0163-769X/03/$20.00/0
Printed in U.S.A.

Endocrine Reviews 24(6):765–781
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doi: 10.1210/er.2000-0026

Insights into G Protein Structure, Function, and Regulation

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In multicellular organisms from *Caenorhabditis elegans* to *Homo sapiens*, the maintenance of homeostasis is dependent on the continual flow and processing of information through a complex network of cells. Moreover, in order for the organism to respond to an ever-changing environment, intercellular signals must be transduced, amplified, and ultimately converted to the appropriate physiological response. The resolution of the molecular events underlying signal response and integration forms the basis of the signal transduction field of research. An evolutionarily highly conserved group of molecules known as heterotrimeric guanine nucleotide-binding proteins (G proteins) are key determinants of the specificity and temporal characteristics of many signaling processes and are the topic of this review. Numerous hormones, neurotransmitters, chemokines, local mediators, and sensory stimuli exert their effects on cells by binding to heptahelical membrane receptors coupled to heterotrimeric G proteins. These highly specialized transducers can modulate the activity of multiple signaling pathways leading to diverse biological responses. *In vivo*, specific combinations of Gα- and Gβγ-subunits are likely required for connecting individual receptors to signaling pathways. The structural determinants of receptor-G protein-effector specificity are not completely understood and, in addition to involving interaction domains of these primary acting proteins, also require the participation of scaffolding and regulatory proteins. (*Endocrine Reviews* 24: 765–781, 2003)

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Abbreviations: AC, Adenylyl cyclase; AGS, activators of G protein signaling; GAP, GTPase-activating protein; GRIN, G protein-regulated inducer of neurite outgrowth; PI_3 kinase, phosphoinositide 3-kinase; PLC, phospholipase C; RGS, regulator of G protein signaling.

I. Introduction

WHEN A LIGAND such as a hormone, neurotransmitter, or glycoprotein interacts with a heptahelical receptor on the surface of the cell, the ligand either stabilizes or induces a conformation in the receptor that activates a heterotrimeric G protein (composed of α-, β-, and γ-subunits) on the inner membrane surface of the cell (1). In the inactive heterotrimeric state, GDP is bound to the Gα-subunit. Upon activation, GDP is released, GTP binds to Gα, and subsequently Gα-GTP dissociates from Gβγ and from the receptor (Fig. 1). Both Gα-GTP and Gβγ are then free to activate downstream effectors. The duration of the signal is determined by the intrinsic GTP hydrolysis rate of the Gα-subunit and the subsequent reassociation of Gα-GDP with Gβγ (1, 2). This article will review current knowledge and recent progress in defining the molecular mechanisms that regulate the activity and specificity of G protein signaling cascades. In addition, we will briefly discuss the use of dynamic experimental approaches that are likely to provide new insights into G protein regulation in the future.

II. G Protein Structure

The solution of crystal structures for inactive (GDP-bound), active (GTP-bound), and transition state (GDP-ALF_4^-) $G\alpha_t$ (3–5) or $G\alpha_i$ (6), as well as structures for the inactive heterotrimeric complexes (7, 8), has provided the framework for understanding the biomechanics of G proteins as molecular switches. For a detailed discussion of the

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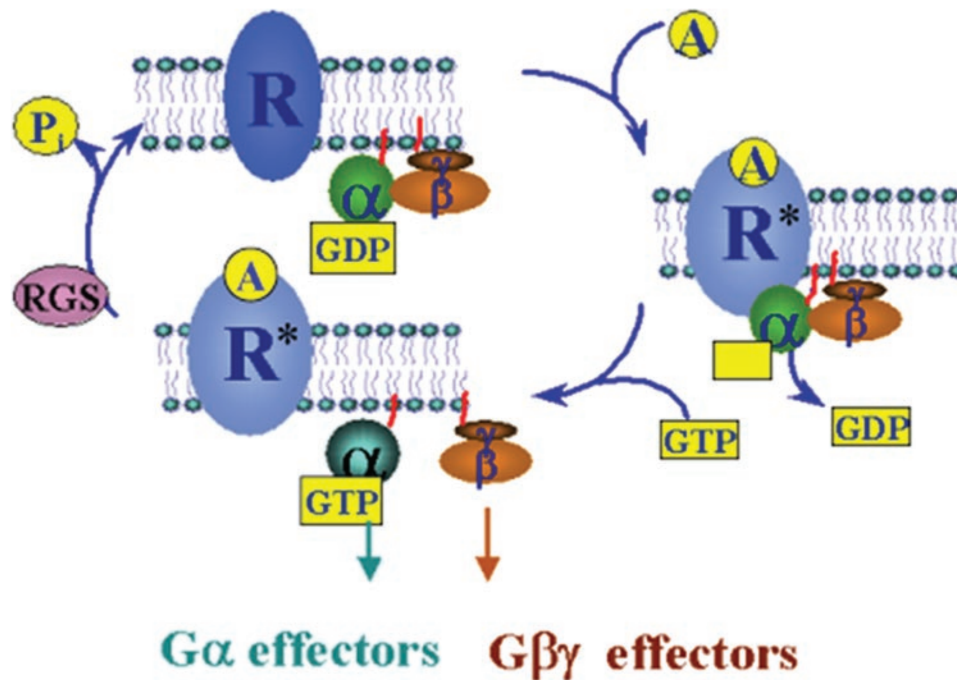

FIG. 1. Receptor-mediated G protein activation. The interaction of an endogenous ligand with its cell surface receptor (R) facilitates the coupling of the activate receptor (R*) with intracellular heterotrimeric G proteins. The R*-G protein coupling promotes the exchange of GDP for GTP on the Gα-subunit. Gα-GTP then dissociates from Gβγ and R*. Both subunits are free to modulate the activity of a wide variety of intracellular effectors. Termination of the signal occurs when the γ-phosphate of GTP is removed by the intrinsic GTPase activity of the Gα-subunit, leaving GDP in the nucleotide binding pocket on Gα. Gα-GDP then reassociates with Gβγ and the cycle is complete. RGS proteins accelerate the intrinsic GTPase activity of Gα-subunits, thereby reducing the duration of signaling events.

specific intramolecular contacts within heterotrimeric G proteins, the reader is referred to reviews in Refs. 2 and 9. Herein, we will briefly discuss key structural and functional features common to all heterotrimeric G proteins to understand the framework and interpretation of recent work in this field.

According to current knowledge, 16 genes encode for Gα-subunits, five genes encode for Gβ-, and 12 genes encode for Gγ-subunits (10). Classically, G proteins are divided into four families based on similarity of their α-subunits: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Table 1). Gα-subunits contain two domains: a GTPase domain that is involved in the binding and hydrolysis of GTP and a helical domain that buries the GTP within the core of the protein (Fig. 2A). The helical domain is the most divergent domain among Gα families and may play a role in directing specificity of receptor- and effector-G protein coupling. Comparison of $G\alpha_t$-GDP and $G\alpha_t$-GTPγS crystal structures has revealed the presence of three flexible regions, designated switches I, II, and III, which become more rigid and well ordered in the GTP-bound active conformation (3, 4). Little is known about the structure of the extreme amino (N-) and carboxy (C-) terminal domains of Gα-subunits because in the isolated G protein crystal structures solved thus far, the N and C termini of Gα were either removed from the protein or disordered (3–6). However, in two separate crystal structures of heterotrimeric complex, the N-terminal helix is ordered by its interaction with the β-propeller domain of Gβ (Refs. 7 and 8 and Fig. 2A). Biochemical studies suggest that these terminal regions play a key role in the activation process and in directing specific protein-protein interactions, as is discussed in the following section.

The Gβ-subunit of heterotrimeric G proteins has a β-propeller structure containing seven WD-40 repeats (Ref. 7 and Fig. 2A). The Gγ-subunit interacts with the Gβ-subunit through an N-terminal coiled coil and makes extensive contacts along the base of the Gβ-subunit (Ref. 7 and Fig. 2A). The Gβγ-dimer binds to a hydrophobic pocket present in Gα-GDP. GTP binding to Gα removes the hydrophobic pocket and reduces the affinity of Gα for Gβγ (4).

III. Molecular Basis for G Protein Activation

The rate-limiting step in G protein activation is the release of GDP from the nucleotide-binding pocket. GDP is spontaneously released from the heterotrimeric G protein at a rate that varies depending on the Gα-subunit. For example, the $G\alpha_o$ GDP release rate (k_{off}) is 0.19 min^{-1} whereas the $G\alpha_{i2}$ release rate is 0.072 min^{-1} (11). However, the inactive state of the Gα-subunits is controlled by Gβγ binding. Higashijima *et al.* (12) showed that in the absence of Mg^{2+}, Gβγ increases the affinity of $G\alpha_o$ for GDP about 300-fold. GDP release is greatly facilitated by receptor activation of the G protein (13). Mutations (14–16) of residues in the critical TCAT guanine nucleotide-binding motif present in the β6-α5 loop of the GTPase domain (4) enhance receptor-independent spontaneous GDP release. Iiri *et al.* (14) identified such an activating mutation in $G\alpha_s$ ($A^{366}S$) in male patients with pseudohypoparathyroidism and gonadotropin-independent precocious puberty. Enhanced GDP release was also observed when similar mutations were generated in $G\alpha_i$ [$A^{326}S$; (15)] and $G\alpha_o$ [$C^{325}S$; (16)] suggesting that this region serves as a com-

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TABLE 1. Classification of Gα-subtypes and their effectors

Family	Subtype	Effector
G_s	$G\alpha_{s(S)}$[a]	↑ AC
	$G\alpha_{s(L)}$[a]	↑ GTPase of tubulin[b]
		↑ src[b]
	$G\alpha_{olf}$	↑ AC
G_i	$G\alpha_{i1}$	↓ AC
	$G\alpha_{i2}$	Rap 1 GAP
	$G\alpha_{i3}$	GRIN 1 and 2
	$G\alpha_{oA}$[a]	↑ GTPase of tubulin[b]
	$G\alpha_{oB}$[a]	↑ src[b]
	$G\alpha_z$	Ca^{2+} and K^+ channels[b]
	$G\alpha_{t1}$	↑ cGMP-PDE
	$G\alpha_{t2}$	
	$G\alpha_g$	Unknown
G_q	$G\alpha_q$	↑ PLCβs
	$G\alpha_{11}$	↑ Bruton's tyrosine kinase ($G\alpha_q$)
	$G\alpha_{14}$	
	$G\alpha_{15 \text{ or } 16}$	
G_{12}	$G\alpha_{12}$	↑ NHE-1[b]
		↑ PLD[b]
	$G\alpha_{13}$	↑ p115RhoGEF
		↑ iNOS[b]

PDE, Phosphodiesterase E; iNOS, inducible nitric oxide synthase; NHE, Na^+/H^+ exchanger; PLD, phospholipase D; GEF, guanine nucleotide exchange factor.

[a] Two splice products of $G\alpha_s$ and $G\alpha_o$ genes.

[b] See Refs. 169 and 228–234.

mon mediator of GDP release. Posner *et al.* (15) also demonstrated that GDP release can occur without inducing a large conformational change in Gα.

In addition to the TCAT motif, most recent work has identified residues within the helical domain as well as within the N- and C-terminal domains of Gα-subunits that are also integral mediators of spontaneous GDP release. For example, in $G\alpha_t$ mutation of three residues located in the inward-facing surface of the α5-helix causes a dramatic increase of basal nucleotide exchange rate in addition to enhanced receptor-catalyzed nucleotide exhange rate (17). Mutation of five residues within the switch IV helical domain in $G\alpha_s$ decreases the rate of GDP release, GTPγS binding, and GTP hydrolysis (18) and disruption of contacts between the helical and GTPase domains also influences basal GDP dissociation rates (19, 20). By fluorescently labeling the C-terminal residue Cys^{347} of a $G\alpha_t/G\alpha_i$ chimera, Yang *et al.* (21) determined that the C terminus moves into a more hydrophobic environment upon AlF_4^- activation. The authors suggest that this movement may reflect an interaction between the C terminus and the α2-β4 loop of $G\alpha_t/G\alpha_i$. In addition, these divergent terminal domains have been implicated as the source of variation in the intrinsic GDP release rates among Gα-subunits. Substitution of 31 N-terminal residues of a $G\alpha_{t/i}$ chimera (low intrinsic exchange rate) with corresponding 42 residues of $G\alpha_s$ (high intrinsic exchange rate) significantly enhanced the nucleotide exchange rate (22). This same group also reported that disruption of a specific contact between Val^{30} (N terminus) and Ile^{339} (C terminus) alters the rate of GTPγS binding, which was inferred as an indirect index of GDP release. Hence, structural interactions between N and C termini of $G\alpha_t$ are important to the maintenance of a slow GDP release rate for $G\alpha_t$.

Receptor-mediated GDP release is dependent on the ability of the receptor to interact with the G protein and trigger conformational changes in Gα that cause release of GDP. Comparing the crystal structure with biochemical data, we can deduce that the receptor contacts Gα at a site that is more than 20 Å away from the guanine nucleotide binding site (1, 21), thus working at a distance to release GDP. Current theory is that receptor contact with the C terminus of the Gα-subunit leads to conformational changes that are propagated through Gα to the GDP binding site (1, 21). However, the requirement for Gβγ in receptor-G protein interaction and G protein activation suggests that Gβγ may actively participate in GDP release by opening an exit route for the guanine nucleotide to leave the complex (23). The heterotrimer contains a prominent cavity between Gα and Gβγ that is believed to be oriented toward the plasma membrane (7, 24). Activated loops of the receptor might use this cavity to tilt Gβγ away from Gα causing the contacts between Gα and Gβγ to be disrupted, including contacts near switch I and the β3-α2 loop in Gα, the potential exit route for the nucleotide. In this way the receptor could use Gβγ as a lever to release GDP (25). Ala substitutions in Gβ at the Gα-Gβγ interface near the GDP exit route inhibit receptor-induced GDP/GTP exchange without affecting Gα-Gβγ binding (26). Thus, the Gβγ-dimer is not merely a passive binding partner with the sole purpose of stabilizing Gα but, rather, Gβγ actively participates in receptor-mediated G protein activation.

IV. Structural Determinants of Receptor-G Protein Specificity

For the purposes of this review, we will limit ourselves to a discussion of regions within Gα and Gβγ that have been determined to mediate receptor-G protein specificity. For a thorough review of specific sites on heptahelical receptors, which direct receptor-G protein coupling specificity, the reader is referred to Refs. 27 and 28 for reviews. The extreme C terminus of Gα (in particular the last five residues) has been established as an important mediator of receptor-G protein interaction (23, 29–31). For example, ADP ribosylation of residue −4 by pertussis toxin uncouples G_i/G_o proteins from receptors (32). Phosphorylation of a tyrosine residue at −4 in $G_{q/11}$ was shown to be required for coupling to metabotropic glutamate receptors (33) although this has not been demonstrated in intact cells. In addition, the requirement of phosphorylation at the tyrosine residue of $G_{q/11}$ cannot be generalized as M_1 muscarinic receptors, and thrombin receptors were shown to couple readily to $G_{q/11}$ proteins in reconstitution experiments (34, 35). Many examples of mutations in this region that alter receptor-G protein specificity have been also reported (36–38). In addition, several investigators have generated sequence-specific C-terminal peptides or antibodies targeting the C-terminal domain to study receptor-G protein interaction. Antibodies recognizing Gα C-terminal domains block receptor-G protein signaling (39). Instead, sequence-specific C-terminal synthetic peptides either stabilize the active agonist-bound form of the

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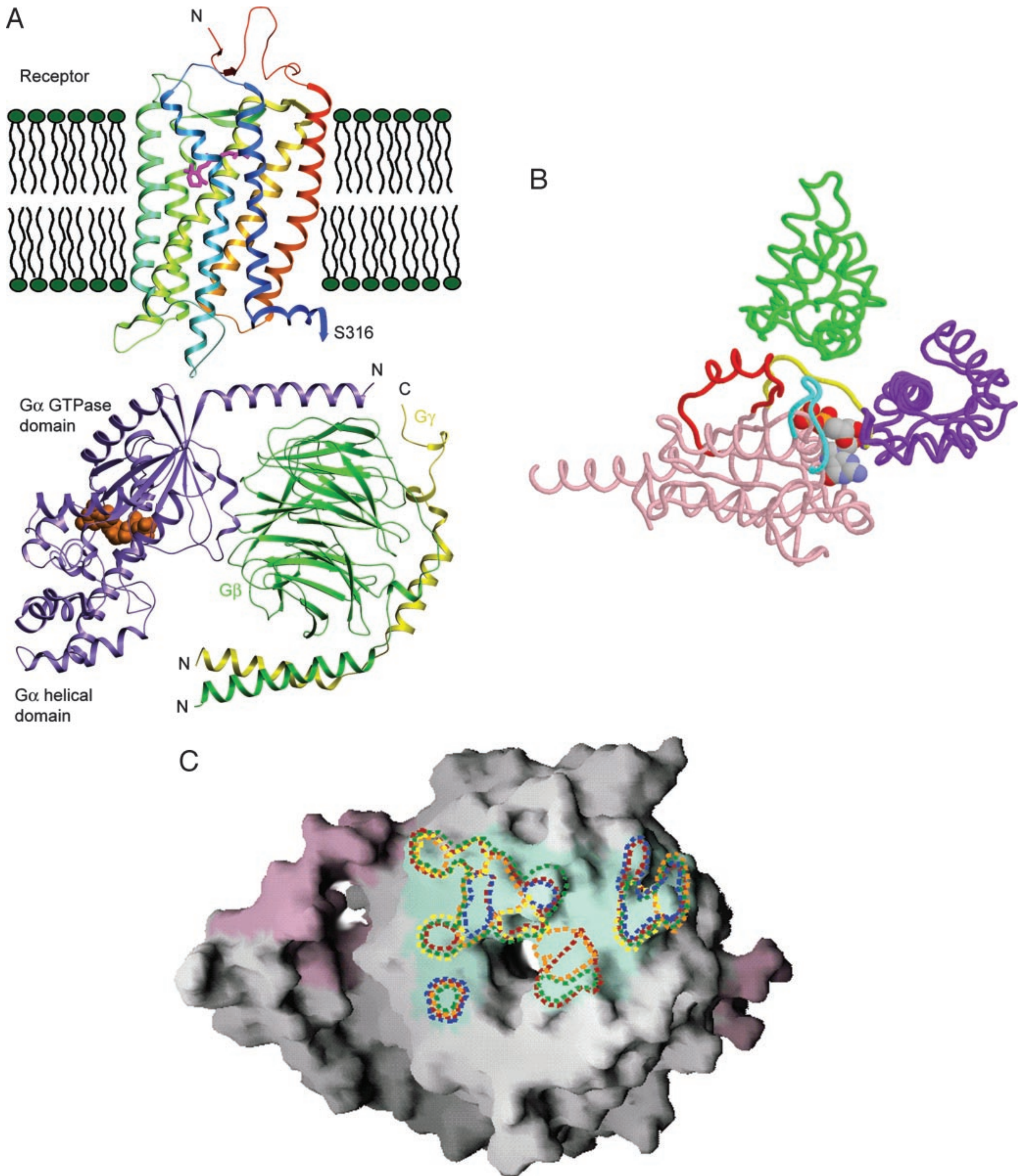

FIG. 2. Schematic diagrams of G_t, the RGS4-$G\alpha_{i1}$ complex, and effector contact sites on Gβ. A, Ribbon diagrams depicting the probable membrane orientation of heterotrimeric G_t. The refined rhodopsin structure is from Ref. 240 [Protein databank (PDB) file 1F88]. $G\alpha_t$ (*purple*); GDP molecule (*red*); Gβ (*green*); Gγ (*yellow*); rhodopsin helices [color gradient from *red* (N terminus) to *navy blue* (C terminus)]; the retinal molecule within rhodopsin (*magenta*). Diagrams were generated using coordinates from PDB files (1GOT and 1BOK) and visualized with WebLab ViewerPro. B, Ribbon diagram depicting the RGS4/$G\alpha_{i1}$ complex. RGS 4 (*green*); $G\alpha_{i1}$ (*red*); $G\alpha_{i1}$ β-sheets (*cyan*); GTP molecule (*magenta*). Diagrams were generated using coordinates from PDB file 1AGR and visualized with WebLab ViewerPro. C, Solvent-accessible surface model of $G\beta_1\gamma_1$ highlighting residues identified as important mediators of effector interaction (26). The crystal coordinates of $G\beta_1\gamma_1$ [PDB entry 1TBG] were used to generate a surface model of the dimer in Graphical Representation and Analysis of Structural Properties. Gβ

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receptor mimicking the G protein (40–42) or serve as competitive inhibitors of receptor-G protein interface (43). Although blocking peptides are commonly interpreted as evidence of a direct receptor-G protein contact site, peptides may also stabilize or disrupt regions of the protein that transmit conformational changes to the guanine nucleotide binding motif and thereby indirectly affect receptor-mediated G protein activation.

The C terminus is not the only region directing receptor-G protein interactions. Several Gα-subunits possess identical or nearly identical residues within the extreme C-terminal domain yet exhibit differential coupling to receptors. For example, within the last 11 amino acids of Gα_{i1} and Gα_t, only a single residue is divergent, yet the serotonin$_{IB}$ receptor fails to couple to Gα_t and readily couples to Gα_i. Investigation into the molecular determinants of this specificity indicated that two residues within the α4-helix of Gα_{i1} are critical mediators of this receptor-G protein coupling profile (44, 45). Key residues for coupling specificity have been also identified within the N terminus (36, 39, 46), the α2-helix, and α2-β4 loop regions (47, 48) as well as within the α4-helix and α4-β6 loop domain (44, 48, 49). Segments of Gβ- and Gγ-subunits may also contribute to the receptor interacting surface of heterotrimers (46, 50–53). Using a peptide specific for α-helical residues in Gα_s, Krieger-Brauer *et al.* (54) blocked β-adrenergic receptor-mediated activation of both Gα_s- and G$\beta\gamma$-effectors. In contrast, a C-terminal sequence-specific peptide for Gα_s only prevented Gα_s-mediated effector activation, suggesting that the extreme C terminus of Gα_s is required for Gα-mediated signaling but is not critical for β-adrenergic receptor recognition and dissociation of Gα from G$\beta\gamma$ (54). Together, these studies suggest that the relative importance of the C terminus for directing receptor-G protein interactions may be dependent on Gα and receptor subtypes. Receptor-G protein specificity is clearly not mediated solely by one structural feature of Gα-subunits but appears to result from a network of specific contacts between the receptor and G protein which differs for each Gα-subunit and for each receptor and results in a large number of possible combinations that can bring remarkable specificity into a system with only a few central players. As suggested by Blahos *et al.* (36), one of the difficulties in isolating the specific determinants of receptor-G protein coupling has been that G protein coupling may still occur even when interactions at certain contact points are weak, absent, or negative if these frailties can be overcome by a stronger interaction at other contact points or when regions that may weaken coupling are removed from either the receptor or the G protein.

V. Receptor-Independent Activators of G Protein Signaling (AGS Proteins)

A novel class of signaling proteins, termed AGS proteins, has been identified (55, 56). AGS proteins activate heterotrimeric G proteins independently of receptor activation. The mechanism for AGS activation differs among members of this family. AGS1 has been found experimentally to promote GTPγS binding. AGS2 selectively associates with G$\beta\gamma$, whereas AGS3 binds to Gα and exhibits a preference for GDP-Gα *vs.* GTP-Gα. AGS3 has been shown to prevent the reassociation of G$\beta\gamma$ with the Gα-subunit and function as a guanine dissociation inhibitor for Gα_i-subunits (57). AGS3 contains a G protein-regulatory motif. This G protein regulatory motif or GoLOCO repeat is an approximately 20-amino acid domain found in several proteins that interact with and/or regulate G proteins, *e.g.*, AGS3, the Partner of Inscuteable and its mammalian homolog, LGN, Purkinje cell protein 2, and Rap1 GTPase-activating protein (GAP). The physiological role of these proteins *in vivo* remains to be determined, but one possible role for these proteins may be in the regulation of G proteins that do not reside near the plasma membrane and cannot be activated directly by receptors, *e.g.*, G proteins in the Golgi that regulate vesicular trafficking (58). Little is known about the role of this pool of G proteins, and the discovery of AGS proteins may stimulate research into a new dimension of heterotrimeric G protein signaling.

VI. The Receptor-Gα Protein Interface as a Therapeutic Target

Traditionally, the extracellular surface and transmembrane domains of G protein-coupled receptors have served as a target for the development of drugs that can selectively activate or inactivate specific cellular pathways. However, some receptor isoforms, such as the dopamine D_{2L} and D_{2S} receptors, and the D_4 receptor variants differ only on the intracellular surface of the protein (59, 60) and cannot be readily distinguished by targeting the ligand-binding site. Moreover, many receptors promiscuously couple to several G protein subtypes in what may be a tissue- or cell-specific phenomenon. Therefore, additional therapeutic targets will certainly be required to more specifically influence intracellular signaling events. One avenue being explored by our laboratory and others is the use of peptide inhibitors that target the receptor-G protein interface (43, 61, 62). Currently, these peptides represent either Gα C-terminal-specific sequences or peptides isolated from a combinatorial library based on C-terminal Gα-sequences and screened for high-affinity receptor binding (31). These studies are based on the idea that the C terminus of Gα-subunits serves as a key receptor contact site and mediator of receptor-G protein specificity. In the short term, these peptides may provide useful tools for exploring specificity of G protein-mediated signaling.

The delivery of peptide inhibitors represents a challenge to the therapeutic use of these tools. Possible delivery

(*gray*); Gγ (*pink*). The area on Gβ that is covered by Gα in the G protein heterotrimer crystal structure is highlighted in *light green*. The effector-interacting residues on Gβ are circled with *colored dashed lines* as follows: β-adrenergic receptor kinase (*orange*); PLCβ_2 (*red*); AC II (*green*); K^+ channel (*blue*); Ca^{2+} channel (*yellow*). Gα-GDP, when bound to G$\beta\gamma$, covers all these distinct yet partially overlapping effector interaction regions on Gβ and, thus, blocks G$\beta\gamma$ regulation of all the effectors. [Figure 2C reprinted with permission from Ford *et al.*: *Science* 280:1271–1274, 1998 (26). © 1988 American Association for the Advancement of Science.]

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systems include the use of inducible retroviral minigene vectors (64), incorporation of peptides into liposomes (65), or the fusion of peptides to a viral peptide sequence that carries the C-terminal peptide into the cell (66). Alternatively, peptidomimetics may prove to be more stable and bioavailable. Selective targeting to specific organs is likely to prove beneficial, because Akhter *et al.* (67) have demonstrated that transgenic mice selectively expressing a Gq C-terminal minigene in the myocardium exhibit a marked inhibition of α_{1B}-adrenergic receptor-mediated inositol phosphate production and blockade of cardiac hypertrophy. The identification of peptide inhibitors with high affinity for specific receptor subtypes and/or variants would also allow for more selective inhibition of signaling pathways. Despite the significant hurdles, targeting the receptor-G protein interface will clarify the complex coordination of players in signaling cascades and may prove therapeutically useful in the future.

VII. Gα Interaction with Effectors

Once Gα-GTP has dissociated from the G$\beta\gamma$-dimer, Gα can directly interact with effector proteins to continue the signaling cascade. The specific effector proteins activated by Gα are dependent on the Gα-subtype and are summarized in Table 1. Well-defined Gα effectors, such as adenylyl cyclase (AC) and phospholipase C (PLC), have been the topic of several excellent reviews (68, 69).

Overall, several patterns emerge upon examination of the Gα-effectors. First, each Gα-family activates a distinct profile of effectors. The molecular basis for this divergence has not been completely elucidated. Cocrystallization studies of Gα_s and the catalytic domains of AC have identified specific contacts within Gα_s at the α2-helix (SII) and the α3-β5 loop (70). In addition, the α4-β6 loop of Gα_s also plays a role in AC activation (71). Sunahara *et al.* (72) demonstrated that GDP-bound Gα_s can also stimulate AC, albeit with a lower potency than the GTP-bound α-subunit. These data are intriguing because they suggest that reassociation of Gα with G$\beta\gamma$ is required for the complete termination of Gα_s signaling, and inhibition of reassociation could prolong both Gα- and G$\beta\gamma$-mediated signaling. In addition, G$\beta\gamma$ could serve to prolong signaling because it can block the PLCβ-mediated acceleration of GTPase activity at Gα_q-proteins (73). Second, within a family, each Gα-subunit exhibits a differential profile of effector activation. For example, Gα_{i2} is required to inhibit forskolin-stimulated AC activity whereas Gα_{i3} serves to inhibit Gα_s-activated AC (74). In addition, α_1-adrenergic receptors elevate intracellular Ca^{2+} by two distinct mechanisms that are dependent on the Gα-subunit coupled to the receptor: Gα_q releases Ca^{2+} from the endoplasmic reticulum whereas Gα_{11} activates a nonselective cation channel (75). Third, some Gα-subunits have only one identified effector, such as cGMP phosphodiesterase for Gα_t whereas others more promiscuously couple to several effector proteins. Lastly, effectors for some Gα-subunits have yet to be definitively identified, and the search for novel Gα-effectors is a rapidly growing area of research. A number of proteins that directly interact with Gα-subunits have been identified, yet further evidence awaits as to whether guanine nucleotide binding to Gα regulates the activity of these proteins *in vivo* in response to receptor activation. Nonetheless, some of the most recent studies identifying novel putative Gα-effectors are discussed below.

Using a yeast-two-hybrid screen, Jordan *et al*. (76) identified direct interactions between Gα_o and Rap1 GAP, Gz GAP, and RGS17. This group also determined that Rap1 GAP interacts with Gα_i-proteins but not with Gα_q or Gα_s. However, receptor-mediated activation of these proteins was not demonstrated. Interestingly, Rap1 GAP interacted preferentially with GDP-bound Gα_o, suggesting that Gα_o-GDP may sequester Rap1 GAP away from Rap1, resulting in a sustained activation of MAPK. These findings reveal a novel mechanism of G protein function that is dependent on GDP-liganded G proteins. G$\beta\gamma$-subunits might then be considered as inhibitors of Gα-GDP proteins (and vice versa).

In search of Gα_z-effectors, Chen *et al.* (77) screened a cDNA expression library using phosphorylated Gα_z-GTPγS as a probe. This group identified two proteins that interact with Gα_z and named them GRIN1 and GRIN2 for G protein-regulated inducer of neurite outgrowth 1 and 2. Both GRIN1 and GRIN2 bound to activated G proteins (Gα_o, Gα_i, and Gα_z) and were identified in neural tissue, but the regulatory mechanism for neurite growth is unknown.

The Ca^{2+} binding protein calnuc (nucleobindin) is a potential effector for Gα_{i3} and Gα_s (78, 79). The binding of calnuc to Gα_{i3} has been shown to be Ca^{2+} and Mg^{2+} dependent (80). This ion dependence has not been shown explicitly for Gα_s, probably because calnuc undergoes a conformational change after Ca^{2+} binding (81) that could be necessary for G protein interaction.

Bruton's tyrosine kinase (*Btk*) has been identified as a novel effector for Gα_q proteins because Gα_q activates *Btk* both *in vitro* and *in vivo*, and this activation is required for receptor-mediated stimulation of p38 MAPK (82). However, the generalization of these results to other G_q family members remains to be determined.

Although a role for G$\alpha_{12/13}$-proteins had been established in several physiological events such as stress fiber formation, cellular transformation, regulation of Na^+/H^+ exchange, modulation of inducible nitric oxide synthase expression, and regulation of Erk and c-jun kinase activity (83), direct interaction of G$\alpha_{12/13}$ with effector proteins has been established recently, when Hart *et al*. (84) identified p115RhoGEF as a direct effector for Gα_{13}. A RGS protein, p115RhoGEF, is also shown to serve as a GAP for both Gα_{12} and Gα_{13}. However, only activated Gα_{13} is able to stimulate p115RhoGEF to trigger GDP/GTP exchange on the small molecular weight G protein Rho. In addition, the cytoskeletal-associated protein radixin has been found to interact with Gα_{13} (85) whereas an interaction between Gα_{12} and heatshock protein 90 is required for Gα_{12}-induced serum response element activation, cytoskeletal changes, and mitogenic response (86).

VIII. G$\beta\gamma$ Interaction with Effectors

Initially, G$\beta\gamma$ was thought to facilitate the completion of intracellular information transfer passively by binding to Gα

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and hastening the return of the heterotrimer to the plasma membrane, thereby preventing noise or spontaneous Gα activation in the absence of receptor stimulation (87). This belief changed when G$\beta\gamma$ was shown to activate a K^+-selective ion channel (I_{KACh}) in cardiac atrial cells (88). Today, G$\beta\gamma$ is known to interact with and activate several effectors, including PLCβ2 and β3 (89, 90), ACs (91), β-adrenergic receptor kinase (92), phosphoinositide 3-kinase (PI_3 kinase) (93, 94), components of the MAPK cascade (95), and K^+ and Ca^{2+} channels (88, 96–98) (Table 2). As the list of G$\beta\gamma$-effectors continues to grow, recent attention has turned toward examining the mechanisms responsible for G$\beta\gamma$-specific signaling.

At present, five different Gβ-subunits and 12 different Gγ-subunits have been identified (10, 99–102), meaning that if G$\beta\gamma$-dimers formed randomly, there would be 60 possible combinations. Although, in general, most Gβ-subunits can dimerize with most Gγ-subtypes, biochemical studies have demonstrated exceptions to the rule. For example, Gβ_2 dimerizes with Gγ_2 *in vitro* but not with Gγ_1, and Gβ_3 does not dimerize with either Gγ_1 or Gγ_2 (103–105). Likewise, Gβ_5 dimerizes poorly with Gγ_2 in a yeast-two-hybrid assay (106), but other studies suggest that these dimers can form *in vitro* (107) and can activate PLCβ_2 (102). Conversely, Gβ and Gγ combinations that were excluded in *in vitro* assays (104) display functional effects when transfected into cells (108). Although G$\beta\gamma$-dimers of varying composition may form *in vivo* as well, G$\beta\gamma$-dimer combinations may also exhibit cell type or tissue specificity. For example, in the retina the primary G$\beta\gamma$-dimer is G$\beta_1\gamma_1$ whereas G$\beta_1\gamma_2$ is the most common dimer formed in the brain (109, 110).

A. G$\beta\gamma$-Dimer composition directs effector and receptor coupling

What is the physiological significance of the formation of different G$\beta\gamma$-dimers? Although it was previously thought that G$\beta\gamma$-dimers were for the most part interchangeable, current research indicates that G$\beta\gamma$-dimer composition determines the quality and efficiency of effector activation and may mediate receptor-G protein coupling specificity similar to Gα-subunits. For example, when nine unique dimers of Gβ_1 or Gβ_2 with G$\gamma_{(1, 2, 3, 5 \text{ or } 7)}$ were tested for the ability to activate various PLCβ isoforms, all dimers could activate the various PLCβ isoforms except retinal-specific G$\beta_1\gamma_1$ (111, 112). Likewise, G$\beta_1\gamma_1$ was markedly less effective at stimulation of ACII and inhibition of ACI than other G$\beta\gamma$ dimer combinations (111, 112). A comparison of G$\beta_1\gamma_2$ with G$\beta_5\gamma_2$ demonstrated that G$\beta_5\gamma_2$ is a much weaker inhibitor of ACI, ACV, and ACVI. In addition, G$\beta_1\gamma_2$ stimulated ACII activity, whereas G$\beta_5\gamma_2$ inhibited the activity of this enzyme (113). In contrast, both G$\beta_1\gamma_2$ and G$\beta_5\gamma_2$ activated PLCβ_2 with similar potency and efficacy (114). Finally, the rank order for Gβ-subtype inhibition of voltage-dependent N-type Ca^{2+} currents differs from enzyme activation [$G\beta_1 = G\beta_2 > G\beta_5 \gg G\beta_3 = G\beta_4$; (115)], and this potency difference may be related to the ability of the various Gβ-subunits to physically interact with the $L_{I\text{-}II}$ loop of the Ca^{2+} channel (115). Together, these data demonstrate that the primary sequence of the Gβ-subunit is a major determinant of effector coupling efficiency and specificity. Isolation of the structural features responsible for effector variation remains to be completely determined. Recently, Mirshahi *et al.* (116) have shown that Ser^{67} in Gβ_1 is part of a functional domain that regulates several different effectors whereas other residues of the β-propeller seem to direct the effector specificity.

With respect to receptor-G protein coupling specificity, both G$\beta_1\gamma_2$ and G$\beta_5\gamma_2$ can couple Gα_q-proteins to endothelin B and M_1 muscarinic receptors. However, G$\beta_1\gamma_2$ but not G$\beta_5\gamma_2$ promotes endothelin B receptor-Gα_i-protein interaction (107). Thus, the G$\beta_5\gamma_2$-dimer specifically couples Gα_q-proteins to receptors (117). With the exception of Gβ_5, the identity of the Gβ-subunit does not currently appear to be a critical determinant of receptor-G protein specificity. For example, A_1 adenosine receptors couple equally well to Gα_i-proteins containing G$\beta_1\gamma_2$-, G$\beta_1\gamma_3$-, G$\beta_2\gamma_2$-, or G$\beta_2\gamma_3$-dimers as measured by reconstitution of high-affinity agonist binding (118). In contrast, G proteins containing a farnesylated γ-subunit coupled less efficiently to the A_1 receptor, suggesting that lipid modification of the G$\beta\gamma$-dimer can influence receptor-G protein coupling efficiency (52).

B. Structural determinants of effector specificity

Unlike Gα-subunits, the conformation of G$\beta\gamma$-dimers does not significantly change whether G$\beta\gamma$ is in the inactive heterotrimeric complex or in the free active state. One notable exception to this idea is that phosducin binding to G$\beta\gamma$ induces a conformational change primarily in blades 1 and 7, thus preventing G$\beta\gamma$ association with additional effectors (119). Once dissociated from Gα, G$\beta\gamma$ can interact with a number of effectors. Using alanine scanning mutagenesis, our laboratory (26) and others (120, 121) previously identified residues on Gβ that contact Gα and that mediate a number of effector interactions including ion channels, PLCβ_2, and ACII (Fig. 2C). Regions important for ACII interaction map roughly to blades 2, 3, and 5, whereas the N-terminal interface of Gβ interacts with G protein-activated, inwardly rectifying potassium channels, 1 and 4 (26). In addition, point mutations either on the Gα interacting face of

TABLE 2. Effectors regulated by G$\beta\gamma$ dimers

Effector	Regulation
PLCβs	Stimulation
AC I	Inhibition
AC II, IV, and VII	Stimulation
K+ channels (GIRK1, 2, 4)	Stimulation
Ca^{2+} channels	Inhibition
G protein receptor kinase	Recruitment to membrane
PI_3 kinase	Stimulation
Bruton's tyrosine kinase	Stimulation
Tsk tyrosine kinase	Stimulation
Protein kinase D	Stimulation
Calmodulin	Inhibition of calmodulin kinase
Tubulin	Increased GTPase activity
Dynamin I	Increased GTPase activity
Shc phosphorylation[a]	Indirect activation of MAPK (?)
Raf-1 protein kinase	Sequestration of G$\beta\gamma$
Ras exchange factor[a]	Indirect activation of MAPK (?)
KSR-1	Sequestration of G$\beta\gamma$

GIRK, G protein-activated inwardly rectifying potassium channel; ?, unknown.

[a] See Refs. 235 and 236.

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blades 1–4 or mutations in the outer loops of blades 2, 6, and 7 inhibit PLCβ_2 activity (26, 120); whereas, PLCβ_3 is inhibited by point mutations within blades 2 and 5 (121). Therefore, each effector contacts a unique but overlapping set of residues on Gβ, and some of these sites also represent Gα interacting sites. These studies are consistent with the idea that interaction with α precludes G$\beta\gamma$ binding to effector proteins. Mutational studies continue to reveal the molecular basis for effector interaction as well as the structural basis for variations between G$\beta\gamma$-subunits in effector coupling efficiency. However, one key question yet to be resolved is how G$\beta\gamma$ activates a particular effector once freed from Gα, in a cytoplasmic milieu full of potential partners. Signaling specificity could be brought about by factors such as discrete subcellular localization of effectors, compartmentalization of scaffolding components, and cell type-specific expression of signaling molecules (122). The formation of signaling networks that bring together specific receptors, G proteins, regulatory proteins, enzymes, and substrates is a hot area of research and will likely reveal key factors regulating signaling specificity.

C. Novel G$\beta\gamma$-effectors

At the current discovery rate of G$\beta\gamma$-effectors, the final tally of proteins that interact with the G$\beta\gamma$-dimer is likely to exceed that for Gα-subunits (68). As shown in Tables 1 and 2, G$\beta\gamma$- and Gα-subunits interact with a number of common effectors, such as PLCβ, Bruton's tyrosine kinase, and certain types of ACs. These effector interactions can be independent, synergistic, or antagonistic. For example, G$\beta\gamma$-subunits potentiate ACII activation by Gα_s, but inhibit Gα_s-stimulated ACI activity. In addition, G$\beta\gamma$-dimers interact with a number of novel effectors that are not regulated by Gα-subunits. These novel effector interactions expand the role of G proteins in the regulation of various cellular processes and are briefly discussed below.

Putative G$\beta\gamma$-effectors recently identified include protein kinase D (123), PI$_3$ kinase (93, 94), tubulin (124), KSR-1 (125), dynamin I (126), Raf-1 protein kinase (127), Tsk protein kinase (128), and calmodulin (129) (see Table 2 and references therein). Although previous data suggest that G$\beta\gamma$-effectors bind to an overlapping domain on Gβ-subunits, additional studies also indicate that G$\beta\gamma$ binding to one particular effector does not necessarily preclude G$\beta\gamma$ interaction with a second effector protein. For instance, G$\beta\gamma$ binding to calmodulin does not prevent G$\beta\gamma$-mediated stimulation of PLCβ (129). The ability of G$\beta\gamma$ to simultaneously regulate different effectors suggests that the G$\beta\gamma$ conformation is not disturbed upon effector binding. One notable exception to this idea is that phosducin binding to G$\beta\gamma$ induces a conformational change primarily in blades 1 and 7 preventing G$\beta\gamma$ association with additional effectors (119). Likewise, G$\beta\gamma$ interaction with the protein kinase KSR-1 prevents G$\beta\gamma$-mediated stimulation of MAPK (125). However, the mechanism responsible for this exclusivity remains to be elucidated. As mentioned before, Chidiac and Ross (73) showed that G$\beta\gamma$ could prevent the acceleration of the GTPase activity of Gα_q by PLCβ, which implies a dual role for G$\beta\gamma$ because it can stimulate PLCβ activity directly and indirectly (through prolonged activation of Gα_q).

Although most G$\beta\gamma$-effectors are believed to directly interact with the Gβ-subunit, a role for the Gγ-subunit has also been suggested. Using a yeast-two-hybrid screen with the protein kinase KSR-1, Bell *et al.* (125) identified Gγ_2, Gγ_3, and Gγ_{10} as interacting proteins. The C terminus of Gγ-subunit seems to play a direct role in modulating PLCβ functions (130). To date, no specific Gβ or Gγ binding domain has been identified, although an intriguing number of G$\beta\gamma$ interacting proteins contain pleckstrin homology domains. Future research is likely to identify an increasing number of G$\beta\gamma$-effector proteins. Recently, our laboratory found that the receptor for activated C kinase 1 and the dynein intermediate chain interact with the G$\beta_1\gamma_1$-dimer (131). G$\beta\gamma$ can inhibit neurotransmitter release independently of second messenger formation and ion channel modulation, perhaps by direct interaction with the exocytotic fusion machinery, because both syntaxin 1B and SNAP25B are G$\beta\gamma$ binding partners (132).

D. Additional role for Gβ_5

Gβ_{1-4} share 80%–90% sequence homology and are ubiquitously expressed (133). In contrast, Gβ_5 shares only about 50% identity with the others and is preferentially expressed in the central nervous system (134). Gβ_{1-4} are entirely particulate proteins, whereas Gβ_5 can exist both in the soluble and membrane fractions (134), and the N-terminal domain of Gβ_5 is significantly longer than the other Gβ-subunits. Although this region is important for Gγ interaction (135), Gβ_5 can dimerize with Gγ, form functional heterotrimers with Gα, and interact with a number of effectors in response to receptor activation (102, 113, 114, 117, 134). However, unlike other Gβ-subunits, Gβ_5 can readily dissociate from Gγ under low-stringency conditions and is stable in solution without being complexed to Gγ (136, 137). Free Gβ_5 has been shown to interact with certain GAPs known as regulators of G protein signaling (RGS proteins) through a G protein Gγ-subunit-like domain (138, 139). The G protein Gγ-subunit-like domain is a 64-amino acid region (34% identical to Gγ_5) that is present in RGS6, RGS7, RGS9, RGS11, and the *Caenorhabditis elegans* RGS protein EGL-10 (140). Gβ_5 binding to RGS proteins enhances the ability of the proteins to accelerate the GTPase activity of Gα-subunits (141). In addition, Gβ_5 binding to RGS6, -7, and -11 allows for the selective inactivation of Gα_o (140) and may localize these RGS proteins within the cytosolic compartment (142). Is Gβ_5 always associated with an RGS protein *in vivo,* or does it shuttle between RGS proteins and Gγ-subunits? In native preparations, RGS9 exists in a tight complex with the long splice variant of Gβ_5 (Gβ_{5Long}) in vertebrate photoreceptors (138). The Gβ_{5L}-variant was absent from the retinal tissue of RGS9-deficient mice despite the presence of normal levels of Gβ_5 mRNA (143). In contrast, Gβ_{5Short} protein levels were normal in knockout mice. Therefore, RGS9 may be required for the translation or stability of Gβ_{5L} in photoreceptor cells whereas Gβ_{5S} may be free to interact with Gγ-subunits (143). Other questions yet to be resolved include: does Gβ_5 interact with other proteins outside the RGS family? Do free Gγ-subunits

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have a signaling role on their own? What is the brain-specific role for $G\beta_5$? The discovery of $G\beta_5$ independent of $G\gamma$ has clearly disproved the previous dogma that $G\beta$-subunits associate only with $G\gamma$, and that only through this association do they elicit a physiological response.

IX. Molecular Basis for G Protein Inactivation

A. Intrinsic GTPase activity

As previously mentioned, the duration of G protein-mediated effector activation is dependent on the intrinsic GTPase activity of the $G\alpha$-subunit. Like the intrinsic GDP release rates, intrinsic GTP hydrolysis activity varies among $G\alpha$-subunits (83). For example, the catalytic rate constant value for GTP hydrolysis for $G\alpha_z$ is approximately 200-fold lower than that of $G\alpha_s$ and $G\alpha_o$ (83, 144, 145). The GTPase domain is highly homologous among $G\alpha$-subunits and the side chain of a conserved arginine residue (Arg^{174} in $G\alpha_t$; located within the helical domain) forms hydrogen bonds with oxygens of the α- and γ-phosphates and the β-γ phosphate bridging oxygen. This Arg residue plays a key role in GTP hydrolysis. Thus, mutations of either this Arg or residues contacting it have been reported to alter the GTPase activity of $G\alpha$-subunits (2, 146). Because of the conserved nature of the GTPase domain of $G\alpha$-subunits, the determinants of $G\alpha$-hydrolysis variability are likely to lie in the divergent helical domain and within the N and C termini or be the result of subtle flexibility and conformational changes among $G\alpha$-subunits. The mechanisms responsible for variations in GTP hydrolysis rates have not been studied in detail. Research in this area has focused instead on identifying proteins that directly interact with $G\alpha$-subunits to regulate their intrinsic GTPase activity. Some of these key studies are discussed below. For the interested reader, detailed descriptions of the mechanism of GTP hydrolysis can be found elsewhere (2, 9).

B. Gα Interaction with GTPase-activating proteins (GAPs)

Several years ago, researchers noted that the intrinsic GTPase activity of $G\alpha$-subunits occurs *in vitro* at a much slower rate than can account for the observed deactivation rates of G protein-controlled processes (147, 148). Therefore, speculation mounted that, *in vivo*, an additional protein was rapidly terminating signal transduction, returning the system to an agonist-responsive state. In mammals, the $G\alpha$-effectors $PLC\beta$ and the γ-subunit of phosphodiesterase ($P\gamma$) were two of the earliest identified GAPs for $G\alpha_q$ and $G\alpha_t$, respectively (149–151). Most recently, Scholich *et al.* (152) have determined that the effector ACV serves as a GAP for $G\alpha_s$. Thus, after activation by $G\alpha$, an effector can feed back on the activated $G\alpha$-subunit and significantly reduce the duration and amplitude of the signal generated.

In addition to effector-mediated feedback inhibition, RGS proteins enhance the GTPase activity of $G\alpha$-subunits, thereby reducing the duration and amplitude of both $G\alpha$- and $G\beta\gamma$-mediated cellular responses (153–156). RGS proteins share a common approximately 125-amino acid domain termed the RGS box (157, 158). To date, more than 30 mammalian RGS proteins have been identified (156, 158–160), each containing 23 conserved hydrophobic residues at the core of the RGS domain (155, 156, 159, 161). *In vitro*, the RGS core domain is both necessary and sufficient for GAP activity. However, *in vivo* this is not the case. Our laboratory and others (162, 163) have demonstrated that in native retinal preparations, RGS9 requires effector activation for the full expression of RGS GAP activity. Likewise, the core RGS domain of RGS16 can stimulate $G\alpha_o$ GTP hydrolysis *in vitro* but requires additional N-terminal residues for functional activity *in vivo* (164). These studies suggest that *in vivo* the noncatalytic domains regulate RGS GAP activity through interactions with cellular factors. Only two such factors have been identified to date, $G\beta_5$ and phosphodiesterase $E\gamma$ (141, 151, 165). Noncatalytic domains of RGS proteins have also been suggested to mediate signal transduction pathway specificity and subcellular targeting of RGS proteins (154, 166).

GAPs for heterotrimeric G proteins accelerate GTP hydrolysis in a manner that differs from that observed with monomeric G protein GAPs. For example, Ras GAP inserts a catalytic Arg residue into the active site that participates in the hydrolysis step (2). However, this Arg finger is provided by the helical domain in heterotrimeric G proteins and mediates intrinsic GTP hydrolysis as discussed above (2). In contrast, RGS proteins bind to the switch regions on $G\alpha$ and thereby stabilize the $G\alpha$ transition state toward GTP hydrolysis (167). The mechanism for effector-mediated GAP activity has not been clearly delineated. By analogy, effector-mediated GAP activity may also occur through a similar stabilizing mechanism. However, differences in activity between effector GAPs and RGS GAPs have been observed. For example, Mukhopadhyay and Ross (168) demonstrated that RGS4 produces a 2-fold greater acceleration of the $G\alpha_q$-bound GTP hydrolysis rate in comparison to $PLC\beta$, but $PLC\beta$ is 100 times more potent than RGS4. Although these findings might suggest different mechanisms of GAP activity for effectors and RGS GAPs, $G\beta\gamma$ can inhibit the GAP activity of both $PLC\beta_1$ and RGS4 (73). This is consistent with the idea that $G\beta\gamma$, effectors, and RGS proteins bind to the same region on $G\alpha$, namely the switch regions of the GTPase domain. Thus, RGS proteins can act as effector inhibitors as well as GAPs. On the other hand, we and others recently determined that the effector $P\gamma$ enhances the GAP activity of the regulator of G protein signaling 9 (RGS9) core domain by increasing the affinity of the RGS9 domain for a $G\alpha_{t/i}$ chimera (163, 170). These studies suggest that RGS proteins may be regulated through their participation in a signal transduction complex that may include receptors and effectors and may be localized near the plasma membrane. A similar suggestion was proposed by Chidiac and Ross (73). Our laboratory has also determined that the α-helical domain of $G\alpha_t$ (a $G\alpha_i$ family member) is a key molecular determinant of the selectivity that the RGS9 core displays as a GAP for $G\alpha_t$ *vs.* $G\alpha_i$ (163). Therefore, RGS protein affinity and GAP activity for various $G\alpha$-subunits may be mediated, at least in part, by the primary structure of the $G\alpha$-subunit as well as by the sequence of the RGS box. Further in-depth discussion of RGS proteins can be found in one of several reviews on this topic (154–156, 161, 166).

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X. Regulation of G Protein Function by Covalent Modification

G protein signaling cascades are also regulated by posttranslational modification of the G proteins themselves, which includes phosphorylation and/or acylation of Gα- and G$\beta\gamma$-subunits. Phosphorylation of Gα-subunits by protein kinase C inhibits signal transduction through Gα_i family members (171–174). For Gα_z, stoichiometric phosphorylation occurs at N-terminal Ser^{16} and Gα_z-GDP is the preferred substrate (175, 176). Protein kinase C phosphorylation of Gα_z prevents heterotrimer formation (175) and inhibits GAP activity of RGSZ1 (177). Thus, phosphorylation could significantly prolong G$\beta\gamma$-effector activation while reducing Gα-effector stimulation. The GDP-bound forms of Gα_t and Gα_s are also kinase substrates (178, 179), and phosphorylation of Gα_{12} prevents interaction with G$\beta\gamma$ (180). Phosphorylation of Gβ- (181) and Gγ-subunits has also been reported (182), and phosphorylation of G$\beta_1\gamma_{12}$ inhibits G$\beta\gamma$-mediated AC activation without altering the activation of PLCβ (183). Thus, phosphorylation cannot only dissociate Gα- and G$\beta\gamma$-mediated signaling, but it also regulates the selective modulation of particular G$\beta\gamma$-effectors.

A. G protein lipidation

In addition to phosphorylation, Gα-subunits are lipidated (myristoylated and palmitoylated) at their N termini. N-myristoylation results from cotranslational addition of the saturated 14-carbon fatty acid myristate to a Gly residue at the second position after the removal of the initiating Met by the enzyme methionine amino-peptidase (184). A stable amide bond links the myristate to the protein. Hence, this myristoylation is essentially an irreversible modification. Only Gα-subunits of G$_i$ family are myristoylated (see Refs. 174 and 185–187 for review). In addition, all G protein Gα-subunits, except Gα_t, contain the posttranslationally attached saturated 16-carbon fatty acid palmitate and some Gα-subunits (Gα_q, Gα_{11}, Gα_{13}, and Gα_{16}) are palmitoylated at multiple sites (see Refs. 174, 188, and 189 for review). Palmitoylation of proteins results from the esterification of Cys thiol groups by palmitate. Due to its unstable character, palmitoylation is readily reversible and subject to regulation (188, 190). As yet, palmitoylation cannot be accurately predicted based on primary sequence. However, palmitoylation occurs frequently in proximity to other lipid modifications such as myristoylation or prenylation.

The Gγ-subunit when dimerized with Gβ is isoprenylated posttranslationally. The 15-carbon isoprenoid farnesyl (Gγ_1, Gγ_8, and Gγ_{11}) or the 20-carbon isoprenoid geranylgeranyl (other Gγ-subunits) is attached via a stable thioether bond to a Cys residue located in the C-terminal CAAX box of Gγ, followed by the proteolytic removal of the C-terminal three amino acids and then the carboxyl methylation of the new C terminus (191). The X residue in the CAAX motif is a major determinant of the isoprenyl group. If X is a Ser, Met, Gln, or Ala, the proteins are farnesylated, whereas Leu at this position results in geranylgeranylation (see Ref. 192 for review). Carboxymethylation of the C terminus of Gγ appears to modulate the affinity of the membrane attachment (193).

B. The role of lipid modifications in G protein membrane association and consequent signaling functions

One clear function of fatty acid acylation is to serve as a hydrophobic membrane anchor. For the G$_i$ family of Gα-subunits that are both myristoylated and palmitoylated, both modifications contribute to the membrane association. Removal of the palmitoylation site while preserving myristoylation results in a partial shift in localization from the membrane to the cytoplasm (194–197). Likewise, mutation of the N-terminal Gly on Gα, which abolishes myristoylation, also inhibits palmitoylation and similarly shifts protein localization (184, 198, 199). Shahinian and Silvius (200) have recently proposed a "kinetic membrane trapping" model for G proteins to account for this localization dependence on both lipid modifications. Within this two-signal model of membrane binding, myristoylation and palmitoylation cooperate to target Gα_i-subunits to the plasma membrane. Myristoylation serves as the initial signal bringing the protein to the membrane, and palmitoylation is the second signal that further secures this interaction. In addition, palmitoylation may specifically target G proteins to the plasma membrane rather than to intracellular organelle membranes (174, 195, 201). Consistent with this two-signal model, in the case of myristoylation-defective mutants of Gα_z and Gα_o, the prenylated G$\beta\gamma$-subunit can substitute for myristoylation and carry the α-subunit to the plasma membrane where it can be palmitoylated and fulfill its signaling activity (174, 202).

For Gα-subunits that are modified solely by palmitate (Gα_s, Gα_q, Gα_{12}, and Gα_{13}), mutations that prevent palmitoylation markedly impair membrane association (203–206). In addition, G$\beta\gamma$ appears to be a crucial prerequisite for membrane anchoring and palmitoylation of Gα_s and Gα_q (207). However, by enzymatically depalmitoylating Gα_q, Hepler *et al.* (208) have determined that Cys residues rather than palmitoylation *per se* are critical determinants of Gα_q-mediated signal transduction. Because most studies investigating the role of palmitoylation have relied on mutating Cys residues, further studies are needed to determine whether the significance of palmitoylation itself has been overestimated thus far. Indeed, a paper by Fishburn *et al.* (209) used a mutant G$\beta\gamma$ complex, which mislocalized to the mitochondrial membrane, to investigate the relative contributions of protein-protein interactions *vs.* lipid modifications in controlling membrane targeting of Gα_z. Using this approach, these authors determined that Gα_z interaction with G$\beta\gamma$, rather than palmitate, directs specific targeting of G protein Gα-subunits to membranes.

Lipid modifications also regulate protein-protein interactions. For example, N-myristoylation of Gα modulates G$\beta\gamma$ (210) and effector interactions (211), and palmitoylation increases the affinity of Gα_s for G$\beta\gamma$ (212). In addition, palmitoylated G$\alpha_s\beta\gamma$ is more resistant to thioesterase cleavage of palmitate than free palmitoylated Gα_s (212). Palmitoylation can also inhibit the interaction of GzGAP (an RGS protein) with Gα_z (213). Thus, the palmitoylation state of G proteins can affect their ability to serve as signaling molecules. As part of a feedback mechanism, palmitate turnover can also be regulated by receptor activity (196, 214).

The addition of the prenyl group to the Gγ-subunit plays

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a central role in the membrane association of the G$\beta\gamma$ complex (for review see Ref. 215). Although not required for G$\beta\gamma$-dimer formation, isoprenylation of Gγ is necessary for productive interaction of G$\beta\gamma$ with other proteins including Gα (111) and effectors such as AC (216–218), PLC (217, 218), and PI_3 kinase (217, 219, 220) as well as with receptors (52).

XI. Advances for the Future: Investigating the Dynamic Nature of G Protein Signaling

The resolution of crystal structures for active, inactive, transition state of Gα (3–6) has provided a basis for understanding G proteins as molecular switches for signaling pathways. These studies also provide a framework for conducting structural, functional, and biochemical experiments that can extend our understanding of G proteins along with their various signaling partners. Because only a few G proteins have been crystallized to date (see Table 3), interpretations and conclusions from these structures may not reflect the full complexity of subunit combinations. Moreover, the static nature of such structures may actually limit our understanding of the dynamic nature of G protein signaling. To more accurately assess G protein interactions with receptors, effectors, and regulators of G protein signaling, it will be necessary to take advantage of new techniques that can provide insights into the complex nature of G protein activation. A few of these techniques are described below.

Fluorescence spectroscopic techniques continue to play an important role in determination of G protein conformational changes. In particular, fluorescence resonance energy transfer (221) provides a real-time measurement of activation, deactivation, and protein-protein interactions under basal and stimulated conditions. Fluorescence resonance energy transfer involves attachment of different fluorescent donor and acceptor probes at known residues. Changes in tertiary structure as a result of binding or activation, which result in the donor fluorophore coming into close proximity to the acceptor fluorophore, result in a quenching of donor emission and a simultaneous increase in acceptor emission as energy is transferred. This can be measured as a ratio between donor and acceptor emission in specific timed intervals, resulting in a real-time measurement of dynamic changes in protein conformation that is both sensitive and specific to labeled regions of the proteins. For example, Remmers (222) used a fluorescently labeled GTPγS analog, *N*-methyl-3′-*O*-anthranoyl-GTPγS, to measure conformational changes in heterotrimeric G proteins upon nucleotide binding. G protein intrinsic Trp fluorescence decreased whereas *N*-methyl-3′-*O*-anthranoyl-GTPγS fluorescence increased upon binding the nucleotide analog. In conjunction with stopped-flow fluorescence measurements, the kinetics of the binding reaction can also be determined. Stopped-flow fluorescence itself has long been used to measure binding kinetics and has been used recently to measure GAP activity as a result of RGS proteins binding to activated Gα-subunits (223).

TABLE 3. G protein crystal structures

PDB code	Structure	Resolution (Å)	Ref.
1GOT	Heterotrimeric $G\alpha_t\beta\gamma_t$ complex	2.00	7
1GP2	Heterotrimeric $G\alpha_{i1}\beta_1\gamma_2$ complex	2.30	8
1TAD	$G\alpha_t$-GDP·AIF_4^-	1.70	5
1TAG	$G\alpha_t$-GDP Mg^{2+}	1.80	4
1TND	$G\alpha_t$-GTPγS	2.20	3
1AZT	$G\alpha_s$-GTPγS	2.50	237
1GFI	$G\alpha_{i1}$-GTP	2.2	238
1TBG	G$\beta\gamma$-dimer	2.1	239

PDB, Protein databank.

Spin labeling can also be used to examine changes in protein conformation in real time. This technique requires introduction of a nitroxide side chain at specific residues and electron paramagnetic resonance signal from the nitroxide spin label can detect and report subtle changes in its local environment. It is possible to determine changes in solvent accessibility, dynamics, and intermolecular distances of side chains in solution in real time, yielding information about the time scale and magnitude of structural changes in the labeled region of the protein. Spin pairs can be used to determine changes in the secondary structure of proteins; introduction of spin labels at positions (i) and (i + 4) allows examination of helical structure within proteins. Changes can be measured on a millisecond time scale. Farrens *et al.* (224) successfully employed this technique to determine movements of helices that accompany rhodopsin activation. They found that α-helix C of rhodopsin moves as a rigid unit in relation to α-helix F upon light activation of this receptor. This technique is being further used in studies to determine conformational changes in the N terminus of $G\alpha_i$ upon activation, because these residues are absent or disordered in most high-resolution crystal structures of GDP- or GTPγS-bound form of Gα-subunits (3, 4, 6) (see Table 3) with the exception of the $G\alpha_i$/RGS4 complex. In the crystal structure of RGS4 core domain bound to aluminum fluoride-activated $G\alpha_i$-GDP subunits, $G\alpha_i$ makes two differing sets of contacts with the RGS molecule. One contact is through the $G\alpha_i$-switch region binding to the RGS core domain, whereas the second contact is through the N terminus of the $G\alpha_i$ binding to an adjacent RGS molecule in the crystal. This suggests some type of crystallization artifact, leaving a question as to the relevance of the N terminus present in this 2.8-Å structure. Although it is clear from heterotrimeric structures that G$\beta\gamma$ binding stabilizes an N-terminal α-helix in Gα-subunits, this may change upon activation. Indeed, site-directed spin-labeling studies have shown that $G\alpha_i$ N terminus is dynamically disordered in the GDP-bound form, but adopts a structure consistent with an α-helix upon interaction with G$\beta\gamma$ (225). However, activation of the spin-labeled $G\alpha_i\beta\gamma$ complex by photoisomerized rhodopsin in the presence of GTPγS causes the N-terminal domain of $G\alpha_i$ to revert to a dynamically disordered state similar to that of the GDP-bound form (225).

Another powerful technique for measuring protein-protein interactions in real time is surface plasmon resonance. This technique measures changes in refractive index on the surface of a chemically modified sensor chip as a binding event occurs. The resultant binding curve allows for a quantitative measure of affinity of the binding interaction. Figler *et al.* (226) used this technique to determine the affinities of Gα-subunits for various G$\beta\gamma$ combinations. Current

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advances include development of methods to immobilize vesicles to a sensor chip derivatized with lipophilic alkyl chains, thus anchoring intact vesicles and providing a physical and chemical environment similar to that of cell membranes, which can be used to measure protein-protein interactions of membrane-associated proteins (227).

Computational approaches such as structure prediction and three-dimensional modeling and mathematical techniques such as monte-carlo simulations all provide valuable insights into G protein signaling. More importantly, they are valuable tools that serve to direct further biochemical and functional experiments. These approaches, combined with genetics, can be used to define and examine key components of the signaling pathway, which will both broaden our understanding of the complex nature of G protein signaling and lead to new questions for further investigations.

Structural and functional aspects of heterotrimeric G proteins, their binding partners, and the signaling networks in which they participate are the subjects of intense investigation, and dramatic progress has been made in recent years. The next frontier is to understand how signaling pathways interact with each other to form signaling networks (241). Cells are bombarded with a multiplicity of ligands, and the cellular response is somehow integrated based on all its responses. The experimental approaches to this problem are beginning to be available, but are in their infancy. Certainly, many new approaches to these issues of complexity in cellular signaling will need to be pioneered, and will surely lead to new insights.

Acknowledgments

We apologize to all whose work could not be recognized because of page restrictions. We are grateful to Ms. Simona Ioffe and Ms. Anjela Papassavas for their assistance in gathering information for this review.

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