (Ric) 8A (Tall & Gilman, 2005).

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Table 1. *G protein structures*

PDB[a]	Complex	Missing regions (no. aa) and notes	Res. (Å)	Reference
G-protein subunits				
1TAG	Gαt(GDP)	N (27), C (10)	1·8	Lambright *et al.* (1994)
1GDD	Gαi1(GDP)	N (8), Switches II (16) and III (6)	2·2	Mixon *et al.* (1995)
1BOF	Gαi1(GDP)[b]	N (9), Switches II (14) and III (7)	2·2	Coleman & Sprang (1998)
1AS3	G42V-Gαi1(GDP)	N (8), Switches II (10) and III (6)	2·4	Raw *et al.* (1997)
1ZCB	Gαi/13(GDP)	N (27), Switch II (7), α4/β6 (2)C (5); N from Gαi1	2·0	Kreutz *et al.* (2006)
1TBG	Gβ1γ1	Cγ (3, F)	2·1	Sondek *et al.* (1996)
Gα complexes with GDIs				
1GOT	Gαt/iβ1γ1 heterotrimer	Nα (5), Cα (7), Nγ (8), Cγ (5, F); 216–294 from Gαi1	2·0	Lambright *et al.* (1996)
1GP2	Gαi1β1γ2 heterotrimer	Nα (4), Cα (6), Nγ (7), Cγ (7, G)	2·3	Wall *et al.* (1995)
1GG2	G203A-Gαi1β1γ2 heterotrimer	Nα (4), Cα (6), Nγ (7), Cγ (7, G)	2·4	Wall *et al.* (1995)
1KJY	Gαi1(GDP)·RGS14 GoLoco	Nα (29), Cα (5)	2·7	Kimple *et al.* (2002)
Elements that undergo R*-mediated conformational changes				
1AQG	Gαt C-terminus	aa 340–350	NMR	Kisselev *et al.* (1998)
1LVZ	Gαt C-terminus	aa 340–350 with K340R, C346S	NMR	Koenig *et al.* (2002)
1MF6	Gγ C-terminus	aa 60–71, no F	NMR	Kisselev & Downs (2003)
Activated Gα subunits				
1TND	Gαt(GTPγS)	N (26), C (1)	2·2	Noel *et al.* (1993)
1GIA	Gαi1(GTPγS)	N (33), C (11)	2·0	Coleman *et al.* (1994a)
1CIP	Gαi1(GppNHp)	N (31), C (7)	1·5	Coleman & Sprang (1999)
1AS0	G42V-Gαi1(GTPγS)	N (31), C (10)	2·0	Raw *et al.* (1997)
1GIL	Q204L- Gαi1(GTPγS)	N (33), C (11)	2·3	Coleman *et al.* (1994b)
1BH2	A326S-Gαi1(GTPγS)	N (31), C (8)	2·1	Posner *et al.* (1998)
1SVS	K180P-Gαi1(GppNHp)	N (31), C (7)	1·5	Thomas *et al.* (2004)
1AZT	Gαs(GTPγS)	N (34), C (3)	2·3	Sunahara *et al.* (1997b)

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Complexes with effectors and other binding proteins				
1FQJ	Gαt/i(GDP·AlF4−)·RGS9·PDEγ	Nα (27), Cα (6); 216–294 from Gαi1; RGS box only	2·0	Slep *et al.* (2001)
1AZS	Gαs(GTPγS)·ACV/II[c]	Nα (35), Cα (1); AC VC1 & IIC2 catalytic domains	2·3	Tesmer *et al.* (1997b)
2BCJ	Gαi/q(GDP·AlF4−)·GRK2·Gβ1γ2	Nα (29), Cα (5), Nγ (3), Cγ (1, G); N from Gαi1	3·1	Tesmer *et al.* (2005)
1SHZ	Gα13/i(GDP·AlF4−)·p115RhoGEF	Nα (30), Cα (5); Gα13 HD, Switches I-III; RGS only	2·9	Chen *et al.* (2005b)
2TRC	Gβ1γ1·phosducin	Cγ (3, F)	2·4	Gaudet *et al.* (1996)
1A0R	Gβ1γ1·phosducin	Nγ (1), Cγ (5); F is present	2·8	Loew *et al.* (1998)
1B9X	Gβ1γ1·S73E-phosducin	Cγ (3, F)	3·0	Gaudet *et al.* (1999)
1B9Y	Gβ1γ1·phosducin	Cγ (3, F)	3·0	Gaudet *et al.* (1999)
1OMW	Gβ1γ2·GRK2	Nγ (7), Cγ (G)	2·5	Lodowski *et al.* (2003)
1XHM	Gβ1γ2·SIGK peptide	Nγ (6), Cγ (17, G)	2·7	Davis *et al.* (2005)
Transition-state structures in GTP hydrolysis				
1TAD	Gαt(GDP·AlF4−)	N (26), C (6)	1·7	Sondek *et al.* (1994)
1GFI	Gαi1(GDP·AlF4−)	N (32), C (9)	2·2	Coleman *et al.* (1994a)
1SVK	K180P-Gαi1(GDP·AlF4−)	N (32), C (9)	2·0	Thomas *et al.* (2004)
1ZCA	Gαi/12(GDP·AlF4−)	N (34), C (9); N from Gαi1	2·9	Kreutz *et al.* (2006)
1AS2	G42V-Gαi1(GDP·Pi)	N (31), C (8)	2·8	Raw *et al.* (1997)
1GIT	G203A-Gαi1(GDP·Pi)	N (31), C (6)	2·6	Berghuis *et al.* (1996)
1FQK	Gαt/i(GDP·AlF4−)·RGS9	Nα (27), Cα (5); 216–294 from Gαi1	2·3	Slep *et al.* (2001)
1AGR	Gαi1(GDP·AlF4−)·RGS4	Nα (4)	2·8	Tesmer *et al.* (1997a)

N, Residues missing from amino terminal methionine; C, residues missing from C-terminus or isoprenylation site (Gγ); F, farnesyl; G, geranylgeranyl; HD, helical domain.

[a] The Protein Data Bank (www.pdb.org).

[b] This structure was solved in the presence of Mg^{2+}, distinguishing it from 1GDD.

[c] Several additional structures of this complex have also been solved in the presence of different ligands for AC: 1CJK, 1CJT, 1CJU, 1CJV (Tesmer *et al.* 1999), 1CS4 and 1CUL (Tesmer *et al.* 2000).

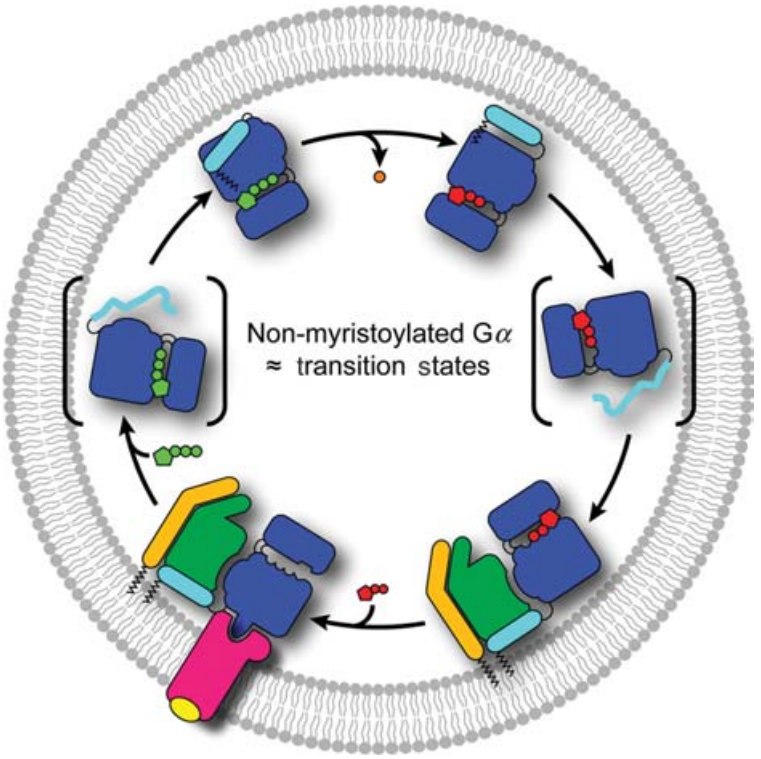


Fig. 3. The GTP-dependent myristoyl switch. Biophysical studies using fluorescence and electron paramagnetic resonance spectroscopy have shown that myristoylation plays a critical role in the structure of the αN-helix (cyan) of Gα subunits. In the non-myristoylated proteins, the N-terminus is disordered in both the GDP-bound and GTP-bound conformations. By contrast, this helix is ordered in the myristoylated proteins. Upon GTP binding to the myristoylated Gα, the N-terminus enters into a more hydrophobic environment than in either the Gα(GDP) or heterotrimeric conformations, suggesting that G-protein activation exposes an intramolecular binding site for the αN-helix. The disordered structures observed in non-myristoylated G proteins may reflect the transition states following activation and prior to heterotrimer formation.

2.2 G-protein $\beta\gamma$ dimer

All six Gβ subunits are ~36 kDa, and share 50–90% sequence identity, with the long and short splice variants of Gβ_5 being the least similar to the other four members of the family (see Section 2.3) (Downes & Gautam, 1999). Although not as dynamic a protein as Gα, Gβ is certainly more attractive with its seven-bladed β-propeller structure formed from seven WD40 sequence repeats (Fig. 2*b*). Each blade is composed of four antiparallel β-strands, where one WD40 sequence contributes the last three strands in one blade and the first strand in the next blade. Consequently, the final blade requires the N-terminus of Gβ to contribute the fourth β-strand, and thereby close the ring. The remaining N-terminal residues adopt an α-helical conformation that forms a coiled coil that is essential for interaction with Gγ or Gγ-like motifs (Fig. 2) (Garritsen *et al.* 1993; Sondek *et al.* 1996). This interaction seems to be important for the proper folding and function of Gβ (Iniguez-Lluhi *et al.* 1992; Higgins & Casey, 1994).

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Members of the Gγ family are all small proteins, between 7 and 8 kDa, that share 30–80% sequence identity (Downes & Gautam, 1999). They are composed of two α-helices connected by a loop. The N-terminal helix interacts with the N-terminus of Gβ, while the C-terminal helix binds to blades 5 and 6 (Fig. 2) (Sondek *et al.* 1996). All of the Gγ subunits undergo post-translational isoprenylation at their C-termini with a 15-carbon farnesyl (Gγ_1, Gγ_8 and Gγ_{11}) or 20-carbon geranylgeranyl (all others) moiety. These isoprenoids are first attached by a stable thioether bond to a cysteine residue located in the C-terminal cysteine-aliphatic-aliphatic-X (CaaX) sequence of Gγ. Then the three C-terminal amino acids are removed, and the new prenylated C-terminus is carboxymethylated (reviewed in Zhang & Casey, 1996). If the X residue in the CaaX motif is serine, methionine, glutamine or alanine, then Gγ is farnesylated, while leucine at this position directs geranylgeranylation (reviewed in Higgins & Casey, 1996). Carboxymethylation increases the affinity of G$\beta\gamma$ for the membrane by neutralizing the negatively charged C-terminus (Fukada *et al.* 1994).

The G-protein β and γ subunits form a functional unit that is not dissociable except by denaturation (Schmidt *et al.* 1992). Although most β subunits can interact with most γ subunits, not all of the 60 possible combinations of dimers form (Clapham & Neer, 1997). For example, Gγ_1 interacts with Gβ_1, but not Gβ_2 despite its high (87%) sequence identity to Gβ_1, and Gβ_2 does bind to Gγ_2 despite its similarity to Gγ_1 (41% identity) (Pronin & Gautam, 1992; Schmidt *et al.* 1992; Garritsen & Simonds, 1994; Downes & Gautam, 1999). Substituting three consecutive residues in the middle of Gγ_1 with homologous residues from Gγ_2, including a leucine that is conserved in all Gγ subunits except for Gγ_1, enables Gγ_1 binding to Gβ_2, however, the reverse substitution does not allow Gγ_2 to bind to Gβ_2, suggesting that additional regions are also important for the specificity of dimer formation (Lee *et al.* 1995a). Several different G$\beta\gamma$ dimers can interact with the same Gα isoform (Graf *et al.* 1992), suggesting that differential expression or subcellular localization may be an important determinant of signaling specificity.

2.3 Unique role of Gβ_5 in complexes with RGS proteins

Gβ_5 is unique among Gβ isoforms. While G$\beta_{1–4}$ share 80–90% sequence identity and are ubiquitously expressed, Gβ_5 shares only 50% identity and is preferentially expressed in the nervous system (Downes & Gautam, 1999). Additionally, G$\beta_{1–4}$ are entirely particulate proteins while Gβ_5 is found in both the particulate and soluble fractions. The N-terminus of Gβ_5 is also significantly longer than the other subunits (Watson *et al.* 1994). Although this region is important for binding Gγ (Garritsen *et al.* 1993), Gβ_5 can still form dimers and functional heterotrimers, as well as interact with a number of effectors following receptor activation (Watson *et al.* 1994, 1996; Zhang *et al.* 1996; Bayewitch *et al.* 1998a; Fletcher *et al.* 1998). Unlike the other Gβ subunits, Gβ_5 can be easily dissociated from Gγ and is stable in solution as a monomer (Cabrera *et al.* 1998; Yoshikawa *et al.* 2000). Interestingly, free Gβ_5 has been shown to form complexes with regulators of G-protein signaling (RGS) proteins, a family of GTPase-activating proteins (GAPs), that contain a Gγ-like (GGL) domain (Levay *et al.* 1999; Makino *et al.* 1999). The GGL domain is a 64-amino-acid sequence that is found in RGS6, 7, 9, 11 and the *C. elegans* RGS protein, EGL-10 (Snow *et al.* 1998). The interaction between these RGS proteins and Gβ_5 enhances their ability to accelerate the GTPase activity (i.e. GAP) of Gα subunits (Kovoor *et al.* 2000). In addition, this interaction allows RGS6, 7 and 11 to selectively GAP Gα_o (Snow *et al.* 1998).

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These unique associations beg the question as to whether $G\beta_5$ interacts with RGS proteins, $G\gamma$ or both *in vivo*. Despite the extensive *in vitro* biochemical evidence, there is little data to support a $G\beta_5\gamma$ complex *in vivo*, although this may be due to the technical difficulties resulting from the somewhat weaker interactions between these proteins than in other dimers (Jones *et al.* 2004). By contrast, in native preparations of vertebrate photoreceptors, the long isoform of $G\beta_5$ ($G\beta_{5L}$) is in a tight complex with RGS9 (Makino *et al.* 1999). When RGS9 is knocked out, $G\beta_{5L}$ cannot be found in the retinal tissue of these animals, despite the presence of normal $G\beta_5$ mRNA levels (Chen *et al.* 2000). The protein levels for the short isoform ($G\beta_{5S}$) were normal in these animals, suggesting that RGS9 may be required for the stability of $G\beta_{5L}$, while $G\beta_{5S}$ is stabilized by interactions with $G\gamma$ or other RGS proteins (Chen *et al.* 2000). Similarly, the protein expression of all GGL-containing RGS proteins is eliminated or substantially reduced in $G\beta_5$ knockout mice (Chen *et al.* 2003). Several other provocative questions are posed by these complexes, including why $G\beta_5$ is specifically enriched in the brain, whether they can form functional heterotrimers *in vivo* and what the physiological roles of the various complexes in signal transduction are. Clearly they are important for the fast inactivation of the photoreceptor response (Chen *et al.* 2000), and roles in opioid and dopaminergic signaling pathways are emerging (reviewed in Jones *et al.* 2004), yet we are only beginning to understand the functional consequences of these interactions.

2.4 Heterotrimer structure

Two crystal structures of G-protein heterotrimers, $G\alpha_{t/i}\beta_1\gamma_1$ and $G\alpha_{i1}\beta_1\gamma_2$, show two sites of interaction between $G\alpha$ and $G\beta\gamma$ (Fig. 2) (Wall *et al.* 1995; Lambright *et al.* 1996). The primary interaction surface is between Switches I and II of $G\alpha$ and residues from 5 of 7 blades on $G\beta$. This switch interface buries $\sim$1800 Å^2 of solvent-accessible surface area, and is composed of hydrophobic residues stabilized by hydrogen bonds and two ion pairs. A second interaction between the N-terminus of $G\alpha$ and blade one of $G\beta$ buries 900 Å^2 of solvent-accessible surface area.

Neither crystal structure provides evidence for $G\alpha$-$G\gamma$ contacts, although the acylated amino and carboxyl termini of $G\alpha$ and $G\gamma$, respectively, would likely be in close proximity to each other, suggesting that their lipids insert cooperatively into the plasma membrane (Bigay *et al.* 1994; Seitz *et al.* 1999). In addition to their membrane-anchoring role, the lipid modifications have been shown to increase the affinity of $G\alpha$ for $G\beta\gamma$, and vice versa, and this effect may be independent of the presence of the membrane (Linder *et al.* 1991; Iniguez-Lluhi *et al.* 1992; Iiri *et al.* 1996). Since these modifications are absent from the two G-protein heterotrimer crystals, additional studies are needed to understand the structural features of these hydrophobic moieties and their effect on $G\alpha$–$G\beta\gamma$ interactions.

Comparing the structures of $G\alpha$(GDP) (Lambright *et al.* 1994; Mixon *et al.* 1995) and the G-protein heterotrimers shows that $G\beta\gamma$ binding significantly alters the conformation of Switches I and II, while the rest of $G\alpha$ remains relatively unperturbed (Fig. 2*c*). By stabilizing these two flexible loops and, in the G_{i1} heterotrimer, forming a new salt bridge between the P-loop and Switch I, $G\beta\gamma$ locks GDP into the nucleotide-binding pocket (Wall *et al.* 1995). This reorganization also appears to disassemble the binding sites for Mg^{2+} and the GTP γ-phosphate, explaining the observation that GTP and $G\beta\gamma$ binding to $G\alpha$ are negatively cooperative (Higashijima *et al.* 1987). In contrast to the rearrangements observed in $G\alpha$ upon heterotrimer formation, significant structural changes are not observed in $G\beta\gamma$.

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2.5 Lipid modifications direct membrane association

One clear function of the fatty-acid modifications on Gα and Gγ is to direct the association of the heterotrimer with the membrane. For Gα_i family members, both fatty acid modifications, myristoylation and palmitoylation, contribute to membrane localization. Mutations that prevent palmitoylation shift the localization of myristoylated proteins from the membrane to the cytoplasm (Degtyarev *et al.* 1994; Mumby *et al.* 1994; Wise *et al.* 1997a; Morales *et al.* 1998). Similarly, mutating the N-terminal glycine to block myristoylation not only increases the amount of soluble protein, but also inhibits palmitoylation of these proteins (Mumby *et al.* 1990; Galbiati *et al.* 1994; Hallak *et al.* 1994; Wilson & Bourne, 1995). These observations support a two-signal membrane-trapping model for anchoring Gα to the plasma membrane (Shahinian & Silvius, 1995). In this model, myristoylation is the first signal that leads to a transient interaction of Gα with the membrane. Palmitoylation is the second signal that serves to securely attach the protein to the membrane. Consistent with the requirement of two hydrophobic moieties for membrane localization, non-myristoylated Gα_i and Gα_o can be trafficked to the membrane by isoprenylated G$\beta\gamma$, where they are subsequently palmitoylated. Since only membranes with the capacity for palmitoylation can trap G proteins in this model, this modification may specifically concentrate the G proteins at the plasma membrane rather than at intracellular organelle membranes that have less palmitoyl acyltransferase activity (Morales *et al.* 1998; McCabe & Berthiaume, 1999; Chen & Manning, 2001). Alternatively, G$\beta\gamma$ may be the primary determinant of Gα localization, as a mutant G$\beta\gamma$ that targets to mitochondrial membranes will recruit Gα_z to the mitochondria independent of palmitoylation (Fishburn *et al.* 2000).

In the remaining Gα family members that are not myristoylated, mutations that prevent palmitoylation can markedly impair membrane association (Wedegaertner *et al.* 1993; Degtyarev *et al.* 1993; Wise *et al.* 1997b; Bhattacharyya & Wedegaertner, 2000). For these proteins, the hydrophobic isoprenyl group on Gγ may act as the first signal, leading to membrane binding and palmitoylation. Mutants of Gα_s and Gα_q that could not interact with G$\beta\gamma$ were localized in the cytosol and were not palmitoylated. When the Gα_q mutant was myristoylated, its membrane localization and palmitoylation were restored (Evanko *et al.* 2000). Additionally, the over-expression of G$\beta\gamma$ was able to rescue the membrane localization of G$\beta\gamma$-binding deficient mutants of Gα_s and Gα_q in a subtype-specific manner (Evanko *et al.* 2001). The two-signal model may also apply to the targeting of G$\beta\gamma$ to the plasma membrane as heterotrimer formation and isoprenylation appear to be required for this process (Evanko *et al.* 2001; Takida & Wedegaertner, 2003). Indeed, the addition of a palmitoylation site to Gγ_2 as a putative second-membrane targeting signal can recover the appropriate plasma membrane localization of G$\beta_1\gamma_2$ in the absence of Gα (Takida & Wedegaertner, 2003).

3. Receptor–G protein complex

3.1 Low affinity interactions between inactive receptors (R) and G proteins

Early studies of receptor–G protein interactions in the visual transduction field demonstrated that transducin (G_t) can bind to dark rhodopsin, albeit at a much lower affinity (Hamm *et al.* 1987). Pure phospholipid vesicles could not bind G_t, while vesicles containing rhodopsin could bind the G protein in both dark and light (Fung, 1983). The dark membrane-binding site saturates at 25% of the rhodopsin concentration (Liebman & Sitaramayya, 1984), and the only protein present at that concentration in washed rod outer segment (ROS) membranes is

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rhodopsin (Hamm & Bownds, 1986). Interestingly, sulfhydryl modification with *N*-ethylmaleimide of what is now known to be the C-terminal cysteine in $G\alpha_t$ does not disturb the interaction between dark rhodopsin and G_t, while it completely blocks binding to the photoactivated receptor (Hofmann & Reichert, 1985). More recently, surface plasmon-waveguide resonance spectroscopy (PWR) has been used to study the interaction between rhodopsin and G_t. These studies indicate that G_t binds to dark rhodopsin with an apparent K_d of 64 nM (Alves *et al.* 2005). Although this is consistent with an earlier result obtained by surface plasmon resonance (SPR) spectroscopy, a similar method (Salamon *et al.* 1996), it is beyond the lower limit of the previously published values for this interaction, ranging from 10 to 0·1 μM (Liebman & Sitaramayya, 1984; Schleicher & Hofmann, 1987). The affinity of G_t for light-activated rhodopsin is ~1 nM (Bennett & Dupont, 1985; Alves *et al.* 2005). A high-affinity interaction between rhodopsin and transducin in the dark suggests that all of the G_t-binding sites in ROS are constitutively saturated, which is a surprising conclusion. Clearly transducin is able to interact with dark rhodopsin; however, much more work is required to explore the structural features and physiological consequences of this interaction.

Similar results have been observed in other receptor systems. For example, G_i co-purified through several steps with the α_2-adrenergic receptor (Cerione *et al.* 1986). The antagonist-bound δ-opioid receptor has ~50-fold lower affinity for G proteins than the agonist-bound receptor, as shown by PWR. Interestingly, GTPγS did not cause dissociation of the antagonist- or inverse agonist-bound δ-opioid receptor–G protein complexes, suggesting that these are R·Gα(GDP)$\beta\gamma$ complexes (Alves *et al.* 2003). Additional support for the formation of these complexes is also provided by kinetic studies of rhodopsin-catalyzed nucleotide exchange in $G\alpha_t$, where a distinct R·Gα(GDP)$\beta\gamma$ complex is required to fit the double displacement (ping-pong) mechanism described (Heck & Hofmann, 2001). In hormone-sensitive cells that contain only a few thousand receptors, preformed receptor–G protein complexes could have a major impact on the kinetics of intracellular events (Hamm *et al.* 1987).

3.2 Receptor activation exposes the high-affinity G-protein binding site

G-protein-coupled receptors have a common body plan with seven transmembrane-spanning α-helices with an extracellular N-terminus, intracellular C-terminus and three interhelical loops on each side of the membrane (Fig. 4). The C-terminal tail contains an eighth α-helix and palmitoylation sites that create a fourth intracellular loop (Palczewski *et al.* 2000). Although the overall sequence homology between receptors is low, there are several highly conserved micro-domains including helix VIII and the palmitoylation sites, as well as the D(E)RY (aspartate/glutamate-arginine-tyrosine) and NPxxYx_5F (asparagine-proline-x-x-tyrosine-x_5-phenylalanine) motifs, which are near the intracellular face of the receptors and play an important role in receptor and G-protein activation (Fig. 4) (reviewed in Kristiansen, 2004). Despite extensive effort, the dark (inactive) conformation of rhodopsin remains the only receptor for which crystal structures have been solved (Palczewski *et al.* 2000; Teller *et al.* 2001; Okada *et al.* 2002, 2004; Li *et al.* 2004). Although these structures shed little light on the G-protein coupling mechanism, they have provided a useful framework for the interpretation of numerous biophysical studies on the conformational changes associated with receptor activation (reviewed in Hubbell *et al.* 2003). Extensive site-directed spin-labeling (SDSL) studies of rhodopsin have shown that photo-activation primarily results in an outward movement of helix VI, thereby opening a crevice in the intracellular face of the receptor (Fig. 4*b*) (Farrens *et al.* 1996). This outward movement appears

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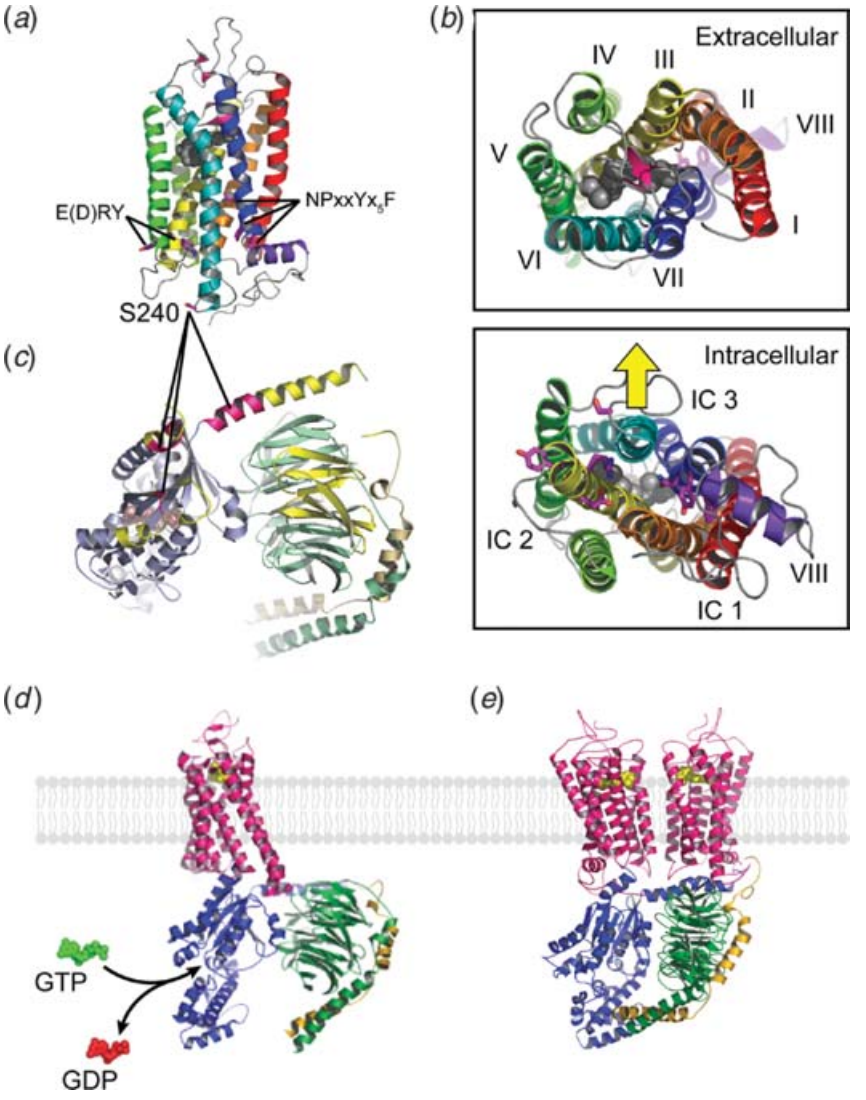


Fig. 4. Receptor–G protein complexes. (*a*) Ribbon model of dark (inactive) bovine rhodopsin (1U19), showing the seven transmembrane-spanning α-helices (red to blue) and 11-*cis*-retinal (gray spheres). Conserved side-chains important for receptor and G-protein activation are shown (magenta), including the E(D)RY motif on helix III (yellow) and NpxxYx_5F motif on helices VII and VIII (blue and purple), as well as S240 in IC 3, which has been cross-linked to several sites on the G protein (lines). (*b*) The extracellular and intracellular faces of rhodopsin are shown. Receptor activation results in an outward movement of helix VI (yellow arrow), which opens a gap in the cytoplasmic face of the receptor, exposing residues critical for G-protein activation, such as the E(D)RY motif on helix III (yellow). (*c*) Ribbon diagram of the heterotrimeric G protein with the C-terminal tail of Gα attached (1AQG), which has been rotated 50° toward the viewer from the putative receptor-binding orientation to visualize the receptor contact sites (yellow and magenta). Receptor contact sites on the G protein have been mapped using a variety of approaches, including chemical cross-linking experiments. Sites that cross-link to residue S240 on the receptor are highlighted (magenta). This type of data will be critical for improving models of the receptor–G protein complex, as it provides constraints for the location of IC 3 relative to Gα. (*d*) Representation of the R*·Gα(O)$\beta\gamma$ complex created by manually docking the G protein onto an activated receptor model based on the rhodopsin crystal structure (1GZM). (*e*) Model of an R_2·Gα(O)$\beta\gamma$ complex based on coordinates generously provided by K. Palczewski (published in Fotiadis *et al.* 2004).

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to be essential for G-protein activation, as disulfide and metal-ion cross-links between helices III and VI prevent light-stimulated nucleotide exchange in G_t (Farrens *et al.* 1996; Sheikh *et al.* 1996). A similar movement has also been observed in other receptors, suggesting that it is an important component of the conformational change leading to G-protein binding (reviewed in Gether, 2000; Gether *et al.* 2002).

3.3 Receptor–G protein interface

The newly formed pocket in the cytoplasmic face of the receptor forms the binding site for the C-terminus of Gα (Fig. 4) (Janz & Farrens, 2004), which is the best characterized receptor contact site in G proteins. Structural information for this region is lacking from G-protein crystal structures, however, as these residues are either absent or disordered in most of them (Table 1). Upon binding light-activated rhodopsin, 11 amino acid C-terminal peptides from $G\alpha_t$ form an α_L-type C-cap motif (Dratz *et al.* 1993; Kisselev *et al.* 1998; Koenig *et al.* 2002). Interestingly, the structure of the activated rhodopsin-bound peptide was similar to that observed in the $G\alpha_{i1}(GDP{\cdot}AlF_4^-){\cdot}RGS4$ structure (Tesmer *et al.* 1997a; Kisselev *et al.* 1998). The angle between the axis of the helix with respect to the membrane normal was determined to be $\sim 40^\circ$ based on the combination of transfer nuclear Overhauser effect and residual dipolar coupling nuclear magnetic resonance (NMR) data (Koenig *et al.* 2002). This same peptide stabilizes the metarhodopsin II (Meta II) signaling conformation of rhodopsin, and will compete with the G_t heterotrimer for binding to the receptor (Hamm *et al.* 1988; Aris *et al.* 2001). Screening a combinatorial library for peptides that could stabilize Meta II demonstrated that the C-terminal seven amino acids of $G\alpha_t$ are the most important for this interaction (Martin *et al.* 1996). Several mutations in this region have been shown to uncouple G proteins from their receptors, including an arginine-to-proline mutation in $G\alpha_s$ first identified in the *unc* clone of S49 murine lymphoma cells that has since been associated with Albright's hereditary osteodystrophy (Sullivan *et al.* 1987; Schwindinger *et al.* 1994; Osawa & Weiss, 1995). Similarly, the pertussis toxin-catalyzed ADP ribosylation of the C-terminal cysteine in $G\alpha_i$ family members also uncouples signaling through G_i-coupled receptors (West *et al.* 1985).

A number of residues in the $\alpha 4/\beta 6$ loop at the C-terminus of Gα have also been implicated in receptor contact by alanine-scanning mutagenesis (Fig. 4*c*) (Onrust *et al.* 1997), chimeric studies (Bae *et al.* 1997, 1999), sequence analysis of conserved residues in Gα subclasses (Lichtarge *et al.* 1996a), chemical cross-linking (Cai *et al.* 2001) and tryptic proteolysis (Mazzoni & Hamm, 1996). Five residues in the $\alpha 3/\beta 5$ loop of $G\alpha_s$, when replaced with the homologous amino acids in $G\alpha_{i2}$, decrease receptor-mediated GTP binding while increasing receptor affinity (Fig. 4*c*) (Grishina & Berlot, 2000). The αN-helix also interacts with the receptor, as shown by peptide competition (Hamm *et al.* 1988), mutagenesis (Onrust *et al.* 1997), chemical cross-linking (Taylor *et al.* 1994; Itoh *et al.* 2001) and Gα chimeras (Ho & Wong, 2000) (Fig. 4*c*).

The G$\beta\gamma$ subunit also binds receptors and is required to stabilize the receptor–Gα interface. The C-terminal 60 amino acids of Gβ can be cross-linked to a peptide corresponding to the third intracellular loop of the α_2-adrenergic receptor (Fig. 4*c*) (Taylor *et al.* 1994, 1996). Alanine-scanning mutagenesis in Gβ identified several mutants with normal heterotrimer formation, but defective receptor-mediated GTPγS uptake, indicating a role for G$\beta\gamma$ in this process, either directly or indirectly through stabilization of the Gα subunit (Ford *et al.* 1998). A farnesylated C-terminal 12-amino-acid peptide of $G\gamma_1$ will stabilize Meta II (Fig. 4) (Kisselev *et al.* 1995). This Gγ peptide forms an amphipathic helix upon interaction with activated rhodopsin

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(Kisselev & Downs, 2003). Other mutational, peptide mapping and biophysical studies have also demonstrated regions of G$\beta\gamma$ that interact with receptors (Phillips *et al.* 1992; Kisselev & Gautam, 1993; Kisselev *et al.* 1994, 1999; Azpiazu *et al.* 1999).

Although there is extensive data describing the receptor binding surface on G proteins, somewhat less is known about the specific point-to-point interactions between these proteins. Generally, the membrane-proximal sections of intracellular loops (IC) 2, 3 and 4 form the primary interaction surface on the receptor. Additional contact sites in the transmembrane helices may also be formed as the Gα C-terminus enters deeply into its binding pocket in R* (Janz & Farrens, 2004). Peptides corresponding to IC loops 2, 3 and 4 block the stabilization of Meta II by $G\alpha_t$ (Konig *et al.* 1989; Marin *et al.* 2000). A reverse substitution approach, where each intracellular loop was first substituted with poly-alanine sequences and then individual alanine residues were mutated back to the native amino acid, demonstrated that residues adjacent to the transmembrane helices in these loops were the most important for G-protein activation (Natochin *et al.* 2003). Chemical cross-linking experiments between rhodopsin and $G\alpha_t$ indicate that residue 240 in IC 3 of rhodopsin is near the amino and carboxyl termini of Gα, as well as the $\alpha 4/\beta 6$ loop (Fig. 4) (Cai *et al.* 2001; Itoh *et al.* 2001). Five residues at the C-terminus of Gα were shown to specifically interact with four non-contiguous residues in IC 3 (Liu *et al.* 1995). Peptides corresponding to IC 3 also cross-link to the N-terminus of Gα and the C-terminus of Gβ (Taylor *et al.* 1994, 1996). Recently, the binding site for the C-terminal peptide of Gα was mapped to a hydrophobic patch on the inner face of helix VI in Meta II based on the ability of the tryptophan-labeled peptide to quench the fluorescence of bimane labels on the receptor (Janz & Farrens, 2004), and both the Gα and Gγ C-termini of G_t interact with IC 4 of rhodopsin (Ernst *et al.* 2000). These data provide important information about specific contacts between receptors and G proteins; however, more constraints are needed to improve our current model of the receptor–G protein interface.

3.4 Structural determinants of receptor–G protein specificity

Relatively few types of G proteins transduce signals from a vast number of heptahelical receptors, thus each member of the G-protein family must be capable of interacting with many different receptors. Furthermore, many receptors are capable of activating multiple G-protein signaling pathways. Regulating the specificity of these interactions is important for proper signal transduction. Therefore, the receptor–G protein interface must also encode important information that determines which G proteins can interact with a particular receptor. Even after identifying many of the contact sites that comprise this interface, the connections that define the coupling between receptors and specific G-protein family members remain unclear.

Each of the contact sites described in the previous section has been shown to contribute to coupling specificity. Carboxyl terminal chimeras of Gα subunits have been frequently used to switch receptor-effector coupling [i.e. replacing the C-terminus of $G\alpha_q$ with that of $G\alpha_i$ results in G_i-coupled receptors that can stimulate phospholipase C, a $G\alpha_q$ effector (Conklin *et al.* 1993)], and many mutations in this region have been shown to alter receptor–G protein coupling (Conklin *et al.* 1996; Kostenis *et al.* 1997c; Blahos *et al.* 1998; Natochin *et al.* 2000). Even single-point mutations in the C-terminus of Gα have been shown to switch receptor coupling (Kostenis *et al.* 1997a). The C-terminal 11 amino acids in $G\alpha_t$ and $G\alpha_i$ are nearly identical, yet the $5\text{-}HT_{1B}$ serotonin receptor activates only G_i through a specific interaction with two amino acids in the α4-helix of this protein (Bae *et al.* 1999). Other key specificity determinants in Gα are the

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N-terminus (Kostenis *et al.* 1997b, 1998), $\alpha N/\beta 1$ loop (Blahos *et al.* 2001), $\alpha 2/\beta 4$ loop (Lee *et al.* 1995b) and the $\alpha 3/\beta 5$ loop (Grishina & Berlot, 2000), and studies suggest that coupling selectivity involves subtle and cooperative interactions among all of these various domains (Slessareva *et al.* 2003). Interestingly, mutations in the $\alpha 2/\beta 4$ and $\alpha 4/\beta 6$ loops in $G\alpha_s$ do not affect binding to the β_2-adrenergic receptor, while mutations in the $\alpha 3/\beta 5$ loop increase receptor binding (Grishina & Berlot, 2000). This may suggest differential determinants of G-protein coupling in different G-protein families. Recently, a mutation in a highly conserved glycine in Linker 1 of $G\alpha$ was shown to increase the activation of $G\alpha_q$ by G_i- and G_s-coupled receptors, demonstrating that sites distant from the receptor–G protein interface may also be important for controlling coupling (Heydorn *et al.* 2004). Further, coupling specificity may involve cooperative interactions between Linker 1 and the C-terminus (Kostenis *et al.* 2005).

In addition to the structural determinants on $G\alpha$, specific isoforms of $G\beta$ and $G\gamma$ have been shown to preferentially interact with specific receptors. For example, both $G\beta_1\gamma_2$ and $G\beta_5\gamma_2$ can couple $G\alpha_q$ to endothelin B and M_1-muscarinic receptors, however only $G\beta_1\gamma_2$ promotes endothelin B receptor binding to $G\alpha_i$ (Lindorfer *et al.* 1998). Additional work showed that $G\beta_5\gamma_2$ specifically couples $G\alpha_q$ to receptors (Fletcher *et al.* 1998). Similarly, the M_2-muscarinic receptor interacted with $G\alpha_o$ heterotrimers with $G\beta_4\gamma_2$, but not $G\beta_1\gamma_2$ (Hou *et al.* 2001). The A_1-adenosine receptor couples equally well to $G\alpha_{i1}$ heterotrimers containing $G\beta_1\gamma_2$, $G\beta_1\gamma_3$, $G\beta_2\gamma_2$ and $G\beta_2\gamma_3$ (Figler *et al.* 1996), however, $G\beta_1$ dimers with $G\gamma_2$ and $G\gamma_3$ had a much faster receptor-catalyzed nucleotide exchange rate compared to dimers with $G\gamma_1$ (Figler *et al.* 1997). A similar study measuring the ability of each of the five $G\beta\gamma_2$ isoforms to couple to the β_1-adrenergic and A_{2a}-adenosine receptors found that $G\beta_4$ coupled the best while $G\beta_5$ coupled the worst. Interestingly, although $G\beta_1$ had a similar potency to $G\beta_4$ in coupling to the β_1-adrenergic receptor, its potency was 20-fold lower when coupling to the adenosine receptor (McIntire *et al.* 2001).

The $G\gamma$ subunit also plays an important role as a determinant of receptor–G protein coupling specificity. Coupling of $G\alpha_{i1}$ to the α_{2a}-adrenergic receptor was significantly affected depending on which of the eight different $G\gamma$ subunits studied was used to form the heterotrimer (Richardson & Robishaw, 1999). Similarly, the M_2-muscarinic receptor does not interact with $G\alpha_o$ heterotrimers with $G\beta_1\gamma_2$, although $G\beta_1\gamma_5$ and $G\beta_1\gamma_7$ could mediate binding to this receptor (Hou *et al.* 2000), and several other studies offer additional examples of $G\gamma$-directed coupling specificity in other receptor systems (Butkerait *et al.* 1995; Jian *et al.* 1999; Lim *et al.* 2001). The principal determinant of this specificity is likely the primary sequence of the C-terminal third of $G\gamma$, independent of the type of lipid modification, as shown by studies with chimeric $G\gamma$ subunits (Jian *et al.* 2001; Myung *et al.* 2005). However, the type of lipid modification also has an effect on receptor coupling, as a geranylgeranyl moiety on $G\gamma_1$ or $G\gamma_2$ increases the affinity of the G protein for rhodopsin (Jian *et al.* 2001) and the A_1-adenosine receptor (Yasuda *et al.* 1996), respectively.

Much of the difficulty in understanding coupling specificity between receptors and G proteins arises from the poor sequence homology of the intracellular loops that comprise the G-protein binding site (reviewed in Wess, 1998). Even closely related receptors that activate the same G protein can have dissimilar intracellular loops, making coupling determinations based on primary structure impossible (Hedin *et al.* 1993), although a recently described statistical approach may help to overcome this difficulty (Muramatsu & Suwa, 2006). Extensive studies have shown that IC 2 and 3 are the most common selectivity determinants in receptors, while occasionally, IC 1 and 4 modulate receptor–G protein interactions (reviewed in Wess, 1997). Frequently, mutations

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in other regions of the receptor distant from the G-protein binding site can affect coupling, suggesting that the global conformation of the receptor or changes in its dynamics may be just as important as specific side-chain interactions in determining receptor–G protein selectivity (Gilchrist *et al.* 1996; Perez *et al.* 1996).

Indeed, the observation that different agonists can affect which G proteins are activated by a given receptor supports a model where specific receptor conformations may be more or less favorable for coupling to specific G proteins (reviewed in Kenakin, 2003; Perez & Karnik, 2005). In one recent example of ligand-directed signaling, structurally different ligands of the CB_1-cannabinoid receptor differentially regulated the binding of the three homologous $G\alpha_i$ proteins to the receptor. Interestingly, while one ligand was shown to behave as an agonist for G_{i1} and G_{i2} and as an inverse agonist for G_{i3}, another ligand was shown to behave exactly the opposite, i.e. as an inverse agonist of G_{i1} and G_{i2} and as an agonist for G_{i3} (Mukhopadhyay & Howlett, 2005). Similarly, the functional responses of protease-activated receptor 1 (PAR1) activation differ whether the receptor is activated by thrombin or an agonist peptide. Mathematical modeling of the downstream signaling pathways indicates that the peptide favors $G\alpha_q$ activation over $G\alpha_{12/13}$ activation by 800-fold, while thrombin-mediated activation of PAR1 does not discriminate between the G proteins (McLaughlin *et al.* 2005). Clearly, specific receptor–G protein coupling results from a complex relationship between specific side-chain interactions between these proteins as well as the agonist-induced tertiary structure of the G-protein binding site.

3.5 Models of the receptor–G protein complex

Recently, computational techniques have enabled investigators to apply this extensive amount of biochemical and biophysical information to the high-resolution structures of rhodopsin and G_t to develop a molecular model of the receptor–G protein complex (Fig. 4) (Slusarz & Ciarkowski, 2004; Fotiadis *et al.* 2004; Ciarkowski *et al.* 2005). Currently, the building blocks available for this complex are the inactive structure of rhodopsin (Teller *et al.* 2001), the GDP-bound transducin heterotrimer (Lambright *et al.* 1994) and the NMR structures of the C-termini of $G\alpha_t$ and $G\gamma_1$ (Kisselev *et al.* 1998; Kisselev & Downs, 2003). The first step in the modeling process is to 'activate' the receptor to provide a binding site for G_t. Two different techniques have been used to develop models of activated rhodopsin. In the first, simplified energy calculations were used to find sterically and energetically reasonable arrangements of the transmembrane helices around all-*trans* retinal. Of these, the arrangement that agreed best with the conformation suggested by SDSL studies of rhodopsin was selected and the non-transmembrane structural elements were reconstructed (Nikiforovich & Marshall, 2003). The second technique used target-driven molecular dynamics simulations to forcefully modify the cytoplasmic side of rhodopsin to meet the experimentally derived distances from SDSL (Slusarz & Ciarkowski, 2004). Both of these methods resulted in reasonable working models for activated rhodopsin that are consistent with experimental evidence.

Since a critical receptor-binding site, the C-terminus of $G\alpha$, is absent in both structures of the heterotrimer (Wall *et al.* 1995; Lambright *et al.* 1996), it must be attached using the NMR structure of the C-terminal peptide bound to activated rhodopsin (Kisselev *et al.* 1998). Next, the G protein was visually docked onto a model of the activated receptor (based on driven molecular dynamics simulations described above). The topology and electrostatic potentials of the binding sites significantly limit the possible orientations between the receptor and G protein. This

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particular model highlights three clusters of conserved interactions between the Gα C-terminus the receptor, suggesting that the conserved residues in these proteins form a core interaction surface that may be shared among different receptor–G protein complexes (Slusarz & Ciarkowski, 2004).

According to this model, the cytoplasmic surface of the receptor is not large enough to accommodate both the Gα C-terminus in its binding pocket and the Gγ C-terminus (Slusarz & Ciarkowski, 2004). One possible resolution is that receptors function as dimers, and increasing experimental evidence suggests that this is the case (reviewed in Javitch, 2004; Milligan, 2004), including the demonstration by neutron scattering of an $R_2{\cdot}G\alpha\beta\gamma$ assembly between a leukotriene B_4 receptor (BLT1) dimer and G_i (Baneres & Parello, 2003). Rhodopsin also seems to be arranged in paracrystalline rows of dimers in native disk membranes as shown by atomic force microscopy (AFM) (Fotiadis *et al.* 2003, 2004; Liang *et al.* 2003). A model of the rhodopsin dimer was constructed by packing the rhodopsin crystal structure into the unit cell constraints determined by AFM (Liang *et al.* 2003; Fotiadis *et al.* 2004). The interface between the two rhodopsin monomers is between helices IV and V in this model, as other orientations were excluded due to geometrical or energetic limitations. The outward movement of helix VI during receptor activation was modeled as a 90° rotation of the helix about its long axis perpendicular to the membrane, which swings the intracellular half of this kinked helix away from the transmembrane bundle. After adding the missing termini onto the G protein, the G protein was docked on a rhodopsin dimer where one monomer had been 'activated'. In the resulting structure, the Gα C-terminus is inserted into the pocket formed upon receptor activation, the N-terminal helix of Gα occupies a groove that runs between the two monomers and the farnesyl group at the C-terminus of Gγ interacts with the inactive receptor in the dimer (Fig. 4*e*) (Fotiadis *et al.* 2004). An alternative model based on a different orientation of rhodopsin monomers within the rows of dimers was also proposed (Ciarkowski *et al.* 2005). However, the issue of the dimerization of rhodopsin-like heptahelical receptors remains a contentious one, where some maintain that receptor monomers are the functional unit in G-protein coupling (Chabre & le Maire, 2005).

The structural complementarity between the cytoplasmic face of dark rhodopsin and G_t was also recently explored using computational techniques to sample the rotational and translational space of several models of G_t with respect to rhodopsin (Fanelli & Dell'Orco, 2005). The best 4000 solutions were winnowed down based on broad distance constraints from experimental data and then the remaining solutions were subjected to cluster analysis followed by energy minimization of the representative models in each cluster. All of the parameters converge on the same rhodopsin–G_t complex, placing the Gα_t C-terminus in a shallow pocket at the center of the transmembrane helices where it is almost accessible to the conserved arginine in the E(D)RY motif that is important for G-protein activation. Interestingly, the architecture of the best complex between monomeric rhodopsin and G_t was also one of the best-scored solutions when G_t was docked to models of rhodopsin dimers or oligomers (Liang *et al.* 2003). The authors go on to propose that G_t may catalyze the structural changes from metarhodopsin I (Meta I) to Meta II, and that activation of both of these proteins may be concurrent processes in a supramolecular complex (Fanelli & Dell'Orco, 2005).

Although a provocative model, its physiological implications in phototransduction are less clear. In the typical operating range of rhodopsin, with approximately one photoactivated receptor per disc membrane, the probability of hitting a preformed complex would be only 10%, and stimulation of a preformed complex would only seem to activate one G protein. In order to achieve the tremendous amplification of the visual signal, a sufficiently fast exchange of G

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proteins on rhodopsin seems necessary for the efficient excitation of the photoresponse (Hamm *et al.* 1987).

Current molecular models of the receptor–G protein complex meet most of the important constraints established by the experiments cited in the previous section, and therefore provide a reasonable framework for the design and interpretation of future studies. Particularly important information will come from additional distance constraints between the receptor and G protein, such as novel cross-links or interprobe measurements from fluorescence or electron paramagnetic resonance spectroscopy (EPR). Also, models of the complex were first assembled from models of the components, the inactive receptor and C-termini of Gα and Gγ, and any improvement in the determination of these structures will enhance our ability to model the complex. Finally, the modeling process does not yet take into account conformational changes in the G protein, which are necessary to cause GDP release from Gα, because little is known about specific receptor-mediated structural rearrangements in this protein.

3.6 Sequential interactions may form the receptor–G protein complex

An alternative hypothesis to account for the widely separated receptor-binding sites on the C-termini of Gα and Gγ is that these regions interact sequentially with the activated receptor (Herrmann *et al.* 2004). Kinetic light scattering was used to follow the association of maltose-binding-protein (MBP) tagged peptides corresponding to the wild-type Gα_t C-terminus, two high-affinity homologs, and the farnesylated Gγ_1 C-terminus to photolyzed rhodopsin. The binding of the Gα_t and Gγ_1 constructs was mutually exclusive, and free Gα peptide could compete off MBP-Gγ and vice versa. In the holo G protein, at least one lipid modification was necessary for rhodopsin binding, and no binding was observed in the absence of lipid modifications, despite binding of the MBP-Gα C-terminal peptide. When the C-terminus of Gα was replaced with a high-affinity sequence, the authors noted the same kinetics of complex formation. Combined with the previous observation that the C-terminus of Gα cannot confer binding to the non-acylated heterotrimer, the authors conclude that the Gα C-terminus is not involved in the initial encounter between the receptor and G protein. They propose a model where G$\beta\gamma$ shields the Gα C-terminus until the farnesylated C-terminus of Gγ interacts with the receptor. Once the C-terminus of Gα is exposed, it interacts with the receptor, leading to GDP release. This sequential fit mechanism is compatible with receptor dimers, but it does not require dimerization in order to explain previous observations about receptor contact sites on the G protein.

4. Molecular basis for G-protein activation

Activated receptors bind to G proteins and catalyze GDP release from Gα, which is the rate-limiting step in G-protein activation (Higashijima *et al.* 1987). Until GTP binds, a high-affinity complex between the receptor and G protein is formed (Rodbell *et al.* 1971; Emeis *et al.* 1982; Bornancin *et al.* 1989). Based on the current models of this interaction, the nucleotide-binding pocket is some 30 Å away from the nearest receptor contact site (Fig. 4*d*) (Bourne, 1997; Hamm, 1998; Slusarz & Ciarkowski, 2004; Fotiadis *et al.* 2004; Ciarkowski *et al.* 2005). Thus, the receptor must induce a conformational change in the G protein to cause GDP release over this distance. Several potential mechanisms for receptor-catalyzed GDP release have been proposed, mainly based on the effects of various mutations on the basal and receptor-catalyzed nucleotide

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exchange rates of the G protein. To date, there is little direct evidence of specific receptor-mediated conformational changes in G proteins, and the molecular mechanisms for receptor-catalyzed nucleotide exchange remain one of the most important unanswered questions in the field of G-protein signaling.

4.1 Potential mechanisms of receptor-catalyzed GDP release

Tracing structural elements from known receptor contact sites to the nucleotide-binding pocket highlights several potential routes that may transmit the conformational changes causing GDP release (Fig. 5*a*). Perhaps the most obvious connection is the α5-helix, which links the C-terminus of Gα to the β6/α5 loop containing the guanine ring-binding TCAT motif. Mutations in this motif greatly enhance spontaneous GDP release (Thomas *et al.* 1993; Iiri *et al.* 1994; Posner *et al.* 1998), including the activating mutation in $G\alpha_s$, A366S, that causes pseudo-hypoparathyroidism and gonadotropin-independent precocious puberty in male patients (Iiri *et al.* 1994). A homologous mutation in $G\alpha_{i1}$ also greatly enhances GDP release (Posner *et al.* 1998). No significant structural differences were observed in the crystal structure of the $G\alpha_{i1}$-A326S heterotrimer compared to the wild-type protein, but the GDP-binding site was only partially occupied. The authors suggest that GDP release can occur without large conformational changes in Gα (Posner *et al.* 1998). However, inspection of the crystal structures seems to require a significant structural rearrangement to free the GDP buried between the GTPase and helical domains. Unfortunately, crystallography cannot elucidate the structural transition that occurred to release GDP from $\sim$70% of heterotrimers in this crystal. The crystal lattice could be working to reinforce the more stable, GDP-bound conformation in the nucleotide-free heterotrimers, which is why no significant structural differences were observed.

Several mutagenesis studies have implicated the α5-helix in receptor-catalyzed nucleotide exchange. Alanine-scanning mutagenesis of $G\alpha_t$ identified three residues on the inward face of the helix with substantially increased rates of basal and receptor-catalyzed exchange, and several residues on the outward face of this helix that decreased catalyzed exchange (Marin *et al.* 2001b). A similar study based on proline-scanning mutagenesis of this helix generally resulted in mutants with increased rates of basal exchange and decreased rates of catalyzed exchange (Marin *et al.* 2002). A flexible five-glycine linker inserted between the extreme C-terminus and the α5-helix only modestly decreased binding to the receptor, but severely reduced receptor-mediated nucleotide exchange, possibly due to disruption of the connection between the receptor contact site and GDP-binding pocket. Doubling the length of the α5-helix by repeating its sequence generated a mutant whose receptor coupling was only modestly reduced. This mutant argues that preserving the rigid connection between the C-terminus and GDP-binding pocket is important for exchange, and further, that the α5-helix may be sufficient for coupling, as extending the helix increases the distance between the receptor and the protein core of Gα, thereby disrupting other potential receptor contacts (Natochin *et al.* 2001). Recently, an isoleucine-to-proline mutation at the C-terminus of the α5-helix has been shown to reduce the stimulatory activity of a D_2 dopamine receptor mimetic peptide on nucleotide exchange in G_i (Nanoff *et al.* 2006). In addition to providing a direct connection between the Gα C-terminus and the β6/α5 loop, receptor-induced perturbations in the α5-helix may be transmitted to the GDP-binding pocket *via* the α1-helix and β2- and β3-strands, as suggested by mutagenesis and computational studies (Marin *et al.* 2001b; Ceruso *et al.* 2004). Using SDSL, we have been able to observe receptor activation-dependent conformational changes in the α5-helix at sites distant from the receptor

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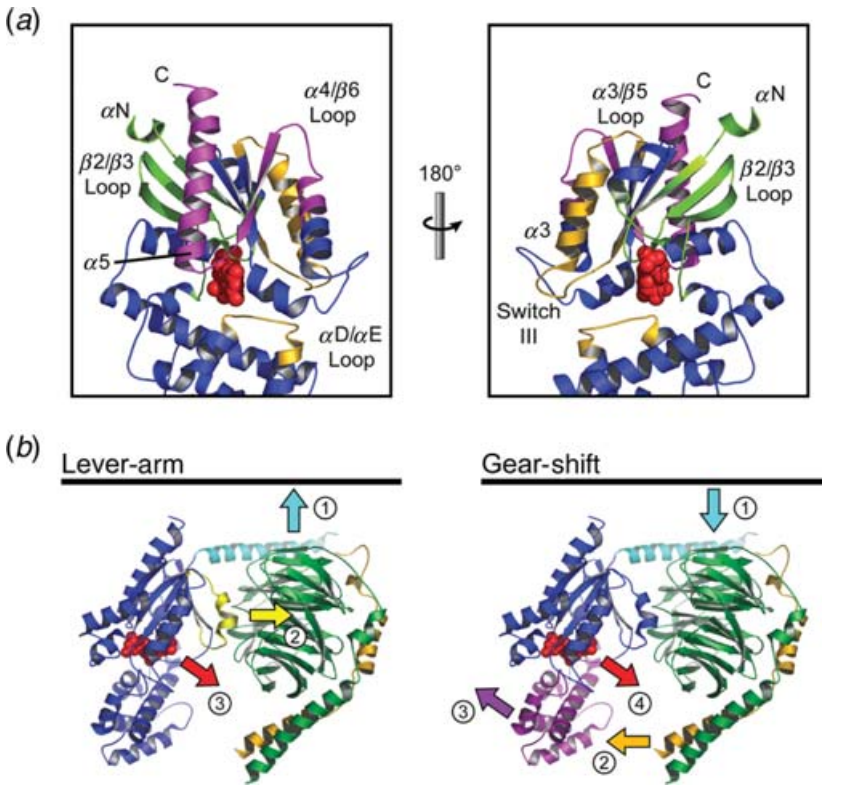


Fig. 5. Proposed mechanisms for receptor-mediated G-protein activation. (*a*) Ribbon model of Gα highlighting structural elements connecting receptor contact sites to the nucleotide-binding pocket. The α5-helix and β6-strand connect the C-terminus and α4/β6 loop to the nucleotide-binding pocket at the β6/α5 loop (magenta). The α3/β5 loop is connect to Switch III by the α3-helix (gold). Perturbations in Switch III may be communicated across the interdomain interface through its interactions with the αD/αE loop (gold). The αN-helix is connected to the P-loop and Switches I and II through its interactions with β-strands 1–3 (green). Additionally, these β-strands also interact with the α5-helix (magenta) and may participate in front-to-back communication between these elements. (*b*) Two models also propose that R* may use G$\beta\gamma$ to catalyze GDP release. In the lever-arm hypothesis, the receptor uses the αN-helix as a lever to pull G$\beta\gamma$ away from Gα (1), thereby prying Switch II away from the nucleotide-binding pocket (2) and causing GDP release (3). The alternative gear-shift model requires that the receptor push G$\beta\gamma$ closer to Gα (1). This allows the N-terminus of Gγ to engage the helical domain (2), thus forcing the binding pocket open due to the reorientation of the two domains (3), leading to GDP release (4).

contact surface. The five-glycine linker uncouples these conformational changes from receptor binding (Oldham *et al.* in press).

Similar to the α5-helix, the β6-strand may connect receptor binding of the α4/β6 loop to the binding pocket at the β6/α5 loop (Fig. 5*a*). Several alanine mutations in this region demonstrated reduced receptor-catalyzed activation, despite normal receptor binding (Onrust *et al.* 1997). The α3/β5 loop in Gα_s appears to be a receptor contact site (Grishina & Berlot, 2000) that may transmit a conformational change along the α3-helix to Switch III. Interactions between Switch III and the αD/αE loop at the domain interface have been implicated in receptor-mediated activation of Gα_s (Grishina & Berlot, 1998). Disruption of these interactions may open the gap between the helical and GTPase domains to release GDP. Interestingly, an arginine-to-glutamate mutation in Switch III has recently been shown to stabilize the nucleotide-free

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conformation of $G\alpha_t$ and, excitingly, this mutant blocks the rhodopsin-catalyzed activation of wild-type G_t (Pereira & Cerione, 2005). If applicable in other systems, this dominant negative mutant will be extremely valuable for the investigation of G-protein coupling.

Several intramolecular contacts with the N-terminal region have also been implicated in nucleotide exchange (Fig. 5*a*). Carboxyl terminal truncations in $G\alpha_o$ and $G\alpha_{i2}$ resulted in reduced affinity for GDP because of impaired interactions with the β1- and β3-strands (Denker *et al.* 1992, 1995). A hydrophobic interaction between the β1-strand and α5-helix in $G\alpha_t$ stabilizes the GDP-bound conformation (Natochin *et al.* 2000). The mutations described in these studies perturb the hydrophobic core of the $G\alpha$ subunit, which may account for their effects on nucleotide exchange more than any specific side-chain interactions. However, through its interactions with the αN-helix and $\alpha N/\beta 1$ loop, the activated receptor may similarly disturb these key stabilizing interactions, reducing the protein's affinity for GDP. Conformational changes in these regions could also affect β-strands 1–3, and thereby alter Switches I, II and the P-loop, all of which form important interactions with GDP.

Two models for receptor-mediated GDP release require that the receptor change the relative orientation of the $G\alpha$ and $G\beta\gamma$ subunits (Fig. 5*b*). In the first model, the receptor uses the N-terminal helix of $G\alpha$ as a lever-arm to pull $G\beta\gamma$ away from $G\alpha$. This would pry Switches I and II away from the nucleotide-binding pocket, resulting in GDP release (Iiri *et al.* 1998). In support of this hypothesis, shortening the αN-helix by four amino acids at its C-terminus to mimic the putative orientation induced by the receptor allows $G\beta\gamma$ to act as an exchange factor for $G\alpha_s$ (Rondard *et al.* 2001). The second gear-shift model proposes that the receptor uses the αN-helix to force $G\beta\gamma$ into $G\alpha$, allowing the N-terminus of $G\gamma$ to engage the helical domain of $G\alpha$. This interaction may cause the interdomain gap between the helical and GTPase domains of $G\alpha$ to open, leading to GDP release (Cherfils & Chabre, 2003).

These models propose that $G\beta\gamma$ plays a homologous role to the guanine nucleotide exchange factors for monomeric G proteins, and that the interaction between $G\alpha$ and $G\beta\gamma$ plays a critical role in G-protein activation. Indeed, several alanine mutations at the switch interface of $G\beta$ are defective in receptor-catalyzed nucleotide exchange despite normal heterotrimer formation (Ford *et al.* 1998). Furthermore, mutations in the C-terminus of $G\gamma$ have been shown to increase receptor-catalyzed nucleotide exchange (Azpiazu & Gautam, 2001). A small peptide has also recently been shown to increase spontaneous nucleotide exchange in $G\alpha_{i1}$. Structural studies indicate that this peptide dramatically displaces Switch II away from the nucleotide-binding pocket, presumably leading to GDP release, although the nucleotide remains bound in the crystal structure of the complex (Johnston *et al.* 2005). This study supports the lever-arm mechanism of nucleotide exchange; however, additional studies are necessary to test the catalytic role of $G\beta\gamma$ in receptor-catalyzed GDP release.

Bound GDP is buried between the GTPase and helical domains of $G\alpha$, although the direct interactions are formed by the GTPase domain. In order for GDP to be released, some of the contacts between these two domains may likely be broken. Several mutagenesis studies have implicated these contacts in nucleotide exchange; however, the results seem to differ depending on the $G\alpha$ subunit involved. In $G\alpha_s$ and $G\alpha_{i1}$, disrupting these interactions resulted in increased rates of basal or decreased rates of receptor-catalyzed exchange (Warner *et al.* 1998; Grishina & Berlot, 1998; Warner & Weinstein, 1999). The former indicating that an opening of the interdomain cleft reduces GDP affinity, and the latter indicating that the receptor may induce conformational changes across the domain interface through these interactions. However, these connections do not seem to be important for regulating the basal and catalyzed exchange rates in

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$G\alpha_t$ (Marin *et al.* 2001a). Thus, the role of interdomain interactions in G-protein activation remains unclear, but inspection of the crystal structure seems to require a movement of these two domains relative to each other in order to release GDP. Indeed, an interdomain reorientation has been suggested by molecular dynamics simulations of $G\alpha_t$ (Ceruso *et al.* 2004) and glycine-to-proline mutations in each of the interdomain linkers enhances the basal nucleotide exchange rate of $G\alpha$ (Majumdar *et al.* 2004).

Although each of the conformational changes described above is supported by mutagenesis and biochemical data, additional information is needed to understand the structural basis of receptor-mediated G-protein activation. Certainly, a high-resolution crystal structure would answer many questions about the orientation of these two proteins, the sites of interaction between them and the activating conformational changes that lead to complex formation and trigger GDP release. Unfortunately, this complex has proven exceedingly difficult to crystallize, and its structure may not be solved in the near future. In the mean time, different experimental approaches must be employed to refine current molecular models of the receptor–G protein complex and characterize the conformational changes leading to GDP release.

Some knowledge about the nature of conformational changes in the G protein also enables speculation about the molecular trigger causing a particular type of change. Site-directed mutagenesis of residues on the side of the α5-helix opposite the β6-strand significantly increase the basal nucleotide exchange rate of $G\alpha$ (Marin *et al.* 2001b). Perhaps the receptor disrupts these interactions in a similar manner. Assuming the general mechanism of receptor-catalyzed GDP release is conserved among receptor–G protein couplings, sequence analysis of conserved residues in these proteins may further serve to illustrate the important interactions for this process. One model suggests that there is a conserved binding site between the α5-helix and the adjacent $\beta2/\beta3$ loop for the conserved arginine in the D(E)RY motif of receptors, and that this interaction may underlie the mechanism for R*-mediated conformational changes in $G\alpha$ (Oliveira *et al.* 1999). Much work remains to be done, however, to identify the structural basis of G-protein activation, characterize the nucleotide-free conformation of the G protein and incorporate these data into improved models of the receptor–G protein complex.

4.2 GTP-mediated alteration of the receptor–G protein complex

Physiologically, the receptor–G protein complex is transient due to rapid binding of GTP, the concentration of which exceeds that of GDP by several-fold in cells. Binding of GTP to the $G\alpha$ subunit causes a structural rearrangement of $G\alpha$(GTP), $G\beta\gamma$ and the receptor that permits effector interactions. A comparison between the $G\alpha$(GDP) (Lambright *et al.* 1994; Mixon *et al.* 1995) and $G\alpha$(GTPγS) (Noel *et al.* 1993; Coleman *et al.* 1994a) crystal structures reveals conformational changes in the three Switch regions (Fig. 2*c*). This structural rearrangement eliminates the $G\beta\gamma$ binding surface, mediating subunit dissociation. The overall similarity of these crystal structures suggests that separation from the receptor is likely due to a reversal of the conformational changes that led to GDP release. GDP can also cause complex dissociation (Birnbaumer *et al.* 1972), and shares a similar affinity to GTP for the receptor-bound nucleotide-free $G\alpha$ (Heck & Hofmann, 2001). Thus, irreversible G-protein activation relies on the greater concentration of GTP within the cell and a structural rearrangement of $G\alpha$ and $G\beta\gamma$ following its binding. However, there is little direct experimental evidence describing the GTP-induced conformational changes in the receptor–G protein complex.

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Studies of G-protein activation in cells suggest that the receptor and G-protein subunits may remain closely associated following receptor activation *in vivo* (see Section 8.4) (Bunemann *et al.* 2003), so questions as to the structural consequences of GTP binding to this complex remain unanswered. Recent evidence suggests that G$\beta\gamma$ may be necessary to cause the dissociation of Gα from the receptor (Herrmann *et al.* 2004). A mutant Gα_t that has a C-terminal sequence with an increased affinity for Meta II can bind independently of its GDP- or GTP-bound conformational state, but dissociates like the native protein in the presence of G$\beta\gamma$. One explanation for this observation is that the G protein may proceed through a reversed sequence of interactions from those described above (see Section 3.6), leading to complex dissociation, which would require G$\beta\gamma$ (Herrmann *et al.* 2004).

In addition to the conformational changes observed in the Switch regions, GTP binding also causes a conformational change in the myristoylated N-terminus of Gα. Site-directed fluorescence labeling indicates that, while the non-myristoylated N-terminus returns to its dynamically disordered conformation, the myristoylated N-terminus moves into an even more hydrophobic environment in the Gα(GTP) state than in either the Gα(GDP) or heterotrimeric conformations (Fig. 3). These data suggest that the conformational changes associated with GTP binding expose an intramolecular binding site for the N-terminus (Preininger *et al.* 2003). Heterotrimeric G proteins may undergo a GTP-dependent myristoyl switch similar to that described for the small GTPase ARF, where nucleotide exchange is coupled to a conformational change that leads to dissociation from the membrane (Goldberg, 1998). The disordered state of the non-myristoylated N-terminus of Gα(GTP) may reflect the transition state leading to intramolecular binding (Fig. 3) (Preininger *et al.* 2003). Additional studies are necessary to identify the binding site for the N-terminus, such as crystallization of myristoylated Gα or biophysical distance measurements. Also, the structural consequences for the N-terminus of non-myristoylated Gα subunits remain unexplored.

5. Activation of downstream effector proteins

Following subunit dissociation, Gα(GTP) and G$\beta\gamma$ each go on to activate specific downstream effector proteins (for an extensive list, see Kristiansen, 2004). Several crystallographic studies provide a significant insight into the structural basis of G protein–effector interactions (Fig. 6).

5.1 Gα interactions with effectors

The first structural information concerning the mechanism of effector activation came from a comparison of the Gα(GTPγS) (Noel *et al.* 1993; Coleman *et al.* 1994a) and Gα(GDP) (Lambright *et al.* 1994; Mixon *et al.* 1995) structures. Significant structural differences were identified in three flexible loops, Switches I–III, near the γ-phosphate binding site. Upon GTP binding, these flexible loops become ordered in a structural rearrangement that disrupts the G$\beta\gamma$-binding site to form a new surface for effector interactions.

Each Gα family member interacts with effector proteins in a highly specific manner. Classical Gα effectors include photoreceptor cGMP phosphodiesterase (PDE) (Gα_t sequesters the inhibitory PDEγ subunit), adenylyl cyclase (AC) (Gα_s activates, Gα_i inhibits), phospholipase Cβ (PLCβ) (Gα_q) and p115RhoGEF (Gα_{13}). Structures of representative effector complexes from each Gα subfamily have been solved, Gα_s·AC (Tesmer *et al.* 1997b), Gα_t·PDEγ·RGS9 (Slep *et al.* 2001), Gα_{13}·p115RhoGEF (Chen *et al.* 2005b) and Gα_q·G-protein-coupled receptor kinase 2

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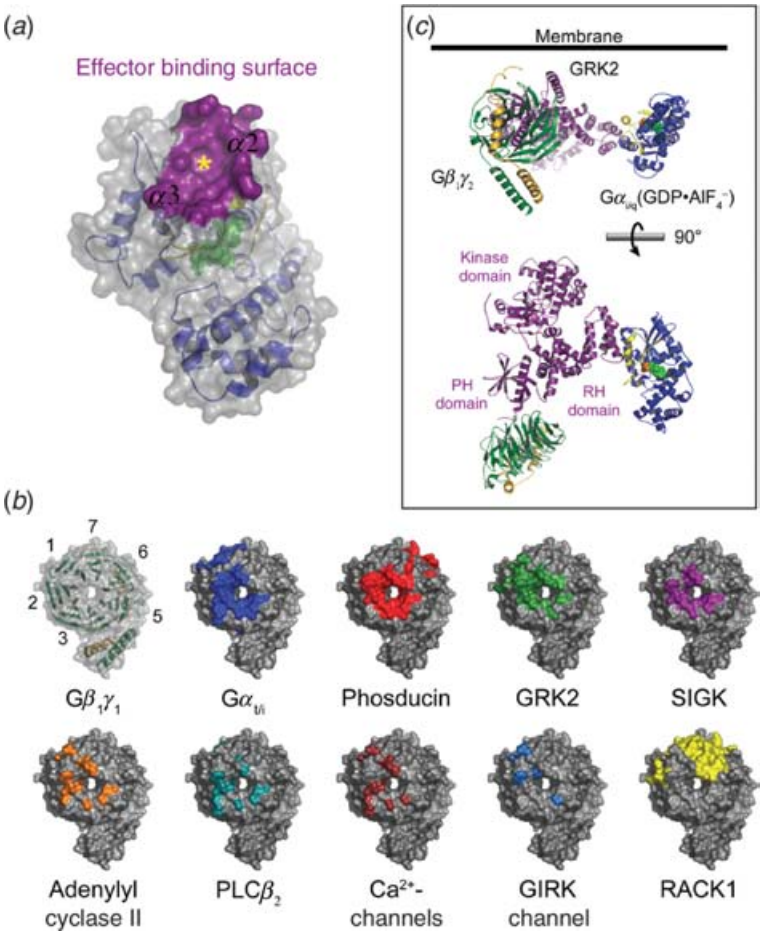


Fig. 6. Subunit interactions with effector proteins. (*a*) Surface rendering of $G\alpha_t$(GTPγS) with the PDEγ binding site highlighted (purple). In all of the effector-Gα crystals that have been solved to date, the effector proteins interact with a hydrophobic pocket (yellow asterisk) lined by the α2- (Switch II) and α3-helices (adapted from Tesmer *et al.* 2005). (*b*) Surface renderings of $G\beta_1\gamma_1$ (1GOT) highlighting the unique, but overlapping binding sites of several interacting proteins. The surfaces in the top row were determined by crystallography, and the surfaces in the bottom row were determined by alanine-scanning mutagenesis in $G\beta_1$ as well as peptide competition studies (RACK1). (*c*) The recently solved structure of the $G\alpha_{i/q}$·GRK2·$G\beta_1\gamma_2$ complex (2BCJ) clearly illustrates the structure of effector–subunit interactions. Moreover, this complex suggests the importance of macromolecular complexes for organizing signal transduction pathways *in vivo*.

(GRK2)·G$\beta\gamma$ (Tesmer *et al.* 2005), demonstrating a localized effector-binding surface on Gα. In each complex, hydrophobic side-chains from the effector insert into a pocket formed between the N-termini of the α2- (Switch II) and α3-helices (Fig. 6*a*). Additional contacts are made between the C-termini of these helices and the $\alpha2/\beta4$ and $\alpha3/\beta5$ loops that determine effector specificity. The tertiary structures of these interacting regions are well conserved, with the exception of the $\alpha3/\beta5$ loop in $G\alpha_s$, suggesting that in most subfamilies effector specificity is determined by the primary sequence of the Gα subunit (Tesmer *et al.* 2005). The specificity of $G\alpha_s$ is regulated by backbone conformation as well as primary sequence (Sunahara *et al.* 1997b). The recently solved structure of the $G\alpha_q$·GRK2·G$\beta\gamma$ complex also reveals an interesting 105°

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rotation of $G\alpha_q$ that brings Switch I, Switch II, Linker 1 and the $\alpha B/\alpha C$ loop in closest proximity to the membrane surface 30 Å away (Fig. 6*c*) (Tesmer *et al.* 2005). Both the $G\alpha_t$·PDEγ·RGS9 and $G\alpha_{13}$·p115RhoGEF structures would allow a similar reorientation of $G\alpha$, but the $G\alpha_s$·AC structure would not. Unfortunately, the N-terminal helix of $G\alpha_q$ is disordered in this structure, and its role in complex formation is unclear. Myristoylation of the N-terminus of $G\alpha_{i1}$ is important for the inhibition of AC (Gallego *et al.* 1992; Taussig *et al.* 1993; Dessauer *et al.* 1998) and at least one N-terminal cysteine in $G\alpha_q$ is necessary for normal activation of PLCβ_1 (Wedegaertner *et al.* 1993) regardless of its palmitoylation state (Hepler *et al.* 1996). Additional studies are needed to dissect the role of the N-terminus and its fatty-acid modifications in the modulation of effector interactions. In addition to the effector-binding site illustrated by the crystal structures, several studies indicate that the $\alpha 4/\beta 6$ loop of $G\alpha$ is important for PDEγ binding to $G\alpha_t$ (Rarick *et al.* 1992; Liu *et al.* 1999) and the activation of AC by $G\alpha_s$ (Berlot & Bourne, 1992).

The structural features of these interactions also underlie several other observations regarding effector activation. First, the GDP-bound $G\alpha$ subunit has some ability to interact with effectors, although with 20- to 100-fold reduced potency than the GTP-bound protein (Skiba *et al.* 1996; Sunahara *et al.* 1997a). These data suggest that re-association of $G\alpha$ with $G\beta\gamma$ is required for the complete termination of G-protein signaling. Second, even closely related $G\alpha$ subunits activate unique complements of effector proteins. For example, $G\alpha_{i2}$ and $G\alpha_{i3}$ specifically inhibit forskolin- and $G\alpha_s$-stimulated activation of AC, respectively (Ghahremani *et al.* 1999). Similarly, the α_1-adrenergic receptor elevates intracellular Ca^{2+} through two distinct mechanisms by coupling to $G\alpha_q$ and $G\alpha_{11}$ (Macrez-Lepretre *et al.* 1997). Finally, some $G\alpha$ subunits have only one identified effector, like $G\alpha_t$ (PDEγ), while some couple to several effector proteins, like $G\alpha_{12}$ [several RhoGEFs, PLCε and protein phosphatase 5, among others (reviewed in Riobo & Manning, 2005)]. Certainly not all $G\alpha$ effectors have been identified, and the search for novel proteins that interact with $G\alpha$ subunits is an active area of research.

5.2 $G\beta\gamma$ interactions with effectors and regulatory proteins

Initially, $G\beta\gamma$ was thought to facilitate the termination of the G-protein signal by passively binding to $G\alpha$ and hastening the return of the heterotrimer to the plasma membrane, thereby reducing spontaneous activation of $G\alpha$ in the absence of receptor stimulation (Neer, 1995). This perspective changed when $G\beta\gamma$ was shown to activate a K^+-selective ion channel in cardiac atrial cells (Logothetis *et al.* 1987). Since that time, many other effectors of $G\beta\gamma$ have been identified, including PLCβ_2 and β_3 (Katz *et al.* 1992; Li *et al.* 1998), AC (Tang & Gilman, 1991), β-adrenergic receptor kinase (βARK) (Pitcher *et al.* 1992), phosphoinositide-3-kinase (Stephens *et al.* 1994), mitogen-activated Protein kinase pathway components (Inglese *et al.* 1995) and K^+ and Ca^{2+} ion channels (Logothetis *et al.* 1987; Reuveny *et al.* 1994; Ikeda, 1996; Herlitze *et al.* 1996). Novel effectors are being discovered all the time (Blackmer *et al.* 2001, 2005; Dell *et al.* 2002; Spiegelberg & Hamm, 2005; Gerachshenko *et al.* 2005).

Many mutational and structural studies demonstrate that effector proteins share unique, but overlapping binding sites on $G\beta\gamma$ (Fig. 6*b*). Alanine-scanning mutagenesis in $G\beta$ identified several residues that mediate interactions with PLCβ_2, ACII, βARK and ion channels (Ford *et al.* 1998; Panchenko *et al.* 1998; Li *et al.* 1998). For example, the ACII interaction surface maps roughly to blades 2, 3 and 5, while the N-terminal interface of $G\beta$ interacts with G-protein-activated inwardly rectifying K^+ (GIRK) channels 1 and 4 (Ford *et al.* 1998). Point mutations in

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blades 1–4 or the outer loops of blades 2, 6 and 7 inhibit PLCβ_2 binding (Ford *et al.* 1998; Panchenko *et al.* 1998), whereas the interaction between PLCβ_3 and G$\beta\gamma$ is blocked by point mutations within blades 2 and 5 (Li *et al.* 1998). Crystal structures of G$\beta\gamma$ in complex with the regulatory protein phosducin (Gaudet *et al.* 1996; Loew *et al.* 1998), the effector GRK2 (Lodowski *et al.* 2003; Tesmer *et al.* 2005) and a small peptide that blocks these interactions (Davis *et al.* 2005), as well as competition studies using Gβ peptides that block the interaction between G$\beta\gamma$ and the receptor for activated C kinase 1 (RACK1) (Chen *et al.* 2005a), further demonstrate that G$\beta\gamma$-binding proteins use a unique complement of residues within a common binding surface to mediate binding (Davis *et al.* 2005). Moreover, this surface is also the binding site for the Switch II region in Gα subunits, and thus, Gα subunits are effective inhibitors of G$\beta\gamma$–effector interactions.

The overall structure of G$\beta\gamma$ is preserved in all of the crystal structures solved thus far, although the interstrand loops move significantly upon complex formation to form an appropriate binding site for specific interacting proteins. The dynamics of these loops probably enable Gβ to bind to such a variety of structurally diverse targets. An additional structural change is observed in the G$\beta_1\gamma_1$·phosducin structures (Gaudet *et al.* 1996; Loew *et al.* 1998; Gaudet *et al.* 1999), where a cavity is introduced between blades 6 and 7 that may represent a putative farnesyl-binding pocket for Gγ C-terminus (Loew *et al.* 1998). Phosducin sequesters G$\beta_1\gamma_1$ away from the membrane in photoreceptor cells to attenuate G-protein signaling, and this phosducin-induced switch in the Gγ farnesyl moiety provides the structural basis for membrane dissociation. This structural change in G$\beta\gamma$ may be a general mechanism by which G$\beta\gamma$-binding proteins regulate the localization of G$\beta\gamma$ and attenuate signaling through Gα subunits, however, it has not been observed in other complexes thus far.

Downstream signaling through G$\beta\gamma$ may also be regulated through the specific composition of the dimer. All of the possible dimers formed from combinations of Gβ_1 or Gβ_2 with Gγ_1, Gγ_2, Gγ_3, Gγ_5 or Gγ_7 could activate PLCβ isoforms except the retinal-specific dimer, G$\beta_1\gamma_1$ (Iniguez-Lluhi *et al.* 1992; Ueda *et al.* 1994). Similarly, G$\beta_1\gamma_1$ was less effective at stimulating ACII and inhibiting ACI than other dimer combinations (Iniguez-Lluhi *et al.* 1992; Ueda *et al.* 1994). Comparing G$\beta_1\gamma_2$ with G$\beta_5\gamma_2$ demonstrates that G$\beta_5\gamma_2$ is a much weaker inhibitor of ACI, ACV and ACVI, and G$\beta_1\gamma_2$ stimulates ACII while G$\beta_5\gamma_2$ inhibits it (Bayewitch *et al.* 1998a, b). Later studies showed that the remaining Gβ subunits activate ACII with a potency similar to Gβ_1, and Gβ_2 and Gβ_4 inhibited ACI as well as Gβ_1, while Gβ_3 was 10-fold less potent (McIntire *et al.* 2001). Interestingly, the specificity of these subtype interactions differ among effectors, as both G$\beta_1\gamma_2$ and G$\beta_5\gamma_2$ activate PLCβ_2 with similar potency and efficacy (Zhang *et al.* 1996). Additionally, the potency of G$\beta\gamma$ inhibition of voltage-dependent N-type Ca^{2+} currents differs depending on the identity of the Gβ subunit in the dimer (Ruiz-Velasco & Ikeda, 2000). Similarly, GRK2 can be recruited to the plasma membrane by Gβ_1 and Gβ_2, but not Gβ_3, to initiate receptor desensitization (Daaka *et al.* 1997). Together, these data indicate that the Gβ isoform is a major determinant of effector coupling specificity.

In addition, the Gγ isoform and, in particular, its lipid modification has been shown to affect effector coupling. The native geranylgeranylated G$\beta_1\gamma_2$ is more effective in activating PLCβ than farnesylated G$\beta_1\gamma_1$ and G$\beta_1\gamma_{11}$ (Myung *et al.* 1999). If the prenyl groups are reversed on these proteins, farnesylated G$\beta_1\gamma_2$ becomes less able to activate PLCβ, while the activity of the geranylgeranylated G$\beta_1\gamma_1$ and G$\beta_1\gamma_{11}$ increases (Myung *et al.* 1999). Similarly, farnesylation of G$\beta_1\gamma_2$ decreased its ability to activate ACII, but geranylgeranylation of G$\beta_1\gamma_1$ and G$\beta_1\gamma_{11}$ was unable to restore their activity, suggesting that primary sequence as well as the prenyl

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modification are important determinants of effector activation (Myung *et al.* 1999). Similarly, altering the position of the geranylgeranyl by insertions, deletions or mutations in the C-terminus of $G\gamma_5$ disrupted interactions with PLCβ (Akgoz *et al.* 2002). Most recently, studies with Gγ chimeras have shown that the N-terminus of $G\gamma_2$ is a better activator of ACII than the N-terminus of $G\gamma_1$. Also, the geranylgeranylated C-terminus of $G\gamma_2$ is a more potent activator of PLCβ_2 and a more potent inhibitor of ACI than the geranylgeranylated C-terminus of $G\gamma_1$ or farnesylated C-terminus of $G\gamma_2$ (Myung *et al.* 2005). Clearly, the composition of the G$\beta\gamma$ dimer plays an important role in coupling to specific effectors. Areas of future research include identifying the structural features responsible for this specificity and characterizing many of these interactions in systems *in vivo*.

6. G-protein inactivation

6.1 Intrinsic GTPase activity of Gα

Termination of the G-protein signal is achieved upon the intrinsic hydrolysis of GTP to GDP by the Gα subunit and re-association of the heterotrimer (Fig. 7). Structural insight into the mechanism of GTP hydrolysis in Gα subunits was provided by crystal structures of Gα(GDP) bound to aluminum fluoride (Sondek *et al.* 1994; Coleman *et al.* 1994a). Aluminum fluoride is a strong activator Gα and binds with GDP in the active site as a tetracoordinate AlF_4^- ion (Coleman *et al.* 1994a; Sondek *et al.* 1994). In the crystal structures of Gα(GDP·AlF_4^-), the fluoraluminate is in a square planar configuration, with the oxygen of the β-phosphate and a water molecule as transaxial ligands, forming a tetragonal bipyramid (Coleman *et al.* 1994a; Sondek *et al.* 1994; Sprang, 1997). Inspection of this transition state active site reveals the functional roles of several residues important for GTPase activity, including a conserved glutamine that polarizes and orients the nucleophilic water molecule and a conserved arginine that stabilizes the developing negative charge on the leaving group to facilitate its release (Fig. 7*a*) (Noel *et al.* 1993; Coleman *et al.* 1994a). Due to its important role in GTP hydrolysis, ADP-ribosylation of this arginine by cholera toxin abolishes the GTPase activity of $G\alpha_s$ and $G\alpha_t$ (Freissmuth & Gilman, 1989; Van Dop *et al.* 1984), as do mutations of this residue (Landis *et al.* 1989; Coleman *et al.* 1994a; Sprang, 1997). Mutations that slow GTP hydrolysis lead to constitutive activation of Gα, which has been shown to cause sporadic endocrine tumors (Lyons *et al.* 1990) and McCune–Albright syndrome (Weinstein *et al.* 1991). Two mutations in the nucleotide-binding pocket, a glycine-to-valine substitution in the P-loop and a glycine-to-alanine mutation in Switch II, stabilize a $G\alpha_{i1}$(GDP·P_i) complex that is associated with a substantial conformational change in Switch II (Fig. 7*a*) (Berghuis *et al.* 1996; Raw *et al.* 1997). An additional lysine-to-proline mutation in Switch I accelerates a conformational change in the Switch regions, but decreases the rate of GTP hydrolysis, suggesting that this transition is an obligatory step leading to bond breakage (Thomas *et al.* 2004). Thus, the dynamics of the Switch regions may be critical for GTP hydrolysis.

Recently, structures of $G\alpha_{12}$(GDP·AlF_4^-) and $G\alpha_{13}$(GDP) highlight a novel conformational change that is likely coupled to GTP hydrolysis in this family of Gα subunits (Fig. 7*c*) (Kreutz *et al.* 2006). A comparison of these two structures indicates that the αD/αE1 loop puckers upon binding AlF_4^-, and that a glutamate residue in this loop acts as a spring to rotate the helical domain away from the GTPase domain upon GTP hydrolysis. This conformational change may facilitate dissociation of these Gα subunits from effector proteins or influence the rates of

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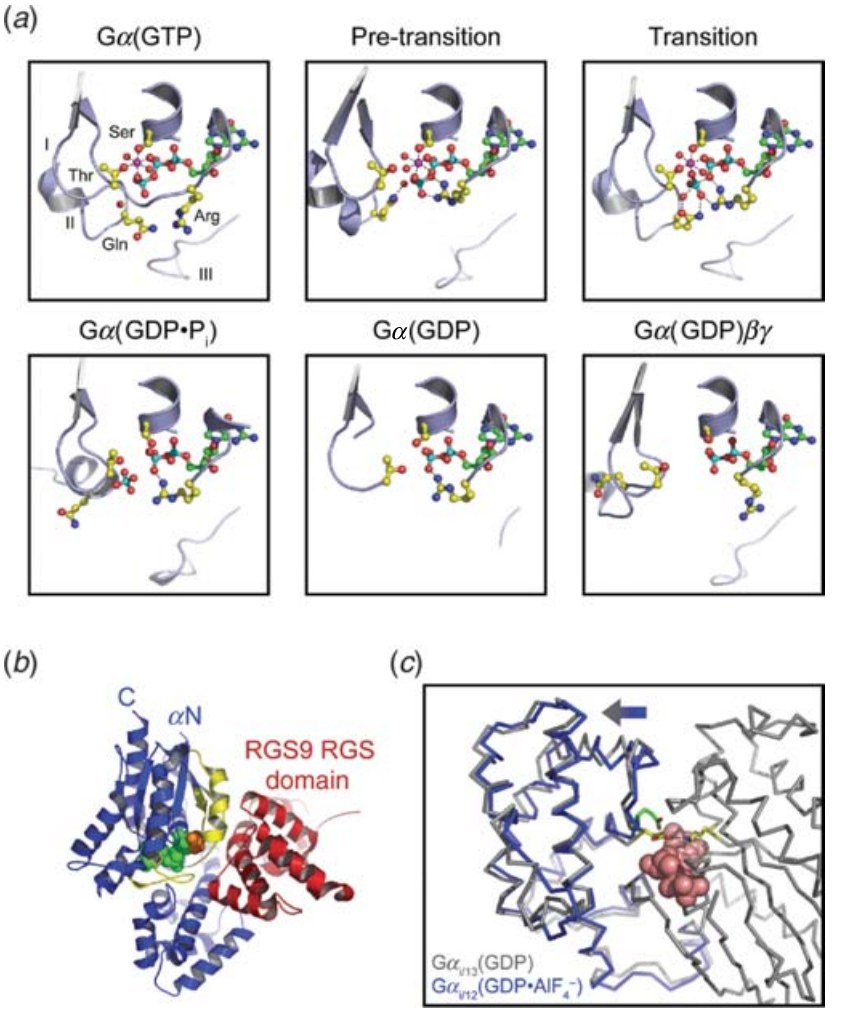


Fig. 7. Mechanisms of GTP hydrolysis by Gα. (*a*) Crystallographic snapshots of GTP hydrolysis. Four important residues for stabilizing the transition state are shown (yellow side-chains), including highly conserved arginine (Arg) and glutamine (Gln). In the Gα(GTP) conformation (1GIA), serine (Ser) and threonine (Thr) coordinate Mg^{2+}, and the nucleophilic water molecule is observed approaching the γ-phosphate (loop between Arg and Thr is removed in subsequent panels for clarity). Prior to bond breakage, a conformational change occurs in Switches I and II that orients Gln and Arg toward the γ-phosphate [based on Gα_t(GDP); 1TAG] in a pre-transition state complex. The transition state is stabilized by Gln and Arg (1GFI), and a conformational change in Switch II is observed in Gα(GDP·P$_i$) following bond breakage (1AS2). The switch regions are disordered in Gα_{i1}(GDP) (1GDD), and G$\beta\gamma$ binding significantly remodels the nucleotide binding pocket (1GP2). (This series of snapshots was based on the discussion presented in Thomas *et al.* 2004). (*b*) Structure of the RGS9 RGS domain binding to Gα_t(GTP·AlF$_4^-$) (1FQK). The RGS proteins enhance the basal GTP hydrolysis rate of Gα subunits by stabilizing the transition state. Structures of these complexes indicate that the RGS domain itself does not directly interact with the nucleotide, unlike the GAPs of monomeric G proteins. (*c*) The recently solved structures of G$\alpha_{i/12}$(GTP·AlF$_4^-$) (1ZCA) and G$\alpha_{i/13}$(GDP) (1ZCB) suggest that GTP hydrolysis leads to an 8·5° rotation of the helical domain away from the GTPase domain in this family of Gα subunits (arrow). This rotation is apparently due to an activation dependent 'spring' in the αD/αE loop mediated by a glutamate residue (stick side-chains) unique to the Gα_{12} family. In the active conformation, this glutamate (green) puckers the αD/αE loop. Following GTP hydrolysis, this residue forms a salt bridge with an adjacent lysine across the binding pocket (yellow).

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nucleotide exchange or hydrolysis, however, additional studies are needed to appreciate the consequences of this unique structural feature of the $G\alpha_{12}$ family.

The intrinsic GTP hydrolysis activity varies among $G\alpha$ family members, where the $G\alpha_s$ (3·5 min^{-1} at 20 °C) and $G\alpha_i$ (1·8–2·4 min^{-1} at 20 °C) family members have higher GTPase activity than members of the $G\alpha_q$ (0·8 min^{-1} at 30 °C) or $G\alpha_{12}$ (0·2 min^{-1} at 30 °C) families (except $G\alpha_z$, 0·05 min^{-1} at 30 °C). The structural reasons for this difference are not clear, but may include differences in the helical domains, N- and C-termini or subtle fluctuations in backbone dynamics (Fields & Casey, 1997; Cabrera-Vera *et al.* 2003). The mechanisms responsible for the variations have not been studied in detail, but rather research in this area has focused on the identification and characterization of the increasing number of proteins that regulate the intrinsic GTPase activity of $G\alpha$ subunits.

6.2 GTPase-activating proteins

Initially, the hydrolysis of GTP was thought to be an unregulated function of $G\alpha$ subunits that offered intrinsic control of the duration of the G-protein signal. However, it was soon noted that the intrinsic GTPase activity of many purified $G\alpha$ subunits *in vitro* was much slower than the deactivation rates of G-protein signaling processes *in vivo*, suggesting that some GTPase accelerating factor was lost during purification (reviewed in Ross & Wilkie, 2000). The first two GAPs identified were the $G\alpha$ effectors PLCβ_1 ($G\alpha_q$) and PDEγ ($G\alpha_t$) (Arshavsky & Bownds, 1992; Berstein *et al.* 1992). More recently, it has been demonstrated that ACV is a GAP for $G\alpha_s$ (Scholich *et al.* 1999). Thus, there is a common paradigm that effectors, after activation by $G\alpha$, can negatively regulate the amplitude and duration of the G-protein signal.

In addition to effector-mediated feedback inhibition, another class of GAPs has been identified, the regulators of G-protein signaling. These proteins share a common 120-amino-acid RGS domain that forms a bundle of nine α-helices that binds to the Switch regions on $G\alpha$. This domain enhances the GTPase activity of $G\alpha$ by stabilizing the transition state conformation and orienting the $G\alpha$ backbone to stabilize the nucleophilic water molecule (Fig. 7*b*) (Tesmer *et al.* 1997a; Slep *et al.* 2001). However, the RGS domain itself does not directly take part in the chemistry of hydrolysis, unlike the GAPs for small G proteins that contribute an extrinsic catalytic arginine residue to the active site (reviewed in Sprang, 1997). The molecular mechanism for effector-mediated GAP activity has not been clearly determined. These proteins may also stabilize the transition state, but differences in activity between effector GAPs and RGS GAPs have been observed. For example, RGS4 produces a two-fold greater activation of GTP hydrolysis in $G\alpha_q$ over PLCβ, but PLCβ is 100 times more potent than RGS4 (Mukhopadhyay & Ross, 1999). However, G$\beta\gamma$ can inhibit the GAP activity of both of these proteins (Chidiac & Ross, 1999), consistent with the idea that G$\beta\gamma$, effectors and RGS proteins all compete for binding to $G\alpha$. This effect may also be due to G$\beta\gamma$-mediated stabilization of $G\alpha$(GDP), which binds poorly to effectors and RGS proteins. Regardless, RGS proteins could inhibit effector activation in addition to accelerating GTP hydrolysis. Alternatively, the $G\alpha_t$ effector, PDEγ, enhances the GAP activity of RGS9 on $G\alpha_t$ by increasing its affinity for the protein (Skiba *et al.* 1999, 2000), and structural studies indicate that the effectors and GAPs may have evolved to interact with non-overlapping binding sites (Tesmer *et al.* 2005).

Again, the N-terminal domain of $G\alpha$ is an important, but uncharacterized, regulator of $G\alpha$–GAP interactions (Ross & Wilkie, 2000). Proteolysis, phosphorylation and palmitoylation

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(Wang *et al.* 1998; Tu *et al.* 1997; Glick *et al.* 1998) have all been shown to decrease the affinity of Gα for GAPs by 100-fold, while myristoylation increases the affinity of this interaction (Tu *et al.* 1997). Mutations in the αN-helix reduce the basal GTP hydrolysis rate of Gα_z, suggesting that its role may be to maintain the structure of the active site (Wang *et al.* 1999). The helix may also contribute to the binding site for the GAP, although this type of interaction is not observed in either crystal structure (Tesmer *et al.* 1997a; Slep *et al.* 2001). Other regions in both proteins outside of the known molecular interface have also been shown to influence Gα–GAP interactions. For example, the helical domain of Gα_t is a key molecular determinant of RGS9 selectivity of Gα_t over Gα_i (Skiba *et al.* 1999), and flanking regions of RGS4 are important for binding to Gα_q, but not Gα_i. Along with their role as key desensitizers of heterotrimeric G-protein signaling, RGS proteins are recognized as important scaffolds that facilitate the rapid and specific activation and termination of signal transduction pathways mediated by heptahelical receptors (reviewed in Ross & Wilkie, 2000; McCudden *et al.* 2005).

7. Novel regulation of G-protein signaling

The basic model of G-protein signaling can be expanded to include two additional regulatory mechanisms, non-receptor GEFs and guanine nucleotide dissociation inhibitors (GDIs). Several proteins have been identified that increase GTPγS binding to Gα subunits, including GAP-43, β-APP, presenilin 1, PBP/RKIP, Ric-8A and AGS1 (reviewed in Sato *et al.* 2006). These proteins appear to be selective for the Gα_i family, although Ric-8A can also activate Gα_q *in vitro* (Tall *et al.* 2003). Additional work remains before the regulation of the GEF activity of these proteins and its functional consequences are completely understood, although excellent progress is being made. Indeed, Ric-8A has been shown to play a critical role in asymmetric cell division during embryogenesis in *C. elegans* (reviewed in McCudden *et al.* 2005) and AGS1 is important for processing signals in the suprachiasmatic nucleus during the circadian cycle (reviewed in Sato *et al.* 2006).

By contrast to the soluble GEFs, which cause GDP release, the class of GDI proteins inhibit GDP dissociation from Gα_i family members, which can dampen signaling through Gα or prolong signaling through G$\beta\gamma$ (reviewed in McCudden *et al.* 2005; Sato *et al.* 2006). The GDI proteins have been implicated as crucial players in asymmetric cell division during embryogenesis (reviewed in Willard *et al.* 2004; McCudden *et al.* 2005; Sato *et al.* 2006). Most of these proteins contain one or more GoLoco motifs (reviewed in Willard *et al.* 2004). The GoLoco domain is a 19-amino-acid sequence that slows spontaneous GDP release from Gα(GDP) and prevents its interaction with G$\beta\gamma$. A crystal structure shows that the N-terminus of the GoLoco domain of RGS14 forms an α-helix that is sandwiched between Switch II and the α3-helix of Gα_i(GDP) (Kimple *et al.* 2002). The GoLoco peptide extends to the helical domain, and contacts Switch I and GDP. Perturbation of Switch II would preclude coincident binding of G$\beta\gamma$ to this complex. The arginine in the highly conserved aspartate-glutamine-arginine (DNR) sequence in GoLoco motifs, inserts into the nucleotide-binding pocket and interacts with the α- and β-phosphates of GDP and the bridging oxygen atom between them. GoLoco binding also results in the formation of a salt bridge between the P-loop and Switch I that is also observed in the structure of the G_i heterotrimer (Wall *et al.* 1995), which locks GDP into the binding pocket. These two new stabilizing interactions provide the structural basis for the GDI activity of GoLoco-containing proteins (Kimple *et al.* 2002).

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8. New approaches to study G-protein dynamics

Crystallography has provided the foundation for much of our current understanding of G-protein signaling (Table 1). Indeed, these structures are the framework for interpreting virtually any biochemical or biophysical experiment conducted in this field. Fortunately, new structures continue to provide exciting and interesting insights into G-protein function, and several remain on the collective wish list of investigators in the field, including the receptor–G protein complex, activated rhodopsin, any receptor that is not rhodopsin, a myristoylated Gα subunit and a nucleotide-free Gα subunit. However, less structural information is available from current crystal structures about important regions of these molecules, such as the N- and C-termini of Gα. Furthermore, the conformations and behavior of these proteins in solution may differ markedly from the snapshot provided by crystallography. Thus, alternative approaches, such as NMR, fluorescence and EPR spectroscopy, as well as computational techniques, will be necessary to investigate the dynamics of G-protein signaling. When combined with high-resolution crystallographic data, these lower resolution approaches will provide tremendous insight into the structural basis of function in G proteins.

8.1 Nuclear magnetic resonance spectroscopy

Recently, the high yield expression and purification of an isotopically labeled Gα_t chimera has been reported, thereby greatly facilitating the study of this protein with NMR (Abdulaev *et al.* 2005b). The two-dimensional ^{1}H-^{15}N heteronuclear single-quantum correlation (^{15}N-HSQC) spectrum of Gα(GDP) is relatively well dispersed and 340 of 397 cross-peaks are readily identifiable. Upon the addition of AlF_4^-, many of the amide resonances in the spectrum shift, particularly the indole ^{1}HN and ^{15}N resonances of two tryptophan residues in Switch II and the adjacent α3-helix, consistent with the crystal structures of the protein in these conformations (Lambright *et al.* 1994; Sondek *et al.* 1994). After this initial study, the authors measured the ^{15}N-HSQC spectrum of a reconstituted heterotrimer (Abdulaev *et al.* 2005a). Interestingly, the chemical shifts observed upon G$\beta\gamma$ binding are generally similar to those observed in the spectra for the AlF_4^-- and GTPγS-bound Gα subunit, specifically for the three tryptophan residues and C-terminal phenylalanine for which definitive assignments were made, suggesting that these two protein conformations are also similar. A model is proposed whereby G$\beta\gamma$ organizes the C-terminus of Gα and the nucleotide-binding pocket for binding to R* and GTP, respectively. In order to reconcile the NMR results with the crystal structures of these conformations, the authors point out that the conformation of Gα(GDP·AlF_4^-) can be modeled into the structure of the heterotrimer with minimal changes in the interfacial contacts between the proteins. This model rotates the helical domain of Gα closer to the N-terminus of Gγ, similar to the gear-shift model described above. However, it seems extremely unlikely that AlF_4^- can bind in the presence of G$\beta\gamma$, as AlF_4^- binding is highly dependent on a structural rearrangement of Switch II.

This approach has also been used to monitor conformational changes associated with receptor-catalyzed GTPγS binding (Ridge *et al.* 2006). In this study, the authors found that R*-generated Gα(GTPγS) was more conformationally dynamic than that generated independently of R*, suggesting that the increase in protein dynamics may be due to interactions with R* and G$\beta\gamma$. Changes in the NMR spectra during R*-catalyzed exchange continued several hours after GTPγS binding appeared to be complete, which the authors suggest is due to slow dissociation of Gα(GTPγS) and G$\beta\gamma$. Support for this hypothesis comes from the observation that

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a soluble receptor mimic that does not catalytically activate the G protein (Abdulaev *et al.* 2000) is found to form a stable complex with a GTPγS-bound heterotrimer (Ridge *et al.* 2006). The C-terminus, which displays two conformational states in the heterotrimer (Abdulaev *et al.* 2005a), is found exclusively in an 'activated' conformation in the putative R*·Gα(GTP)$\beta\gamma$ complex and the Gα(GTP) conformation (Ridge *et al.* 2006).

Although complete assignment of the cross-peaks and analysis of the solution structures will be necessary before the differences between the crystal structures and the NMR data are completely understood, the current analysis raises some questions. For example, it seems unlikely that the dynamics of a tryptophan residue in Switch II, a region that contributes significantly to the G$\beta\gamma$-binding surface (Wall *et al.* 1995; Lambright *et al.* 1996), would increase upon heterotrimer formation. Indeed, these flexible loops are absent in the structure of Gα_{i1} that lacks either crystal contacts or G$\beta\gamma$ to stabilize this region (Mixon *et al.* 1995). Our own SDSL studies indicate that G$\beta\gamma$ dampens the backbone dynamics of Switch II (N. Van Eps *et al.*, unpublished observations). Moreover, the authors suggest that G$\beta\gamma$ pre-organizes the nucleotide-binding site for GTP, but this role is difficult to reconcile with the well-established negative cooperativity between heterotrimer formation and GTP binding (Higashijima *et al.* 1987), as is the observation that the heterotrimer dissociates slowly following GTP binding. Certainly this effort is in the initial stages, but it has already raised some provocative issues concerning the dynamics of G-protein signaling. Moreover, it is on a trajectory to provide the first high-resolution structural information for the receptor-bound nucleotide-free Gα, which would hopefully expose the molecular mechanism of GDP release.

8.2 Site-directed labeling techniques

Site-directed cysteine mutagenesis enables the specific incorporation of a thiol-reactive, environmentally sensitive probe to monitor local conformational changes virtually anywhere in a given protein. One of the most extensively used applications of this technique has been the incorporation of paramagnetic probes to investigate protein dynamics using EPR. An EPR spectrum indicates the mobility of the side-chain, the solvent accessibility determined by the collision frequency with paramagnetic quenchers in solution and the distance from another paramagnetic center, such as a metal ion or second spin label (reviewed in Hubbell *et al.* 2000). Conventional, continuous-wave EPR can accurately measure distances between 8 Å and 25 Å (Rabenstein & Shin, 1995), while pulsed EPR spectroscopy extends that distance to $\sim$70 Å (Pannier *et al.* 2000). Additionally, EPR spectra are characteristic for different secondary structural features in proteins (Mchaourab *et al.* 1996). Extensive spin-labeling studies of rhodopsin were the first to reveal the conformational changes that accompany receptor activation (Farrens *et al.* 1996). We have adopted this approach to study G proteins, and have characterized the dynamics of the N-terminal helix of Gα as it progresses through the G-protein cycle (Medkova *et al.* 2002). We have also identified a GTP-dependent myristoyl switch in the N-terminus that may regulate subunit and membrane interactions (Preininger *et al.* 2003). Future studies will explore the interaction between spin-labeled Gα and regulatory proteins, including receptors and RGS proteins.

Fluorescence labels may also be incorporated into proteins to probe conformational dynamics, providing much of the same sort of information as EPR. For example, steady-state fluorescence measurements can monitor changes in environmental polarity, the fluorescence anisotropy is related to the mobility of the fluorophore and quenching reagents may be used to

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determine the solvent accessibility of the probe (Mansoor *et al.* 1999). This approach has also been used to explore conformational changes in rhodopsin (Dunham & Farrens, 1999) and the β_2-adrenergic receptor (Ghanouni *et al.* 2001). Additionally, fluorescence resonance energy transfer may be used to determine inter-probe distances between 20 Å and 100 Å (Heyduk, 2002).

Although these two approaches do not provide the atomic resolution of crystallography or NMR, they are important biophysical approaches to the conformational dynamics of proteins and protein complexes that are not amenable to other techniques. Currently, the most important application of these techniques in the study of G-protein signaling is the determination of the receptor–G protein complex. Long-range distance measurements will be especially helpful to define the stoichiometry and orientation of the proteins in the complex and to answer specific questions about the mechanism of nucleotide exchange. For example, distance measurements between the extreme N-terminus (as a surrogate reporter for the spatial orientation of G$\beta\gamma$) and the GTPase domain will test the lever-arm and gear-shift models of G protein activation, while similar measurements between the helical and GTPase domains will directly test the importance of domain separation in GDP release. Furthermore, these distance measurements provide hard data to incorporate into molecular models of the interaction to guide assembly of the receptor–G protein complex *in silico*.

8.3 Mapping allosteric connectivity with computational approaches

Collectively, the group of G-protein crystal structures provides a rich dataset for computational studies of G-protein biophysics. Moreover, the Gα subunit is particularly interesting in that it cycles through at least six limit conformations, Gα(GDP), Gα(GDP)$\beta\gamma$, R*·Gα(O)$\beta\gamma$, Gα(GTP), Gα(GDP·AlF_4^-), Gα(GDP·P_i), in addition to its interactions with a variety of regulatory proteins, including receptors, effectors, GAPs and GDIs, each of which specifically interacts with different surfaces of Gα or different conformations of the protein. One of the fundamental problems in understanding the complex interplay between the occupants of the nucleotide-binding pocket and various regulatory proteins is the identification of the allosteric or intramolecular signaling pathways in Gα subunits. Several elegant computational approaches have been developed to address these fundamental questions in signal transduction.

Two methods are based on the hypothesis that, over the course of evolution, functionally important amino acids in proteins will not be mutated, while unimportant amino acids will vary. In the evolutionary trace analysis (Lichtarge *et al.* 1996b), a multiple sequence alignment is generated for a given family of proteins and the individual members of the family are clustered together with their closest relatives based on relative sequence identity. Then, a consensus sequence is generated for each cluster, and if an amino acid varies at a given position, it is left blank. The consensus sequences for all of the clusters are then aligned to provide the evolutionary trace. A residue is 'conserved' if it is the same in all of the consensus sequences, 'class-specific' if it varies among the consensus sequences, or 'neutral' if it is blank in any one of the consensus sequences. The conserved and class-specific amino acids can then be mapped onto the crystal structure, which will identify functionally important areas of the protein. When applied to heterotrimeric G proteins, the evolutionary trace identified subunit interfaces, receptor and effector interaction sites (Lichtarge *et al.* 1996a).

Statistical coupling analysis (SCA) is an extension of the evolutionary trace (Lockless & Ranganathan, 1999). This method uses a very large multiple sequence alignment to identify

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statistical interactions between any two amino-acid positions in the protein, and is based on the additional hypothesis that functional coupling between any two sites in the protein should mutually constrain the evolution at those two sites. In this approach, conservation is defined as the deviance of amino-acid frequencies at a given position from their average abundance in all proteins in nature. Statistical coupling is the extent to which an amino acid at one site changes in response to a perturbation at another site. This analysis predicted long-range interactions with the ligand-binding site in the PDZ domain family, which were subsequently verified by mutagenesis (Lockless & Ranganathan, 1999). When SCA was applied to the G-protein superfamily, including the monomeric G proteins, a network of amino acids was identified that surrounds the nucleotide-binding pocket and links it to the effector-binding surface at the switch regions (Hatley *et al.* 2003). Interestingly, this connectivity is only observed in the GTP-bound conformation of the G proteins, but not in the GDP-bound conformation. Since mutations in this allosteric network significantly affected the nucleotide sensitivity of the interactions between $G\alpha_s$ and $G\beta\gamma$ or AC, these residues likely form a core allosteric network conferring nucleotide-dependent switching in G proteins (Hatley *et al.* 2003).

Unfortunately, the SCA approach is limited by the large sequence alignment that must be assembled for statistical analysis in order to identify coupling energetics, and it is unable to provide a physical explanation for the coupling between two residues, whether it be ligand specificity, structural stability, folding or intramolecular signaling. An alternative method that follows anisotropic thermal diffusion (ATD) through proteins has recently been described to overcome these limitations in identifying intramolecular signaling pathways (Ota & Agard, 2005). In the ATD method, a protein is energy minimized and equilibrated to 10 K with molecular dynamics, which essentially 'freezes' each atom. Then, a particular region of interest is 'heated' to 300 K, and the propagation of the kinetic energy is followed by the deviation of atoms from their positions in the minimized structure in subsequent dynamics simulations. Interestingly, the energy propagates along defined pathways that highlight the physical connectivity of intramolecular signaling pathways. When this approach was applied to a PDZ domain family member, the results were consistent with the SCA pathway, but provided a more direct physical connection between the binding pocket and distant residues (Lockless & Ranganathan, 1999; Ota & Agard, 2005). This method would be very useful to trace the connectivity between bound nucleotides and protein–protein interaction surfaces in G proteins, particularly the receptor-binding surface. Since the monomeric G proteins were included in the SCA analysis (Hatley *et al.* 2003), connectivity to the receptor-binding site could not be addressed by this method. Finally, this technique provides an interesting new way to explore the current models of the receptor–G protein complex, and the interface between these two proteins.

A different way of examining connectivity within a protein is to use an ensemble-based model of the native state of a given protein to identify the energetic coupling between different structural elements (Pan *et al.* 2000). The protein ensemble is generated through the combinatorial unfolding of a set of predefined folding units, the boundaries of which are incrementally shifted to provide an exhaustive enumeration of that protein's partially folded states (Hilser & Freire, 1996). The Boltzmann weight of each state is determined from the calculated Gibbs energy, which provides the probability of each state in the ensemble. From these probabilities, a residue-specific stability constant may be calculated that indicates the relative probability that a residue is folded *versus* unfolded in the ensemble (i.e. a high stability constant indicates a higher folded probability) (Hilser & Freire, 1996). Connectivity between residues is identified by exploring the ensemble to see if the folded status of a given residue impacts the folded status of another

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residue (Pan *et al.* 2000). This analysis can be extended to look at how the folded probability of any given residue correlates to the probability of a group of residues, like a ligand-binding site, being folded (Pan *et al.* 2000). Again, heterotrimeric G proteins are an excellent system for the application of this technique as it can examine the connectivity between nucleotide and effector binding in a way that is difficult to address experimentally.

8.4 Studies of G-protein function in living cells

Much of our understanding of G-protein structure and function is the result of a vast number of biochemical experiments conducted *in vitro* where the constituents of an assay can be carefully controlled and manipulated. Although this reductionist approach is often the only way to dissect a particular experimental question, it does set aside the basic fact that the G-protein cycle runs inside of cells in the presence of a variety of regulatory and scaffolding proteins that almost certainly influence every one of the reactions and interactions that have been carefully described *in vitro*. Recently, several groups have adopted fluorescence-based approaches to study G proteins in living cells (reviewed in Hebert *et al.* 2006). One method is based on the non-radiative energy transfer from *Renilla reniformis* luciferase attached to the C-terminus of the receptor to a blue-shifted variant of *Aequorea victoria* green fluorescent protein attached to one of the three G-protein subunits (Gales *et al.* 2005). Upon degradation of its substrate, luciferase emits light at a wavelength that will excite GFP when the two molecules are within 100 Å of each other. This bioluminescence resonance energy transfer (BRET) approach detected basal coupling of receptors and G proteins, rapid ligand-induced increases in the association of receptors and G proteins, as well as a slow decrease in BRET reflecting receptor desensitization. Interestingly, the agonist-promoted increase in BRET was highly dependent on the Gα isoform present in the complex, suggesting that this assay provides a new tool to assess the specificity of receptor–G protein interactions *in vivo*.

Fluorescence resonance energy transfer (FRET) is similar to BRET, where the energy donor is a fluorescent protein that is excited by an external light source rather than a chemical reaction. Receptor activation increases FRET between fluorescent proteins attached to the Gα_i helical domain and the N-terminus of Gγ, reaching maximum in 1–2 s, which is five times slower than the rate of receptor activation (Bunemann *et al.* 2003). Somewhat surprisingly, the increase in FRET indicates that the fluorescent proteins are coming closer together following receptor activation, not moving farther apart as would be expected if the subunits dissociated following activation. Energy transfer between Gα and a Gγ subunit tagged at the C-terminus decreased after receptor stimulation, further supporting a conformational rearrangement in the heterotrimer rather than subunit dissociation. This observation runs contrary to biochemical experience, as well as an earlier study using this technique to study G-protein activation in yeast (Janetopoulos *et al.* 2001), which support subunit dissociation. Recent evidence suggests that the discrepancy may be due to the identity of the Gα subunit. Upon extending the FRET studies to include all members of the Gα_i family, Gα_o was the only member to exhibit a receptor activation-dependent decrease in FRET, suggesting subunit dissociation. When a region of the helical domain containing αB/αC loop in Gα_i was replaced with the homologous sequence from Gα_o, the mutant Gα_i demonstrated a decrease in FRET, suggesting that this region was critical for preventing subunit dissociation following activation (Frank *et al.* 2005).

These fluorescence-based techniques have raised some provocative questions regarding G-protein activation in intact cells. Unfortunately, the fluorescent protein tags are relatively large,

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and may have a substantial impact on the structure and dynamics of the system under study. Fortunately, new technologies are being developed that allow the use of a small molecule as an energy donor, rather than a bulky fluorescent protein, and this approach has been used to explore conformational changes in receptors while preserving downstream signaling (Hoffmann *et al.* 2005). Additional studies seem to be necessary before the structural changes responsible for the differences in energy transfer are truly understood. Perhaps these *in vivo* systems might benefit from a brief return to the test tube, where much more detailed biochemical analyses may be carried out to assess the impact of the intracellular milieu and the fluorescent tags on G-protein activation and subunit dissociation.

9. Conclusions

As seems only fitting, many of the unanswered questions about the structural basis for function in heterotrimeric G proteins can be framed in terms of what we do not know from the many crystal structures of these proteins in a variety of conformations and complexes. For example, the N- and C-termini of Gα play key functional roles in mediating its interaction with regulatory proteins, yet limited structural information for these regions is available from crystallography. Furthermore, answers to some of the most important questions in G-protein signaling await a better understanding of the structure of the receptor–G protein complex, including agonist-mediated conformational changes in receptors, point-to-point interactions between the receptor and G protein, the transition leading to GDP release, the structural determinants of R*-G protein specificity and the functional role of receptor dimerization in G protein activation. Although low resolution models have been described that address all of these issues, the strongest evidence in favor of any structural hypothesis comes once it has been captured on film.

In addition to the numerous G-protein crystal structures, a vast amount of biochemical data is available from mutagenesis studies of these proteins. Care must be taken, however, in interpreting the results of these studies, particularly in terms of the structure and function of Gα. For example, the effector-binding sites for AC, RhoGEF and GRK2 on Gα_s, Gα_{13} and Gα_q, respectively, determined by mutagenesis or chimeras (Berlot & Bourne, 1992; Day *et al.* 2004; Vazquez-Prado *et al.* 2004) do not agree with the interaction surfaces evident in the crystal structures of these complexes (Tesmer *et al.* 1997b, 2005; Chen *et al.* 2005b). Although the effects of mutagenesis on these effector interactions were significant, the effects were indirectly coupled to the effector-binding sites. In another example, several Gα_q mutations have different phenotypes depending on which activated background was used, AlF_4^-, Q209L or R183C (Day *et al.* 2004). Clearly, the allosteric network in Gα couples different regions of the protein in ways that are not clearly understood.

Similar care must also be taken in the interpretation of crystal structures, where lattice forces may order flexible regions in non-physiological ways. Additionally, the proteins used in crystallography are often partial representations of the physiological interactions (i.e. a single domain is crystallized with Gα, rather than the holoprotein) or structures of mutants or chimeras themselves. Indeed, each of the structural approaches described here have unique limitations, which can be overcome through the use of multiple approaches to address the structure and function of G proteins.

With these limitations in mind, it becomes important to perform a careful biochemical characterization of any mutants or chimeras used in structural analyses. The most fundamental question is whether the mutant Gα can undergo an activation-dependent conformational change

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upon binding AlF_4^- or GTP, which can be measured by trypsin protection assays or changes in intrinsic fluorescence. If enough protein is available, the rates of GTP binding and hydrolysis should be determined, providing a good measure of how any particular mutation is affecting the basic function of the G-protein switch in comparison to the wild-type protein. In addition to these basic studies, a variety of methods have been described to assess heterotrimer formation, R*-catalyzed GTP binding, effector activation and RGS-catalyzed GTP hydrolysis. Through a careful biochemical characterization, it can be shown that the G protein used in structural studies is either behaving somewhat normally or that the structural result should be interpreted with caution.

Finally, the biophysical techniques described above provide promising alternative approaches to addressing issues in G-protein function. The variety of experimental techniques that have been used to describe the conformational changes in receptors upon activation are now being applied to study each of the structural transitions of Gα as it progresses through the G-protein cycle. When interpreted in terms of the G-protein crystal structures, these studies can provide rather detailed mechanistic information about conformational changes in these proteins. An important contrast to crystallography, however, is that these other methods provide dynamic information on proteins in solution. Indeed, crystallography and solution studies complement each other by enabling high resolution predictions from low resolution information, especially when computational methods are used to facilitate this process. As biophysical studies continue to provide new data on conformational changes in G proteins, we will be able to further enhance our current understanding of the structural determinants of the function, specificity and regulation of G-protein signaling.

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