

Review

G protein $\beta\gamma$ subunits: Central mediators of G protein-coupled receptor signaling

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Abstract. G protein $\beta\gamma$ subunits are central participants in G protein-coupled receptor signaling pathways. They interact with receptors, G protein α subunits and downstream targets to coordinate multiple, different GPCR functions. Much is known about the biology of $G\beta\gamma$ subunits but mysteries remain. Here, we will review what is known about general

aspects of structure and function of $G\beta\gamma$ as well as discuss emerging mechanisms for regulation of $G\beta\gamma$ signaling. Recent data suggest that $G\beta\gamma$ is a potential therapeutic drug target. Thus, a thorough understanding of the molecular and physiological functions of $G\beta\gamma$ has significant implications.

Keywords. G protein-coupled receptor, G protein $\beta\gamma$ subunits, signal transduction, protein-protein interactions.

Heterotrimeric G proteins and signaling by $G\beta\gamma$ subunits

G protein β subunits were first discovered as components of G proteins almost 30 years ago. Despite enormous advances since then, there remain multiple emerging and unanswered questions about the fundamental details of the biochemical roles for $G\beta\gamma$ in GPCR-dependent G protein activation, as well as questions about broader roles in novel signaling mechanisms, physiology and pathophysiology.

Heterotrimeric G proteins consisting of multiple isoforms of distinct $G\alpha$, β and γ subunits mediate the actions of a wide variety of cell surface receptors [1–3]. Receptors catalyze exchange of tightly bound GDP for GTP on the α subunit in a process that requires the complete heterotrimer. In the classical model for G protein signaling, binding of GTP results in activation of the G protein and dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ subunits (Fig. 1A). In recent years, a variety of reports have suggested additional

modes of activation that could either add complexity to the classical model or represent entirely independent mechanisms for heterotrimeric G protein regulation [4–10]. Whatever the mode of G protein activation, the $G\alpha$ and $G\beta\gamma$ subunits both interact with effector molecules, such as phospholipases and ion channels, in a manner that leads to their activation. $G\beta\gamma$ does not have a catalytic site and thus acts as a modulator of G protein signaling through regulated protein-protein interactions. The list of molecules that have been reported to bind to $G\beta\gamma$ continues to grow. While great progress has been made in the understanding of $G\beta\gamma$ structure and function, fundamental mechanisms for molecular recognition and effector regulation by $G\beta\gamma$ have yet to be fully elucidated. Additionally, how all these interactions are coordinated to mediate various G protein signaling processes in cells and tissues is not entirely clear. In this review we will discuss general background concerning $G\beta\gamma$ structure and function with an emphasis on new and emerging mechanisms and approaches for study-

ing $G\beta\gamma$ signaling. These new data are leading to a greater understanding of how $G\beta\gamma$ functions at a mechanistic level and at a coordinated physiological level in cells and tissues. Together, this information could establish a basis for development of future therapeutic interventions.

General structure of the $G\beta\gamma$ subunits

The first high resolution structures of the $G\beta\gamma$ subunits arose from X-ray crystal structures of G protein heterotrimers elucidated by the Sprang group [11], and independently by Paul Sigler's group [12]. Subsequently, the structure of the $G\beta\gamma$ dimer alone was solved [13]. The $G\beta$ subunits fold into a prototypical β -propeller comprised of four-stranded β sheets forming each of the seven blades of the propeller (Fig. 1B). The first 57–70 amino acids N-terminal to the β -propeller comprise an α -helical domain that forms a tight coiled-coil interaction with the γ subunit. The most highly conserved regions of the protein are the β sheets of the propeller and variable loops connect the β strands. Two independent structural elements of the $G\alpha$ subunits interact with different regions of $G\beta\gamma$. The $G\alpha$ N-terminal α helix (yellow, Fig. 1C) interacts with the side of the β propeller at blade 1. The $G\alpha$ switch II region that undergoes conformational changes upon GTP binding interacts with the top of the β propeller (dark blue helix, Fig. 1C). In the structures of $G\beta\gamma$ that have been solved there is very little difference in overall structure, with and without bound $G\alpha$ or other binding partners [13–15]. An exception is a structure of $G\beta\gamma$ bound to phosducin showing a movement of blades 6 and 7 of the β propeller creating a cavity between these two blades [16]. These movements have not been seen in other structures of $G\beta\gamma$ [15] and their significance remains to be determined. The apparent lack of significant conformational changes of the $G\beta\gamma$ subunit upon G protein activation has led to the concept that $G\beta\gamma$ activity, with respect to downstream signaling, is regulated by the mode of $G\alpha$ subunit binding, with activation-dependent changes in $G\alpha$ subunit conformation leading to uncovering a signaling surface on $G\beta\gamma$ [1]. A current debate concerns whether this involves subunit dissociation or subunit rearrangement.

Synthesis and Trafficking of $G\beta\gamma$

G protein $\beta\gamma$ subunits are membrane bound proteins that had been suggested to exist almost exclusively on the plasma membrane (PM) tethered to the mem-

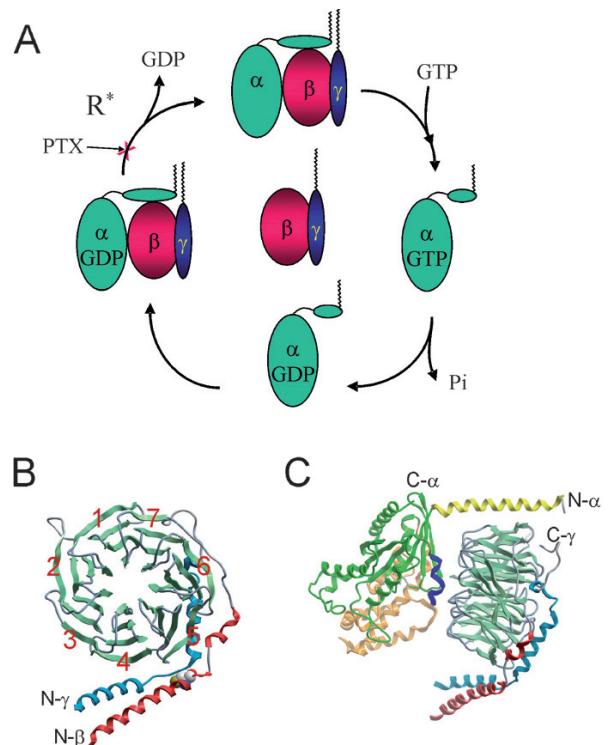


Figure 1. (A) The G protein cycle. The $G\alpha$ subunit bound to GDP interacts with the $G\beta$ subunit with two contacts involving the N-terminal domain and the GTP binding domain. This is the inactive resting state. This complex is a substrate for the activated GPCR (R^*) which catalyzes an increase in the dissociation rate of GDP from the $G\alpha$ subunit leading to the nucleotide free $G\alpha\beta\gamma$ complex. This complex is very short lived in the cell where high concentrations of GTP in the cell bind to the empty nucleotide binding site to drive a conformational change in the $G\alpha$ subunit. This conformational change leads to the active $G\alpha$ GTP subunit, perhaps separated from signaling competent free $G\beta\gamma$ subunits. The $G\alpha$ subunit has the intrinsic capacity to hydrolyze GTP to GDP, allowing reassembly with $G\beta\gamma$ to return to the resting state. Regulators of G protein signaling (RGS proteins) can bind to $G\alpha$ and enhance the rate of GTP hydrolysis. Pertussis Toxin (PTX) modifies the G protein α subunit and prevents interactions of the $G\alpha$ GDP $\beta\gamma$ heterotrimer with R^* . Also shown are myristoyl and isoprenoid lipid groups at the $G\alpha$ N-terminus and $G\gamma$ C-terminus respectively. (B) Ribbon diagram representing the three dimensional crystal structure of the $G\beta\gamma$ subunits. The blades of the propeller are numbered as in Wall et al. [11]. The $G\beta$ N-terminal helix is in red and forms a coiled coil interaction with the $G\gamma$ subunit in blue. (C) Ribbon diagram of the G protein heterotrimer with the $G\beta\gamma$ subunit rotated 90° relative to B. The $G\alpha$ N-terminus is represented as a yellow helix and the $G\alpha$ Switch II region is in dark blue.

brane through post-translational modification. Recent data has led to significant insight into how G protein subunits are synthesized, assembled, processed, and targeted to membranes. A comprehensive review of G protein subunit assembly and trafficking has recently been published [17], but key points relevant to $G\beta\gamma$ assembly and trafficking will be summarized here. $G\beta$ and γ subunits have no transmembrane hydrophobic domains and are synthesized

in the cytoplasm. This process has recently been shown to require specific chaperone proteins. Two chaperones are involved in proper folding and stabilization of the newly synthesized G β subunit. The CCT1 (also known as TRiC) chaperonin complex binds newly synthesized G β but not G γ and is required for folding into the seven bladed propeller structure [18]. Phospholipase like protein (PhLP) appears to act as a co-chaperone with CCT, regulating CCT mediated folding of G β [19]. Once folded, CCT dissociates and PhLP remains bound until assembly with G γ [20]. A potential chaperone specific for G γ subunits has also been recently identified as Dopamine Receptor-interacting Protein 78 (DRiP78). DRiP78 binds to G γ and PhLP and may be required for G $\beta\gamma$ assembly [21]. DRiP78 is localized to the Endoplasmic Reticulum (ER), suggesting that some aspects of the initial assembly process of G γ with G β could occur on the ER.

Once assembled, the G γ subunit is processed at the C-terminus to attach an isoprenoid moiety. G γ has signal sequences that direct prenylation with either a farnesyl or geranyl-geranyl moiety. The first step in the C-terminal processing reaction is covalent attachment of the isoprenoid group to a cysteine that is four amino acids from the C-terminus by either geranyl-geranyl or farnesyl transferase. Once modified with lipid the G $\beta\gamma$ complex is targeted to the ER, where a protease, ras-converting enzyme (Rce1) removes the C-terminal three amino acids from the G γ subunit. Isoprenyl cysteine carboxy methyl transferase (Icmt) catalyzes carboxy-methylation of the C-terminus of G γ to yield the fully modified subunit. Assembly with G α may also occur on the cytoplasmic surface of the ER prior to trafficking to the PM through an unknown mechanism.

Assembly with G α is important for PM targeting of G $\beta\gamma$. It was previously thought that geranyl-geranylation of G γ would be sufficient for PM targeting, but expression of G $\beta\gamma$ alone leads to localization to intracellular membranous structures, primarily ER. However, when coexpressed with G α , G $\beta\gamma$ localizes primarily to the PM. It has been proposed that G α lipid modification provides an additional signal that is required for efficient PM targeting of both G α and G $\beta\gamma$ [22].

Recent data indicates that once G $\beta\gamma$ is fully processed it can translocate to intracellular membranes. Initial work by Berlot and colleagues examined trafficking of G $\alpha_s\beta_1\gamma_7$ complexes after activation by the β_2 AR [23]. Here, the receptor and the G proteins internalize but segregate to different intracellular compartments upon stimulation with isoproterenol. The G protein G α and G $\beta\gamma$ subunits initially show diffuse cytoplasmic distri-

bution followed by colocalization on intracellular vesicles distinct from β AR containing vesicles. Subsequent work by others has suggested GPCR- activation results in translocation of fluorescently tagged G protein γ subunits or tagged β subunits to intracellular membranes such as the Golgi or ER [24]. Distinct families of G γ subunits were found that translocate, whereas other families do not [25]. For example it was found that $\gamma_{1, 5, 9, 10, 11}$ and 13 support M2 receptor-dependent translocation, whereas $\gamma_{2, 3, 4, 7, 8}$ and 12 do not [26]. Differences in translocation appeared to correlate with specific sequences in the C-terminus of G γ . These findings are intriguing and suggest specific functional roles for G γ subunit subtypes although the role of the translocation remains to be defined.

G protein β and γ subunit subtypes

Five different G β subunit and 12 different G γ subunit genes have been identified in the human and mouse genomes [27]. The G β_{1-4} subunits share greater than 80% amino acid sequence identity compared to 50% identity for G β_5 . There is significantly lower identity amongst the γ subunit subtypes. These different subunit subtypes can pair to form unique G $\beta_x\gamma_x$ combinations. The functional significance of the diversity of individual G $\beta\gamma$ subunit combinations is not well understood. Interpretation of phenotypes resulting from knockout of individual G $\beta\gamma$ subunits is complicated by the fact that G $\beta\gamma$ participates in multiple, integrated functional interactions with receptors, G α subunits, and effectors. Thus, the resulting phenotypes can be difficult to attribute to a specific functional interaction. Nevertheless, there is evidence from knockout studies to suggest that specific G $\beta\gamma$ subtypes interact with particular GPCRs. Ribozyme-directed G γ_7 subunit depletion impaired β -adrenergic receptor signaling but not signaling by another Gs coupled receptor, PGE $_1$ [28]. Genetic deletion of specific γ subunits in mice results in specific phenotypes. For example, deletion of G γ_7 resulted in distinct behavioral changes associated with specific loss of cAMP production in the striatum [29], and deletion of G γ_3 results in changes in metabolism resulting in resistance to a high fat diet [30]. In both of these cases loss of the specific G γ subunits also resulted in a loss in specific G α expression, indicating roles for specific G $\alpha\beta\gamma$ combinations in these phenotypes.

One instructive example comes from a recent study of G γ functions in the *Arabidopsis thaliana* plant system. In this plant and other species there is one G α subunit, one G β subunit, and two G γ subunits that share approximately 50% amino acid identity [31–34].

Knockout and overexpression of these two $G\gamma$ subunits allowed for a relatively simple dissection of the functional significance of the two $G\gamma$ subunit isoforms. In this system, many of the functional effects appear to be mediated by $G\beta\gamma$. For example, $G\beta$ plays a specific role in plant pathogen resistance and knockout of *A. thaliana* $G\gamma_1$ increased susceptibility to infection with a pathogenic fungus, while $G\gamma_2$ deletion had no significant effect on this function [35]. Similar specificity was seen with other processes involved in seed germination and root development. Thus in this relatively simple G protein system, clear, distinct roles are observed for different $G\gamma$ subunits. The mammalian system is obviously much more complex with many different $G\gamma$ subunits, where many detailed experiments will be required to sort out their individual specific functions.

To date, there is no specific, satisfactory mechanistic explanation for the selectivity for different $G\beta\gamma$ subunits that is observed in intact cells. Although some selectivity has been observed in some *in vitro* reconstitution systems, the difference between subtypes is generally not dramatic. An exception is $G\beta\gamma$ complexes containing the $G\gamma_1$ subunit, which is strictly localized to the retina as part of the transducin heterotrimer. These complexes are generally less potent for activation of effectors such as adenylyl cyclase (AC) and phospholipase C (PLC) [36, 37] and couple less strongly to GPCRs other than rhodopsin. $G\gamma_1$ is modified with a 15 carbon farnesyl rather than a 20 carbon geranylgeranyl lipid moiety and some of the difference may result from this, but there is also evidence that there are sequence determinants on $G\gamma_1$ that may be partially responsible for differences in efficacy and potency of this subunit [38]. Some other examples of biochemical selectivity for effectors exist but, in general, the differences are not enough to explain the striking differences observed in intact cell systems or *in vivo*.

$G\beta_5$

$G\beta_5$ is clearly an outlier with respect to sequence homology with other $G\beta$ subunits, with 53% identity to the most closely related $G\beta$ subunit. Initial evidence that $G\beta_5$ was a bona fide $G\beta$ subunit was its ability to assemble with $G\gamma$ subunits in transfected cells to activate PLC β_2 [39, 40]. Further analysis of purified $G\beta_5\gamma_2$ complexes revealed that $G\gamma$ was loosely bound and could be separated from the $G\beta_5$ subunit under non-denaturing detergent conditions where other $G\beta\gamma$ combinations are not separable [41, 42]. This $G\beta_5\gamma_2$ complex was initially proposed to be only capable of binding to $G\alpha_q$ [43] but other workers demonstrated interactions with $G\alpha_{i/o}$ -GDP [42, 44].

This latter data indicates that $G\beta_5$ has the determinants to direct $G\beta_5$ containing complexes to GDP-bound $G\alpha$ subunits. Siderovski and colleagues were the first to recognize that members of the RGS7 (R7) subfamily of regulators of G protein signaling (RGS proteins) contained regions with significant homology to $G\gamma$ subunits (ggl domains) and predicted that they could potentially assemble with $G\beta$ subunits [45] (Fig. 2A and B). Biochemical studies demonstrated that R7 family members could form stable complexes with $G\beta_5$ but not other $G\beta$ subunits. In parallel, Slepak and colleagues purified native $G\beta_5$ from bovine retina and identified R7 family members as tightly associated proteins that co-purified [46]. In neither of these studies was $G\gamma$ found to co-purify or form stable complexes with $G\beta_5$ [47]. This leads to a currently unresolved debate as to whether $G\beta_5\gamma_x$ is present in native cells or tissues. It has been suggested that the difficulty in finding $G\beta_5\gamma_x$ in native tissues is due to its inherent instability in detergent that is required to extract $G\beta\gamma$ subunits from native tissue. A recent study examining $G\beta_5$ complex formation with different $G\gamma$ subunits in cells using bifluorescence complementation (BiFC) suggests that $G\beta_5$ slightly prefers $G\gamma_2$ as a binding partner relative to RGS7. These data suggest that in native tissues, if $G\gamma_2$ is present in significant quantity, it would assemble with $G\beta_5$ [44]. On the other hand, other factors such as molecular chaperones may add a level of control to assembly that is not observed in the transfected HEK293 cells.

While the occurrence of this $G\beta_5$ RGS complex is very novel and exciting it is not clear how this complex is regulated and what the functional and physiological role of the complex is in GPCR signaling. Some exciting clues have come with the discovery of a protein, R7BP, that binds to R7 family members [48, 49]. This protein binds to the DEP (for Disheveled, EGL-10, Pleckstrin) homology domain of R7 family members and can regulate the distribution of $G\beta_5$ R7 complexes in cells [48, 49]. R7BP can be palmitoylated near its carboxy-terminus, and regulated palmitoylation depalmitoylation at this site determines the subcellular localization of the R7BP/ $G\beta_5$ /R7 complex [49]. Palmitoylated R7BP targets the complex to the PM where it can efficiently inhibit GPCR mediated processes through its RGS domain, while the depalmitoylated form undergoes nuclear-cytoplasmic shuttling that could be involved in regulation of transcription [49, 50]. Interestingly, the DEP domain of R7 is involved in an intra molecular interaction with the $G\beta_5$ subunit in the $G\beta_5$ /R7 complex [51, 52]. In the recently solved crystal structure of $G\beta_5$ -RGS9 complex, the DEP domain, in conjunction with the ggl-DEP linker, occludes the $G\alpha$ binding site on $G\beta_5$ [52].

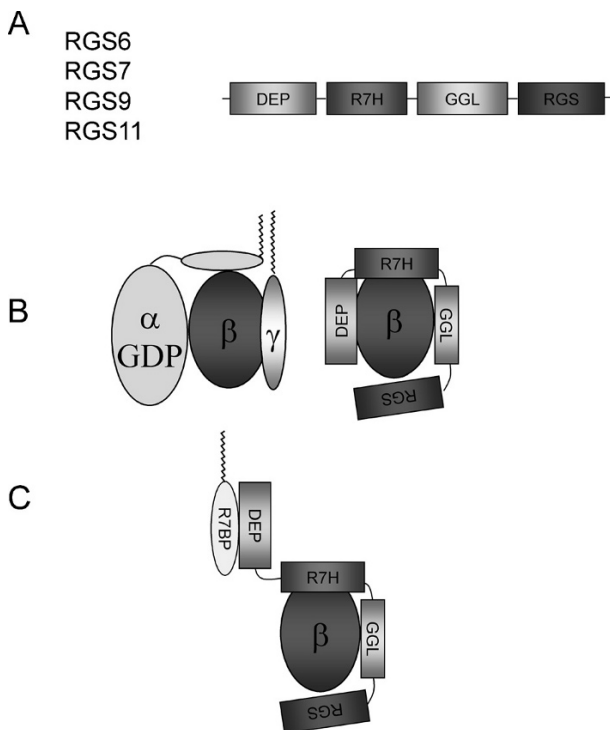


Figure 2. (A) Domain organization of the R7 family of RGS proteins. DEP is the Disheveled, EGL-10, Pleckstrin homology domain, R7H is the R7 homology domain, ggl is the G protein γ like domain, and RGS is the RGS homology domain. (B) Depiction of the interactions of an R7 protein with $G\beta\gamma$ with the ggl domain interacting with $G\beta_5$ instead of $G\gamma$ and the DEP domain potentially interacting with the $G\alpha$ subunit binding site on $G\beta$. (C) Model depicting palmitoylated R7 binding protein bound to the DEP domain of R7. Evidence suggests that this binding could alter DEP domain interactions with $G\beta_5$ to expose binding surfaces on $G\beta_5$. The palmitoylated R7BP could then direct binding to the plasma membrane.

Regulatory mechanisms may exist that “uncap” the $G\alpha$ binding site on $G\beta_5$ to allow productive interactions with $G\alpha$ subunits for receptor catalyzed nucleotide exchange reactions [53, 54]. For example, R7BP could affect this interaction which could, in turn, affect the activity of the $G\beta_5$ /R7 complex (Fig. 2B and C). Binding of R7BP to $G\beta_5$ /R7 complex improves the activity of the complex as a GAP through PM targeting, but additional mechanisms must exist that involve reversible interactions of the DEP domain with $G\beta_5$ to allow receptor-G protein coupling.

While these regulatory mechanisms are emerging for the $G\beta_5$ /R7 complexes, the functional purpose of $G\beta_5$ association with this complex is still unclear. While $\beta_5\gamma_x$ complexes may not be biologically relevant signaling complexes, analysis of the complexes provides information on the molecular determinants that $G\beta_5$ itself may bring to the $G\beta_5$ /R7 complex. For example, studies with $G\beta_5\gamma_2$ indicate $G\beta_5$ is capable of binding $G\alpha$ subunits, interacting with $G\beta\gamma$ regulated

effectors, and participating in receptor mediated G protein nucleotide exchange. That these functionalities exist in $G\beta_5$ suggests that $G\beta_5$ could bring some of these functions to the $G\beta_5$ /R7 complex.

Central functional role in G protein coupled receptor signaling

G protein $\beta\gamma$ subunits are required for GPCR signaling

Early reconstitution studies with receptors and purified G proteins indicate that $G\beta\gamma$ is required for GPCR catalyzed nucleotide exchange [55, 56]. Studies examining muscarinic receptor coupling to $G\alpha_o$ initially suggested that a possible reason for the requirement for $G\beta\gamma$ was that $G\beta\gamma$ was simply required to target $G\alpha_o$ to the membrane and that, once properly oriented at the plasma membrane, $G\alpha$ could productively engage the receptor [57]. While this may be part of the requirement for $G\beta\gamma$ function in receptor coupling, it does not exclude other mechanisms for promoting coupling and cannot, in itself, explain receptor selectivity for particular $G\beta\gamma$ isoforms. Another possibility that is supported in part by structural data is that binding of $G\alpha$ to $G\beta\gamma$ organizes the structure of the $G\alpha$ subunit such that it is a substrate for receptor interactions. Free $G\alpha_i$ subunits are in a distinct conformation relative to the structures in the G protein heterotrimer [11, 12, 58, 59]. For example, in the structure of $G\alpha_i$ -GDP the amino terminus forms a distinct globular domain that adopts an extended helical conformation in direct contact with $G\beta\gamma$ in the $G\alpha_i$ -GDP $\beta\gamma$ complex [58]. The amino terminus of the $G\alpha$ subunit is important for engagement of phospholipid membranes through lipid modifications at the N-terminus. Additionally, amino acids in the $G\alpha$ N-terminus are important for receptor-G protein coupling, suggesting that $G\beta\gamma$ may help present $G\alpha$ in the appropriate conformation to the receptor [60].

GPCRs also interact directly with G protein $\beta\gamma$ subunits. A peptide mimic of the third loop of the α_{2A} adrenergic receptor catalyzed nucleotide exchange on $G\alpha_o$ in a purified system only in the presence of the $G\beta\gamma$ subunits [61]. The peptide was directly crosslinked to the G protein β subunit, and the crosslinking site was mapped to the C-terminus [62]. Other studies have demonstrated direct binding of receptor fragments to $G\beta\gamma$ subunits primarily using glutathione S-transferase (GST) fusion protein binding assays. For example, a portion of the C-terminal tail of the parathyroid hormone receptor bound directly to $G\beta\gamma$ subunits [63]. The specific $G\beta\gamma$

binding site was disrupted in the full length PTH receptor by site directed mutagenesis leading to a loss of downstream signaling to PLC and AC. In other studies with the third intracellular loop of the M3 muscarinic receptor, disruption of the $G\beta\gamma$ binding site did not affect downstream signaling but inhibited receptor desensitization [64]. The data suggest that maintenance of $G\beta\gamma$ binding to this loop facilitated recruitment of G protein coupled receptor kinase (GRK) to the receptor.

Another point of contact between $G\beta\gamma$ and receptors is the C-terminus of the $G\gamma$ subunit. As discussed earlier, $G\gamma$ is prenylated at a C-terminal cysteine. This prenyl modification is required for receptor-G protein coupling but, since it is also required for membrane targeting, it is difficult to determine if the G protein coupling requirement simply reflects the need for $G\beta\gamma$ to be at the membrane or if it is a direct physical coupling between the receptor and $G\gamma$. Evidence for direct physical interactions comes from experiments utilizing prenylated C-terminal peptides from $G\gamma_1$. These peptides inhibit receptor-G protein coupling and alter the activation state of rhodopsin, indicating a direct physical interaction [65]. NMR studies show a specific conformational alteration of the peptide upon receptor activation [66, 67]. In many of the structures of $G\beta\gamma$ this region of $G\gamma$ is disordered, suggesting this region may be conformationally flexible.

Still unanswered is how direct GPCR- $G\beta\gamma$ interactions are mechanistically involved in the G protein activation process. This, in part, comes from our current lack of general understanding of the mechanistic details of GPCR stimulated nucleotide exchange. As discussed, some models of receptor activation portray the $G\beta\gamma$ subunit as a passive participant that scaffolds $G\alpha$ at the membrane, while others indicate an active role. Two models that include $G\beta\gamma$ as an active participant include the lever hypothesis and the gearshift model. In the lever hypothesis, the receptor has been proposed to engage both $G\alpha$ and $G\beta\gamma$ and pry the two molecules apart by pulling on the N-terminus of $G\alpha$, acting as a lever to open up the interface between the switch II region of the $G\alpha$ subunit contacting the $G\beta$ subunit (Fig. 3A). As the subunits rearrange, the $G\beta$ subunit pulls open the nucleotide binding pocket on $G\alpha$, enhancing the off-rate of GDP [68]. One line of evidence in support of this hypothesis is that mutation of amino acids on $G\beta$ that directly interact with $G\alpha$ switch II prevents $G\beta$ -dependent $G\alpha$ activation. In contrast, other data indicates that the Switch II $G\beta$ interface can rapidly “breathe” without full subunit dissociation. This model, depicted in figure 4A, is based on evidence that $G\alpha$ N-terminal interactions and $G\alpha$ Switch II

interactions with $G\beta$ are individually weak but the combined bivalent interaction is strong [69, 70]. Peptides and proteins appear to be able to bind to Switch II binding surface on $G\beta$ during breathing and cause G protein $\beta\gamma$ subunit activation without nucleotide exchange on $G\alpha$, as discussed in section 6 (Fig. 4B and C). Since the $G\alpha/\beta\gamma$ interactions at these surfaces are weak, they may not be sufficiently stable to pry open the nucleotide binding pocket as the subunits reorient relative to one another in the lever model.

The gear shift hypothesis suggests that the receptor increases interactions between the coiled-coil amino terminus of the $G\beta\gamma$ complex and the helical domain of $G\alpha$, pushing the helical and Ras-like domains of $G\alpha$ apart to provide an exit route for GDP (Fig. 3B) [71]. Recent BRET data examining $G\alpha$ - $\beta\gamma$ interactions in intact cells demonstrate that some parts of the helical domain of $G\alpha$ move away from the N-terminus of $G\gamma$ subunits upon activation, but the region linking the helical and GTPase domains move closer to $G\gamma$ -N. The authors suggest that these movements, where parts of $G\beta\gamma$ and $G\alpha$ move closer together during the activation process, are most consistent with the gearshift model for G protein activation [6]. In either mechanism, $G\beta\gamma$ is intimately involved in the process of nucleotide exchange. For a detailed discussion of these models see Oldham and Hamm [60].

If, in fact, there are direct interactions between receptors and $G\beta\gamma$ that are important for the mechanisms of nucleotide exchange and subunit dissociation, it has significant implications for the specificity with which receptors recognize specific $G\beta\gamma$ isoforms. If the sole function of $G\beta\gamma$ were to serve as a scaffold for $G\alpha$, the reported selectivity of receptors for particular $G\beta\gamma$ subtypes would be unlikely to have been observed.

Mechanisms for activation of $G\beta\gamma$ signaling

In addition to its supporting role in GPCR-dependent $G\alpha$ interactions, $G\beta\gamma$ acts to directly regulate downstream signaling in its own right. The first effector found to be activated by $G\beta\gamma$ was the acetylcholine-regulated inwardly-rectifying K^+ channel in atrial myocytes [72]. A key observation in isolated inside-out patches from atrial myocytes was that the acetylcholine driven channel activation was independent of soluble second messengers, suggesting that the subunits of the G_i protein could directly activate the channel. This led to attempts to directly activate the channel in excised atrial membrane patches with $G\beta\gamma$ and $G\alpha$ subunits [72, 73]. After considerable controversy a consensus emerged that $G\beta\gamma$ subunits are the primary mediators of channel activation through

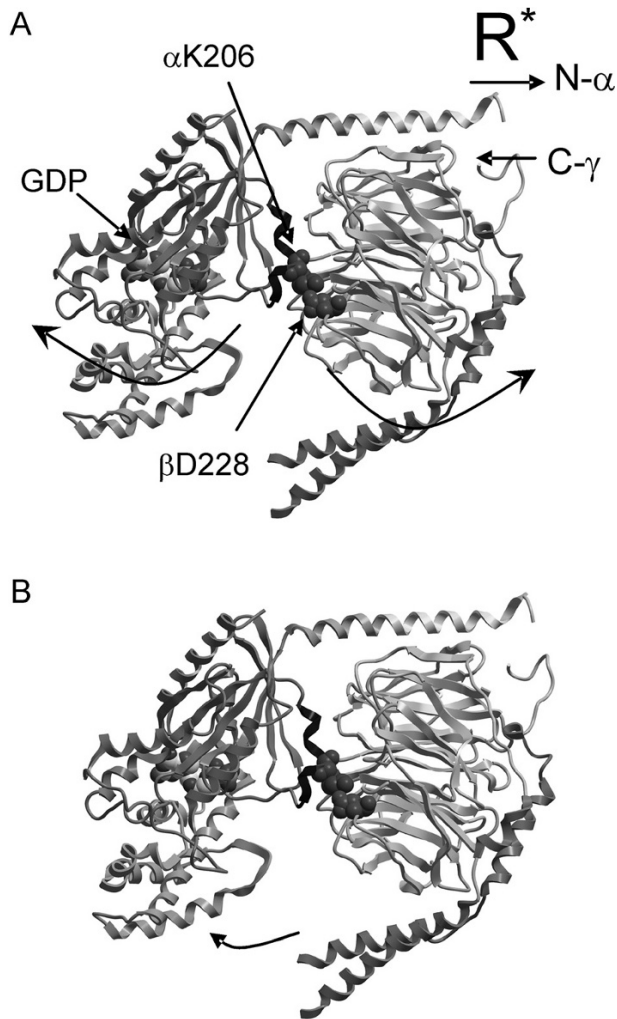


Figure 3. Models for participation of $G\beta\gamma$ subunits in regulation of nucleotide exchange. (A) Lever arm model for $G\beta\gamma$ dependent activation of $G\alpha$ subunit nucleotide exchange. In this model R^* interaction with the N-terminus of the $G\alpha$ subunit and the C-terminus of $G\gamma$ results in $G\beta\gamma$ acting as a lever to pull open the nucleotide binding site on $G\alpha$ to enhance the rate of GDP release. Critical interactions at the $G\alpha$ switch II / $G\beta\gamma$ interface ($G\beta$ D228 and $G\alpha$ K206) are required for $G\beta$ to pull on $G\alpha$ switch II to open the nucleotide binding pocket. (B) Gear shift model for $G\beta\gamma$ -dependent activation of nucleotide exchange. Here the receptor causes the $G\beta\gamma$ subunits to move closer to the $G\alpha$ subunit. This results in a potential physical interaction between the N-terminal helical $G\beta\gamma$ coiled-coil extension and the helical domain of $G\alpha$, leading to opening of the nucleotide binding pocket and release of GDP.

direct binding to the channel [74, 75]. At first, recognition of $G\beta\gamma$ as the primary mediator of channel activation was controversial, in part, because $G\beta\gamma$ mediating direct downstream functions had not been previously demonstrated, and because precedent with adenylyl cyclase demonstrated that $G\alpha_s$ was responsible for direct stimulation of adenylyl cyclase activity. Additional support for the idea that $G\beta\gamma$ is a signal mediator in its own right came from genetic analysis of

the pheromone signaling pathway in yeast, indicating that $G\beta\gamma$ is the key activator of downstream signaling from the G protein coupled pheromone receptor [76]. Today, the list of proteins that interact with $G\beta\gamma$ has expanded to encompass a large number of targets (Table 1). In some cases, these are enzymes or channels and it is clear that $G\beta\gamma$ -binding has a functional effect on the activity of the target. In the case of ERK, $G\beta\gamma$ expression in cells leads to ERK activation, but the direct binding target and the exact mechanism for this activation has not been defined. In many instances the regulation of the target has a clearly documented physiological correlate whereas, in others, the physiological significance of the $G\beta\gamma$ -target interaction is not clear. Overall, however, it is now well established that the $G\beta\gamma$ subunits play major roles in mediating downstream signaling from GPCRs and may be as prevalent as those mediated by $G\alpha$ subunits.

When considering how G proteins are activated by GPCRs, it is the $G\alpha$ subunit that undergoes significant conformational changes upon binding of GTP, suggesting an obvious mechanism for $G\alpha$ activation. So how can $G\beta\gamma$ be “turned on” to propagate a downstream signal? The current model, as discussed earlier, is based on the subunit dissociation model where $G\alpha$ subunits occlude effector binding surfaces on G protein $\beta\gamma$ subunits until activated by binding of GTP. The conformational changes in $G\alpha$ lead to dissociation from $G\beta\gamma$ to expose effector interaction surfaces on $G\beta\gamma$ (Fig. 1). Some evidence for this idea stems from the observation that addition of purified $G\alpha$ GDP to *in vitro* assays of $G\beta\gamma$ -dependent effector activation inhibits effector regulation [72, 77]. Since there is no apparently critical difference in $G\beta\gamma$ subunit structure in either the free or heterotrimeric structure, it suggests that G protein activation does not cause alteration of $G\beta\gamma$ subunit conformation [11–13]. A direct test of the hypothesis that a signaling surface on $G\beta\gamma$ is covered by $G\alpha$ involved alanine substitution of amino acids at the $G\alpha$ subunit-binding surface of the $G\beta$ subunit and testing the purified mutant protein for activation of effectors in various *in vitro* assay systems [78, 79]. Many of these purified $G\beta\gamma$ mutants were unable to efficiently regulate effectors. An important conclusion from this analysis was that each effector utilized this surface with both overlapping and unique subsets of amino acids within the $G\alpha$ subunit binding surface. Complementary studies used a similar mutational approach to map effector binding sites in the blade regions of the $G\beta$ propeller and identified amino acids outside the $G\alpha$ subunit interface important for effector regulation, with some amino acids involved in unique effector interactions [80]. Thus, G protein α subunit activation

Table 1. $G\beta\gamma$ subunit targets¹.

Physiological $G\beta\gamma$ effectors (direct)
inwardly rectifying K^+ channel (GIRK1/GIRK2, GIRK1/GIRK4) [72, 75, 171]
GPCR kinase 2 and 3 [172]
PLC β 1, β 2 and β 3 [77, 86–88, 173]
Adenylyl cyclase (activation), II, IV, VII [174, 175]
Adenylyl cyclase (inhibition), I, III, V, VI [174–177]
N type Ca^{2+} channels [91]
P/Q type Ca^{2+} channels [178]
Phosphoinositide 3 kinase γ [89, 179]
SNAP-25 [180, 181]
P-Rex1 Rac GEF [90]
Proteins regulated by $G\beta\gamma$
PAK (p21 activated kinase) [182]
Raf-1 [183]
Dynamin [184]
Ras GRF [185]
Db1 [186]
Btk kinase [187]
Tubulin/microtubules [188, 189]
Histone deacetylase 5 (HDAC5) [190]
P114 RhoGEF [191]
RGS4 [192]
RGS3 [193]
ATP sensitive K^+ channel [194]
Phospholipase D1 [195]
IP_3 receptor 1 [196]
T type Ca^{2+} channels [197, 198]
FLJ008 Rac/cdc42 GEF [199]
$G\beta\gamma$ binding proteins
RACK I [200]
Group III AGS proteins [4, 131]
AGS2 TcTex1 [201]
AGS7 Thyroid receptor Interacting Protein (TRIP13)
AGS8 KIAA1866
AGS9 Rpn10
$G\beta\gamma$ effectors (indirect)
MAP kinase [202]
PLA_2 [203]

¹ Physiological $G\beta\gamma$ effectors are those proteins that directly bind $G\beta\gamma$ and for which a clear physiologic role for $G\beta\gamma$ interaction has been established. Proteins regulated by $G\beta\gamma$ are proteins known to bind and have activity regulated by $G\beta\gamma$ but for which the physiological role for the interaction has not been established. $G\beta\gamma$ -binding proteins are proteins that bind to $G\beta\gamma$ but where regulation of an activity has not been demonstrated.

likely exposes surfaces on $G\beta$ that form a core site for effector binding but multiple other $G\beta\gamma$ surfaces also participate in effector binding and activation.

Pertussis toxin-sensitive signaling by G protein $\beta\gamma$ subunits

As discussed above, acetyl choline-dependent regulation of the atrial K^+ channel is now known to be through $G\beta\gamma$ binding to the channel. This acetylcholine regulation of the potassium current is inhibited in isolated atrial myocytes by pretreatment with pertussis toxin (PTX) which selectively modifies $G\alpha_i$ family G proteins [81]. Many other processes dependent on $G\beta\gamma$ downstream signaling are also inhibited by PTX. For example, GPCR-dependent PLC activation is

mediated by pertussis toxin-sensitive and -insensitive mechanisms [82, 83]. The PTX-insensitive pathways are primarily mediated by $G\alpha_q$ -dependent activation of PLC β or pathways involving Rho and PLC ϵ [83–85]. PTX-sensitive pathways were presumed to be mediated by members of the $G\alpha_i$ family yet purified PTX-sensitive $G\alpha_i$ family subunits were unable to reconstitute activation of PLC. On the other hand purified $G\beta\gamma$ subunits were able to activate PLC β isoforms *in vitro*, albeit at relatively high concentrations compared to typical activation by G protein α_q subunits [86–88]. This suggested that $G\beta\gamma$ subunits released from G_i heterotrimers were responsible for PTX-sensitive GPCR-dependent PIP₂ hydrolysis. The model for these and other related systems is that PTX-dependent ADP ribosylation of the $G\alpha$ subunits prevents productive interactions between the heterotrimer and receptors preventing nucleotide exchange and activation of $G\alpha_i$, keeping $G\beta\gamma$ sequestered in an inactive state (Fig. 1A). Many of the GPCR-dependent physiological processes inhibited by PTX are mediated by $G\beta\gamma$ subunits rather than $G\alpha$ [72, 89–91]. Fewer examples of PTX-insensitive processes being mediated by the $G\beta\gamma$ subunits have been reported but likely exist [92]. Thus, most $G\beta\gamma$ -dependent signaling appears to arise from G_i proteins.

The apparent specificity of $G\beta\gamma$ -dependent signaling for G_i -coupled receptors presents a conundrum in terms of what regulates the selectivity of $G\beta\gamma$ -dependent processes. In theory GPCR-dependent activation of any G protein subtype would, upon nucleotide exchange-dependent activation of the $G\alpha$ subunit, lead to free $G\beta\gamma$ that could activate any of the $G\beta\gamma$ effectors. Several hypotheses have been proposed to explain the observation that not all GPCRs activate $G\beta\gamma$ -dependent signaling processes. 1) $G\beta\gamma$ subtypes are specifically associated with particular receptors and G protein α subunits and confer effector activation selectivity. The problem with this hypothesis is that specific $G\beta\gamma$ subtypes have not been shown to be selective for particular effectors, so while specific subtypes may be associated with particular receptors; it is not clear how these subtypes would confer selectivity for particular effector pathways. 2) The potency for $G\beta\gamma$ subunit dependent activation of effectors is 10–100 fold lower than for $G\alpha$ subunit mediated effects (see [86] for example). This suggests that activation of receptors that activate $G\alpha_s$ or $G\alpha_q$ would cause activation of $G\alpha$ subunit-dependent effectors at levels of G protein activation that would be significantly lower than that required to release enough $G\beta\gamma$ to activate a $G\beta\gamma$ -dependent effector. So, for example, under conditions required to achieve maximal inositol phosphate release through a G_q -coupled receptor, $G\beta\gamma$ -dependent processes would

not be activated. Since $G\alpha_i$ has a relatively low affinity for AC and G_i proteins are relatively abundant, activation of G_i coupled receptors could release enough $G\beta\gamma$ to achieve significant effector activation. Based on the ideas discussed in the above section, one would still predict that G_i coupled receptor-activation would lead to stimulation of many of the effectors listed in Table 1. Some other ideas that could account for the fact that not all $G\beta\gamma$ -regulated effectors are activated by G_i coupled receptors, or other receptor types, include: 1) Tissue specific expression of some of the components provides some constraint, with some effectors expressed relatively specifically in certain tissues. For example, PLC β 2 and PI3-kinase γ are relatively restricted in expression to monocytic cells, and so would only be activated by GPCRs in these cells, but for other effectors an additional mechanism must exist in cells with multiple GPCRs, 2) Restricting the subcellular location of specific effectors and receptors could impart specificity, 3) Precoupling of receptors G proteins and effectors could confer specificity. For example, GIRK channels have been shown to preferentially form complexes with heterotrimers containing specific $G\alpha_i$ subunits and, while $G\alpha$ subunits do not regulate channel activation, they do bind directly to the channel [93, 94]. If particular $G\alpha$ subunits provide a docking surface for $G\beta\gamma$ targets this could control specificity for specific $G\alpha$ subunit subtypes [95], 4) Simultaneous activation of GPCRs with other receptors could lead to availability of $G\beta\gamma$ in concert with other cellular signals such as phosphorylation, providing a coincidence detection mechanism for activation of specific effectors.

Effector recognition by $G\beta\gamma$

The targets listed in Table 1 that are recognized by $G\beta\gamma$ comprise a diverse array of molecules, many of which are unrelated in terms of structure and sequence. A key question is: What is the nature of molecular recognition that allows $G\beta\gamma$ to interact specifically and productively with this diverse array of targets? We will discuss here what is known about the nature of recognition of targets by $G\beta\gamma$ based on both direct structural visualization of complexes as well as other biochemical analyses.

Three dimensional crystal structures of $G\beta\gamma$ effector complexes: G protein coupled-receptor kinase 2 (GRK2) and phosducin

The structure of $G\beta\gamma$ has been solved in complexes with $G\alpha$ subunits, GRK2, and phosducin. Detailed examination of the nature of the interactions supports the general hypothesis that there are common and

unique interactions amongst various $G\beta\gamma$ targets. This is exemplified best in the co-crystal structure of $G\beta\gamma$ and phosducin [14, 16]. Phosducin, a protein first identified as a regulator in the visual signaling system, binds to $G\beta\gamma$ and is composed of distinct N and C-terminal domains. Both of these domains are required for productive interactions with $G\beta\gamma$. In the three dimensional structure of the complex, the N-terminus of phosducin associates with an area that overlaps with the $G\alpha$ GDP binding site on the top of the β -propeller, while the C-terminus interacts with the sides of the propeller at blades 1 and 7, a region that does not overlap with $G\alpha$ GDP binding [14, 16]. On the other hand the GRK2- $G\beta_1\gamma_2$ interface is dominated by interactions at the $G\alpha$ GDP binding site on the top surface of $G\beta$ [15, 96]. Interestingly while both phosducin and GRK2 have interactions on the top of the β -propeller at the $G\alpha$ subunit interface, the modes of interaction are quite different when compared to $G\alpha$. For example the GRK2 C-terminus is an extended α helix followed by a short C-terminal loop and it is the loop that interacts with amino acids that also contact the $G\alpha$ subunit [97]. In the $G\alpha$ subunit the major contacts with these same amino acids on $G\beta$ are from the switch II α helix region and it is the side chains extending from this helix that interact with $G\beta$. Thus, completely structurally distinct motifs from $G\alpha$ and GRK2 interact with a very similar contact surface on $G\beta$.

Peptide mapping approaches

While crystallography is invaluable in determining protein interaction surfaces, thus far only a limited number of complexes of $G\beta\gamma$ with binding partners have been solved by this method. As an alternative, biochemical methods have been used to map effector binding surfaces. A particularly fruitful approach has been to use synthetic peptides from $G\beta\gamma$ target molecules. Initial studies in this area identified a peptide from type II adenylyl cyclase that binds to $G\beta\gamma$ and blocks $G\beta\gamma$ -dependent regulation of multiple, $G\beta\gamma$ -regulated effectors [98]. The authors used a molecular modeling and chemical crosslinking approach to identify the binding site for this peptide on $G\beta\gamma$ subunits [99, 100]. This binding site mapped to a surface near the $G\alpha$ switch II-binding site on $G\beta\gamma$ subunits and correlates well with the mutagenic mapping analysis of ACII contacts on $G\beta$.

A similar approach was used to map interaction sites between PLC β 2 and $G\beta\gamma$. Initial analysis indicated that a protein fragment containing a region of the catalytic domain could block $G\beta\gamma$ -dependent PLC β 2 activation in transfected COS cells and bound to purified $G\beta\gamma$ *in vitro* [101]. Further analysis with

peptides and chemical crosslinking mapped the binding site for this peptide to two sites on G protein $\beta\gamma$ subunits, one in the switch II binding region and another at the amino terminus of the G β subunit near cysteine 25 (Fig. 1B, spacefilled CPK) [42, 102, 103]. Crosslinking to both of these sites was blocked by preincubation by intact PLC β 2 or PLC β 3 [102]. This indicated that the amino terminus of G β may function as an effector binding site. Mutagenesis of this site in the G β subunit to disrupt PLC interactions actually potentiated G $\beta\gamma$ -dependent activation of PLC β 2, suggesting that binding of this site to PLC β 2 inhibits PLC activity [103]. Interestingly, this site plays a role in activation of PLC β 2 in the presence of AGS8, as will be described in a later section. The observation that the amino terminus of G β is an important interaction site in mammals awaits confirmation by other laboratories, but it correlates directly with an effector binding site identified in yeast G $\beta\gamma$ subunits [104, 105].

Another approach to examining effector binding surfaces on G $\beta\gamma$ and regulation of target molecules has been to use peptides from G β subunits and test them in effector regulation assays. Peptides from different blades of the G β propeller were shown to inhibit G $\beta\gamma$ dependent regulation of type II adenylyl cyclase [99] or PLC β 2 while others stimulated PLC β 2 independent of G $\beta\gamma$ [106]. This led to the concept that there are distinct effector-binding and signal-transfer surfaces on G $\beta\gamma$. This is based on the hypothesis that blocking peptides correspond to binding surfaces that contribute to the energetics of the G $\beta\gamma$ -target binding, but are not involved in altering target activity. On the other hand, activating peptides from G $\beta\gamma$ have been proposed to represent signal transfer surfaces that mediate the activation of the effector. This interesting concept remains to be developed further with mutagenic analysis of intact G $\beta\gamma$ subunits.

Protein Interaction “Hot Spot” on G $\beta\gamma$

Identification of the specific amino acids in G $\beta\gamma$ involved in individual target recognition does not explain the molecular basis for G $\beta\gamma$ -dependent recognition of diverse effector structures. Various G $\beta\gamma$ binding motifs within effectors have been proposed [98] but it has become clear that there is no single consensus sequence or structural motif that mediates binding to G $\beta\gamma$. As an approach to understanding this, G $\beta\gamma$ subunits were used as targets in a random peptide phage display screen in an attempt to identify consensus sequences for binding to distinct surfaces on G $\beta\gamma$ [107]. Multiple, distinct peptides were identified that apparently bound to the same surface on G $\beta\gamma$ based on competition and mutational analysis. This result, where large protein surfaces are subjected

to selection in naïve random peptide-binding screens, and only a small portion of the overall surface mediates binding of diverse sets of peptide sequences, is indicative of a preferred protein binding surface [108, 109]. Combining these data with alanine scanning mutagenesis and structural analysis has led to the concept of energetic “hot spots” that provide key energetic residues for binding at a protein-protein interface, but also have intrinsic physical-chemical characteristics that are optimal for mediating multiple protein-protein interactions [109]. Some characteristics of these surfaces are flexibility and the opportunity for mediating multiple types of chemical interactions (ionic, hydrophobic) without strict geometric requirements for binding [110]. In this way a single binding site can accommodate multiple structural and chemical motifs.

Crystallographic determination of the structure of a phage display selected peptide (SIGK) bound to G $\beta\gamma$ identified the preferred binding surface as a site corresponding to the G α subunit switch II binding region on G β [111]. Alanine substitution of all of the amino acids within 6Å of this peptide binding site defined amino acids required for peptide binding. Each of these alanine substituted mutants was then tested for ability to affect binding of other peptides identified in the original phage display screen. Each of the peptides had a unique pattern of requirements for interactions with specific amino acids within the binding site. This demonstrated that the “hot spot” has the inherent ability to bind multiple binding sequences with unique sets of interactions that can be exploited by natural binding partners and suggests a mechanism for G $\beta\gamma$ interaction with multiple different sequences and structures.

Mechanisms for effector regulation by G $\beta\gamma$

In the previous section, modes of binding and recognition of targets by G $\beta\gamma$ were discussed, but how G $\beta\gamma$ -binding translates into alterations in functional activity of downstream targets has also been investigated by multiple laboratories. Two general mechanisms for effector regulation by G $\beta\gamma$ depend on whether the target is cytosolic or membrane bound. In the case of cytosolic proteins such as PLC β 2 or GRK2, whose substrates are localized to the plasma membrane, a potential mechanism for activation is recruitment to the plasma membrane by membrane-bound G $\beta\gamma$. For other targets, such as adenylyl cyclases or GIRK channels, that are transmembrane proteins, regulation must occur through conformational alteration. While many effectors are activated by G $\beta\gamma$, the potential mechanisms for regulation of each of these

are too numerous to be discussed here. GRK2 and GIRK regulation will be discussed briefly because mechanisms of activation of GRK2 and GIRK have been well studied and represent examples of either translocation-based or allosteric regulation. The mechanism for activation of PLC β by G $\beta\gamma$ is less clear and may, in fact, be regulated by both translocation and allosteric regulation, as will be discussed in greater detail.

Activation of GRK2 by G $\beta\gamma$

An example of an enzyme whose activity is regulated by G $\beta\gamma$ -dependent translocation is GRK2. GRK2 is normally cytoplasmic and there is strong evidence that, during receptor activation, free G $\beta\gamma$ subunits are released that provide a binding site for GRK2. In cooperation with phosphatidylinositol 4,5 bisphosphate (PIP₂), G $\beta\gamma$ subunits recruit GRK2 to the membrane, where it can interact with and phosphorylate activated GPCRs [112]. In addition to its role in membrane recruitment, G $\beta\gamma$ could allosterically modulate GRK2 function. Complexes between G $\beta\gamma$ and GRK2 have been crystallized and the structures solved [15, 96]. Since the GRK2 structure in the absence of G $\beta\gamma$ was not solved, it could not be determined whether G $\beta\gamma$ -binding alters the structure of GRK2. Biochemical analyses suggest subtle rearrangements of the GRK2 structure upon G $\beta\gamma$ -binding, but the functional significance of these alterations are not clear [97].

Activation of GIRK channels by G $\beta\gamma$

In the case of all membrane bound proteins such as ion channels and adenylyl cyclases the activation mechanism requires structural alterations rather than translocation. No direct structural data yet exists that demonstrates specific alterations of effector conformation upon G $\beta\gamma$ binding. For G $\beta\gamma$ -dependent regulation of GIRK, a combination of mutagenic analysis, biophysical studies of channel properties, and homology modeling based on a bacterial voltage-dependent K⁺ channel, have been used to develop a proposed mechanism for G $\beta\gamma$ -dependent activation. The model suggests that G $\beta\gamma$ binding to an intracellular soluble domain of the channel strengthens interactions between the channel and PIP₂ and alters the position of a helix at the mouth of the conductance pore to increase the activity of the channel [113, 114]. For this and other targets the details of conformational changes that occur upon G $\beta\gamma$ binding are unknown and await detailed atomic level structural determination of an effector with and without bound G $\beta\gamma$.

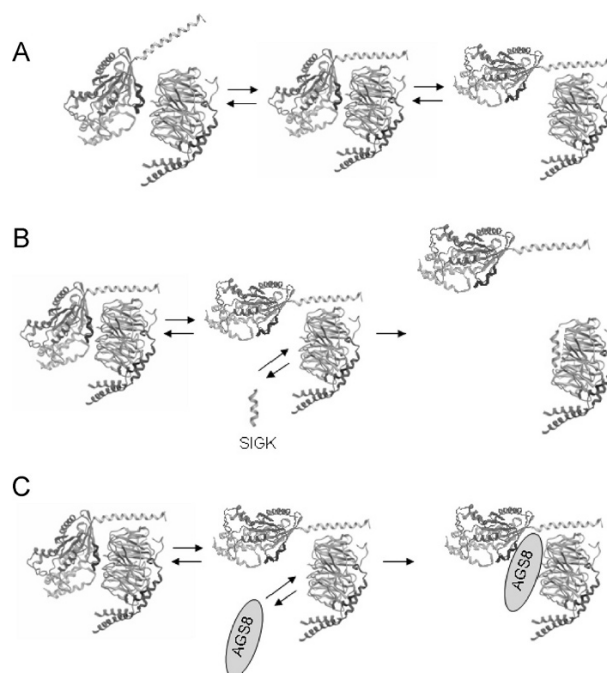


Figure 4. Models for “breathing” of G α /G $\beta\gamma$ interfaces and non-receptor dependent activation mechanisms. (A) In the center is a ribbon diagram depicting G α binding to G $\beta\gamma$ showing the two components of a bivalent interaction of G α with G $\beta\gamma$, the G α N-terminal helix interaction with the side of blade one of the G β propeller and the G α switch II interaction with the top of the G β propeller (see Fig 1.). In this model either of these two interfaces can open and close in rapid equilibrium without subunit dissociation. Only when both contacts are broken simultaneously can subunit dissociation occur. (B) SIGK-dependent subunit dissociation. In this model when the G α switch II G β interfaces open up, SIGK can bind and prevent closure of this interface, resulting in an enhanced rate of subunit dissociation. (C) AGS8-dependent G protein activation. Similar to, B., when the G α switch II G β interface opens AGS8 binds, but since AGS8 can bind to G α and G β , the bivalent interaction of G α with the complex is maintained and subunit dissociation does not occur.

Activation of PLC β by G protein $\beta\gamma$ subunits

Two independent analyses examined whether translocation is necessary for G $\beta\gamma$ -dependent activation of PLC activity. Both found that PLC β has an intrinsic capacity to bind to membrane surfaces that is independent of interactions with G $\beta\gamma$ subunits [115, 116]. In these experiments, G $\beta\gamma$ subunits did not alter the proportion of PLC associated with membrane surface but, at the same time, increased PLC activity. This indicates that one mechanism for activation of PLC isoforms is to alter its enzymatic activity either through conformational alteration of the active site or modulating the orientation of PLC with respect to the membrane surface.

Key to understanding how G $\beta\gamma$ activates PLC that is bound at the membrane is to understand the mode of interaction of G $\beta\gamma$ with PLC. Structures of PLC δ 1 and PLC β 2 have been solved that provide a detailed picture of the domain organization of these enzymes

(Fig. 5A and B) [117, 118]. PLC δ and PLC β 2 share very similar domain structures. Both contain an N-terminal pleckstrin homology domain followed by an EF hand domain, conserved X and Y domains that comprise the catalytic domain and a C2 lipid binding domain. In PLC β 2 the C2 domain is followed by an extended C-terminal domain that interacts with G α_q GTP. The PH domain of PLC δ was deleted in the expressed protein used to solve the PLC δ structure and the C-terminal extension beyond the C2 domain was removed in the protein used to solve the PLC β 2 structure. Early biochemical studies indicated that deletion of the PLC β 2 C-terminus eliminates regulation by G α_q without affecting G $\beta\gamma$ -dependent regulation [119]. While a structure has been solved for a Rac-PLC β 2 complex, no structural data exists as yet for the G $\beta\gamma$ /PLC β complex. On the other hand, biochemical approaches have yielded information about the nature of G $\beta\gamma$ -PLC interactions. Two sites for interaction of G $\beta\gamma$ on PLC β 2 have been proposed, one on the catalytic domain and one on the PH domain. Here, the data supporting these two sites will be presented and the implications with respect to regulation of PLC enzymatic activity by G $\beta\gamma$ will be discussed.

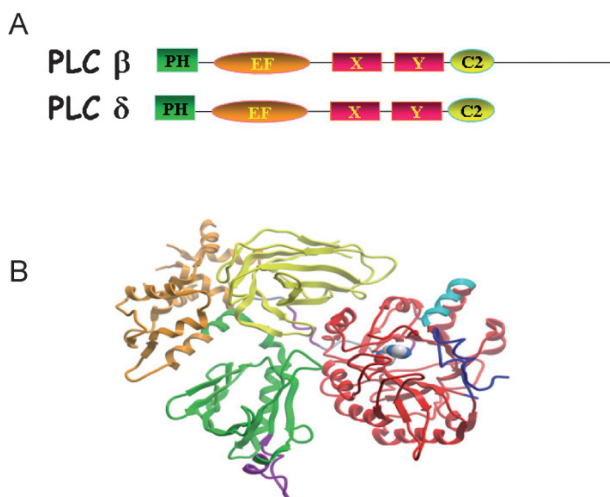


Figure 5. (A) Domain organization of PLC δ and PLC β . Pleckstrin homology domain (PH), EF hand domain (EF), catalytic domain (X and Y), C2 domain (C2). (B) Ribbon representation of PLC β 2 (from coordinates 2FJU) with domains color coded as for A: in turquoise, the helical region of the PLC β 2 catalytic domain (574–583) found to interact with G $\beta\gamma$; in dark blue, the X–Y linker domain that caps the enzyme active site; in purple, a region of the PH domain important for G $\beta\gamma$ -dependent PLC activation; in CPK spacefill, the catalytic histidine required for PIP $_2$ hydrolysis.

The first evidence that the catalytic domain could interact with G $\beta\gamma$ came from a screen of fragments of PLC β 2 for their ability to compete for PLC β 2 activation by G $\beta\gamma$ in transfected tissue culture cells

[101]. Two overlapping fragments from the catalytic Y domain of PLC blocked activation by G $\beta\gamma$ or a G $_i$ coupled C5A receptor but not the G $_q$ coupled α_1 -adrenergic receptor. A GST fusion protein comprising a portion of one of these fragments, L580–V641 within the conserved Y domain, bound directly to purified G $\beta\gamma$ *in vitro*, demonstrating a direct interaction between the catalytic domain of PLC β 2 and G $\beta\gamma$. To further narrow down the interaction region, examination of a homology model of PLC β 2 based on the structure of PLC δ identified surface exposed regions likely to be accessible to G $\beta\gamma$ [102]. Overlapping peptide fragments corresponding to these exposed regions were synthesized and shown to inhibit G $\beta\gamma$ -dependent activation of PLC β 2 in a purified system leading to identification of E574–K583 as a G $\beta\gamma$ binding region on PLC β 2 (Fig. 5B, light blue helix). Direct interaction of these peptides from the PLC β 2 catalytic domain with G $\beta\gamma$ was confirmed by chemical crosslinking to both G β and G γ in a manner that was competed with excess PLC β 2 or PLC β 3 holoenzyme [102]. To confirm that this region was important for PLC β 2 activation in the context of the PLC β 2 holoenzyme, triple alanine substitutions in the PLC β 2 E574–K583 helix inhibited activation of PLC β 2 by G $\beta\gamma$ subunits with minimal effects on PLC basal enzymatic activity [103]. Finally, triple alanine mutation of E574, L575 and K576 disrupted direct binding of purified PLC β 2 to G $\beta\gamma$ [120]. Together, these data strongly suggest that this region of the catalytic domain is involved in direct interactions with the G $\beta\gamma$ subunit and that interaction of G $\beta\gamma$ with these amino acids regulates PLC β 2 activity.

In support of the idea that the pleckstrin homology domain confers binding and activation by G $\beta\gamma$ is the observation that the isolated PH domain from PLC β 2 interacts with G $\beta\gamma$ on membrane surfaces as detected by fluorescence resonance energy transfer [121]. A second key observation is that splicing of the PLC β 2 PH domain onto PLC δ confers the ability of PLC δ to be activated by G protein $\beta\gamma$ subunits [122]. This chimeric PLC bound to lipid membranes with properties similar to PLC β 2, suggesting the activation involved conformational activation rather than membrane translocation. Point mutations in the PH domain of the chimera inhibited G $\beta\gamma$ -dependent activation. On the other hand, in chimeras of PLC β 2 with the PH domain of PLC β 1, there is no substantial loss in activation of the enzyme by G $\beta\gamma$ despite the fact that PLC β 1 is not activated by G $\beta\gamma$ [123]. This suggests that domains other than the PH domain are required for activation of PLC by G $\beta\gamma$, consistent with the observed binding of G $\beta\gamma$ to the catalytic domain. How might G $\beta\gamma$ binding to the PH domain or the catalytic domain of PLC alter enzymatic activity, since

G $\beta\gamma$ can cause enzyme activation without enzyme translocation? Recent studies of Rac-dependent activation of PLC show that Rac can activate PLC β 2 but not PLC β 1 and that this requires interaction with the PH domain [123]. Structures of PLC β 2, with and without bound Rac2, have recently been solved [118]. Rac2 interacts with the PH domain of PLC β 2 but there is no significant conformational difference between the structures of free- and Rac2-bound PLC β 2. This indicates Rac2 may activate PLC β 2 by causing either translocation of PLC β 2 or alterations in interactions of the enzyme with the membrane. In the structures of PLC β 2 solved by Sondek's group, a linker region between the X and Y domains of the catalytic domain is folded back to occlude access of substrate to the enzyme active site (Fig. 5B). This suggests a potential mechanism for activation that involves removal of this inhibitory linker from the active site [124]. Since the linker still occludes the active site in the RacGTP-bound PLC β 2 co-structure, the investigators propose that Rac causes alterations in interactions of the active site with charged lipids in the membrane that lead to displacement from the active site. Overall, the inhibitory linker model could allow for multiple modes of protein binding to achieve increases in enzyme activity. These could involve reorientation of PLC at the membrane that would allow negatively charged lipid head groups to pull this domain from the active site, for proteins to bind directly to this region, or for proteins to bind at a distance to cause conformational alterations that relieve this constraint.

Scarlata and colleagues propose that binding of G $\beta\gamma$ to the PH domain alters the orientation of the PH domain relative to the catalytic domain, allowing the catalytic domain to productively interact with the substrate at the membrane surface [125, 126]. In favor of this hypothesis, measurements of interdomain movements of a PLC δ /PLC β chimera by FRET indicate that G $\beta\gamma$ causes alterations in interactions between the catalytic domain and the PH domain. This mechanism could be operating as an independent mechanism for PLC activation or it could work in concert with direct binding of G $\beta\gamma$ to the catalytic domain. Binding of G $\beta\gamma$ at the catalytic domain, or the PH domain, could alter interactions of the catalytic domain with the membrane that would relieve auto-inhibition or could cause displacement of the linker through conformational alterations in the protein. Further biochemical and structural analysis will be required to determine the validity of these proposed mechanisms.

Receptor-independent mechanisms for activation of G protein signaling through G $\beta\gamma$

An emerging area is non-receptor and nucleotide exchange-independent mechanisms for G protein activation [7, 127]. Some of these mechanisms involve binding of proteins to G α subunits leading to release of free G $\beta\gamma$ subunits, but other proteins and peptides have been recently found that activate G protein $\beta\gamma$ subunit signaling through direct binding to G $\beta\gamma$. Since G $\beta\gamma$ is not thought to undergo conformational changes that could lead to nucleotide exchange on G α or result in subunit dissociation, the mechanisms for action of these molecules that bind directly to G $\beta\gamma$ are not obvious. In most cases, detailed studies of these mechanisms have not been done, but some examples are discussed that shed new light on potential roles of G $\beta\gamma$ in G protein activation are discussed below.

Activation of G $\beta\gamma$ signaling by G $\beta\gamma$ binding peptides SIRK/SIGK

An instructive study is based on the observation that some of the peptides identified through phage display screening that bind to the G $\beta\gamma$ "hot spot" cause activation of G protein dependent signaling pathways in cells. SIRK peptide was discovered in a the phage display screen using G protein $\beta\gamma$ subunits as a target for binding [107]. Despite being discovered in a naïve random peptide screen, the peptide bound to a biologically relevant signaling surface, as demonstrated by its ability to block G $\beta\gamma$ -dependent PLC β 2 and PI3K γ activation *in vitro*. It did not affect G $\beta\gamma$ -dependent inhibition of adenylyl cyclase *in vitro* or inhibition of N-type Ca²⁺ channels in SCG neurons, demonstrating selectivity for inhibition of some G $\beta\gamma$ targets. A surprise came when studying the effects of cell-permeable versions (either tat-modified or myristoylated versions) of SIRK (mSIRK or tatSIRK) and a related peptide SIGK in intact cells. These peptides, predicted to inhibit G protein signaling, rapidly, potently and effectively activated the ERK/MAP kinase pathway in intact cells in a G $\beta\gamma$ -dependent manner [128]. To confirm that G $\beta\gamma$ was the target of these peptides in intact cells, the effects of mSIRK on CHO cells, transfected with mutant G β (β W332A), which does not bind the peptide, were examined. In these cells, with strong constitutive expression of G β (W332A) and G γ ₂, the expressed subunits appear to substitute for a significant proportion of the endogenous G $\beta\gamma$ complexes, and substantially inhibit mSIRK dependent ERK activation [129]. This strongly supports the idea that mSIRK activates G $\beta\gamma$ subunit signaling in intact cells by binding directly to G $\beta\gamma$ subunits. To explain this observation it was proposed that the peptide must be binding to G $\beta\gamma$ in a way that

leads to exposure of $G\beta\gamma$ signaling surfaces involved in ERK activation that are not themselves blocked by binding of the peptide. The direct $G\beta\gamma$ -binding effector responsible for ERK activation is not known, but the effector-binding surface required for activation of the ERK pathway must be different from the peptide binding site and the PLC β 2 binding site. To understand the mechanism of action of these peptides their effects on $G\alpha/\beta\gamma$ interactions were examined. Kinetic and equilibrium analysis indicated that SIRK and SIGK enhanced the rate of G protein subunit dissociation in the presence of excess GDP and the absence of GTP [10, 128]. Additionally, other peptides known to bind to $G\beta\gamma$ and compete for $G\alpha$ subunit interactions did not influence $G\alpha$ subunit dissociation kinetics. This argues that the basis for the effect is not a strict competition for $G\alpha/\beta\gamma$ subunit interactions, since this would be expected to alter equilibrium binding without affecting dissociation kinetics of a preformed complex. The solved structure of SIGK peptide/ $G\beta\gamma$ complex showed SIGK bound to the $G\alpha$ subunit switch II-binding region on $G\beta\gamma$ [111] (See Fig. 1C and Fig. 6). This suggests that SIGK/SIRK should directly compete for $G\alpha$ binding to $G\beta\gamma$. A proposed model for how $G\beta\gamma$ could enhance subunit dissociation that can explain most of the data is depicted in figure 4B. As described previously, the $G\alpha/\beta\gamma$ switch II interface is in a dynamic state of association and dissociation (“breathing”) while overall $G\beta\gamma/\alpha$ interactions are maintained by the $G\alpha$ N-terminal helix. We propose that SIRK/SIGK can insert into this interface during this transient breathing and block this part of the $G\beta\gamma/\alpha$ interaction. The resulting dissociation rate would then only be limited by the off rate for the weakly interacting N-terminal α helix, leading to rapid subunit dissociation. This model fits much of the data and provides supporting evidence that this surface “breathes”. On the other hand, the model predicts that any peptide that binds at this interface and competes for $G\alpha/\beta\gamma$ interactions in an equilibrium experiment should enhance subunit dissociation, which is not what is observed. This discrepancy suggests the effects of the peptide involve a mechanism other than simple competition [10, 111]. Such a mechanism remains to be established.

Overall, these studies highlight a novel potential mechanism for G protein activation that could be exploited physiologically by receptors, by Activators of G protein signaling (AGS proteins), or pharmacologically.

AGS proteins. Activators of G protein signaling (AGS proteins) are a group of structurally distinct proteins discovered in a yeast-based screen for activation of the

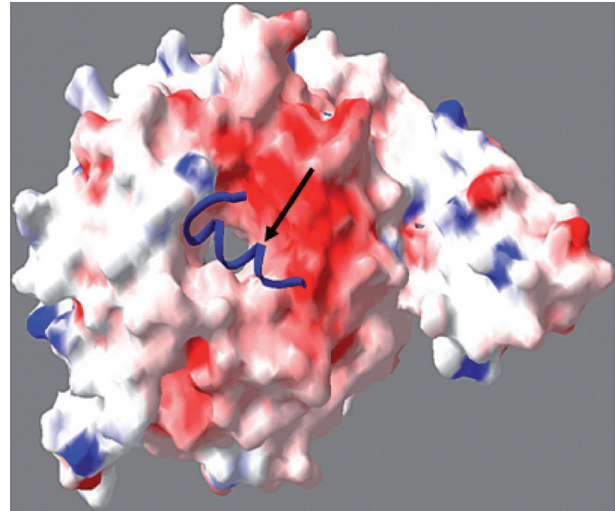


Figure 6. Surface potential representation of $G\beta\gamma$ with SIGK (blue ribbon) bound at the “hot spot”. Blue areas are positively charged, red areas are negatively charged and white areas are neutral.

$G\beta\gamma$ dependent pheromone response pathway [4, 7, 127]. The mechanisms for G protein activation by proteins that bind $G\alpha$ subunits (Class I and Class II AGS proteins) are simple to understand. For example, Class I AGS proteins include DexRas and promote nucleotide exchange on $G\alpha$ subunits, releasing free $G\beta\gamma$ through a mechanism similar to receptors. Class II AGS proteins contain a GPR or Goloco motif that binds to $G\alpha_{i/o}$ family subunits and promotes $G\beta\gamma$ subunit dissociation through a nucleotide exchange-independent mechanism leading to accumulation of free $G\beta\gamma$ subunits that can activate downstream targets. The GPR/Goloco motif in these proteins binds to the switch II region of the $G\alpha$ subunit near the interface between $G\alpha$ and $G\beta\gamma$ subunits [130]. This results in a conformational change in switch II at the $G\alpha/\beta\gamma$ interface, disrupting $G\alpha/\beta\gamma$ interactions and leading to subunit dissociation.

Class III AGS proteins that bind directly to $G\beta\gamma$ are less well investigated or understood. Since $G\beta\gamma$ is not thought to undergo significant conformational alterations, it is difficult to imagine a mechanism that does not involve binding of the AGS protein to the $G\alpha/\beta\gamma$ interface. But if the AGS protein bound to the region on $G\beta\gamma$ at the $G\alpha/\beta\gamma$ interface, it would obscure this critical signaling surface on $G\beta\gamma$ required for activation of target proteins. Thus a conundrum is presented where somehow these activating proteins that bind the $G\beta\gamma$ subunits must relieve the constraints of the GDP bound heterotrimer yet still allow $G\beta\gamma$ to signal downstream.

Some insight into the mechanism of action of these proteins comes from a recent analysis of AGS8. AGS8 was found in the yeast-based screen using a cDNA

library derived from a rat model of transient cardiac ischemia. AGS8 binds to $G\beta\gamma$ subunits but does not significantly affect $G\beta\gamma$ -dependent PLC β 2 activation when $G\beta\gamma$ is transfected into COS cells in the absence of $G\alpha$ subunits [131]. However, AGS8 relieves the inhibition of PLC seen when $G\alpha$ subunits are transfected with $G\beta\gamma$ subunits and $G\beta\gamma$ -dependent PLC activation is inhibited due to formation of the heterotrimer. The AGS8 binding site on the G protein $\beta\gamma$ subunit appears to reside at the $G\alpha/G\beta\gamma$ interface at a site that overlaps with the SIGK binding site. This observation was puzzling since AGS8 did not block PLC activation, yet amino acids at the SIGK binding surface are required for PLC activation. Another surprise is that AGS8 does not promote subunit dissociation or block $G\alpha_{i1}$ subunit binding to $G\beta\gamma$. These observations are difficult to reconcile with the SIGK data demonstrating that peptide binding at this site led to dissociation of $G\alpha$ from $G\beta\gamma$, until it was found that AGS8 could also bind to the $G\alpha_{i1}$ subunit in a nucleotide-independent manner. SIGK promotes subunit dissociation by binding at the $G\alpha/G\beta\gamma$ interface but binds only to $G\beta\gamma$, so $G\alpha$ subunits are released. AGS8 binds both $G\alpha$ and $G\beta\gamma$ resulting in retention of $G\alpha$ subunit binding in the complex (Fig. 4C). Thus, AGS8 binds to the G protein heterotrimer by binding the $G\beta\gamma$ and $G\alpha$ subunits simultaneously and does not cause dissociation of these subunits, yet it activates PLC β 2 signaling by a $G\alpha$ GDP $\beta\gamma$ heterotrimer.

In this AGS8/ $G\alpha/G\beta\gamma$ complex, the mechanism by which $G\beta\gamma$ could activate PLC β 2 is not easily explained based on our current understanding of regulation of $G\beta\gamma$ -dependent signal transduction. A critical $G\beta\gamma$ surface for signaling to PLC β 2 activation is bound to AGS8. In our model, when AGS8 binds to the "hot spot" and forms a signaling complex with $G\alpha$ and $G\beta\gamma$ subunits, the PLC β 2 inhibitory site at the amino terminus becomes a stimulatory binding site. This PLC β 2 binding site was previously identified as an inhibitory site by chemical crosslinking and mutagenesis [103] (discussed in section 7). This implies that AGS8 alters $G\beta\gamma$ conformation or orientation at the membrane to make the bound complex competent for downstream signaling. An alternative model is that AGS8 itself provides binding determinants for PLC binding in conjunction with amino acids at the $G\beta\gamma$ N-terminus that participate in PLC activation. More direct evidence to address these ideas awaits further structural investigation.

These two examples (SIGK and AGS8) of $G\beta\gamma$ -dependent, nucleotide exchange-independent, signaling mechanisms suggest additional modes of G protein activation outside of the well defined classical para-

digm for G protein activation. How these biochemically characterized mechanisms operate in a physiological context remains to be determined. With emerging evidence that receptors bind directly to G protein $\beta\gamma$ subunits these observations may also be relevant to GPCR signaling. It is possible that some receptors, in addition to causing nucleotide exchange, can also promote subunit dissociation that is mechanically independent of the nucleotide exchange process on $G\alpha$ subunits. On the other hand there is increasing evidence that under some GPCR-dependent G protein activation conditions the subunits may not dissociate [5, 6, 132]. The molecular model described for the action of AGS8 suggests potential mechanisms for non-dissociated G protein signaling complexes to promote downstream signaling. Overall, it is clear that the current simple picture of $G\beta\gamma$ as a passive participant in the G protein activation and signaling process needs revision.

NDPK phosphorylation of $G\beta\gamma$

G protein β subunits have been found to be substrates for phosphorylation in a variety of tissues [133, 134]. A model has been developed where transient high-energy phosphorylation of a histidine residue serves as a phosphate donor involved in transfer of phosphate from the $G\beta$ subunit to GDP associated with $G\alpha$ subunits leading to activation of the $G\alpha$ subunit and subsequent signaling in a GPCR-independent manner. The amino acid phosphorylated in $G\beta$ is His 266, and requires nucleotide diphospho (NDP) kinase. Direct reconstitution of phosphorylation with purified NDP kinase has not been achieved, suggesting a requirement for an additional cofactor in the reaction. The significance of this process was unclear until a recent study in cardiac myocytes suggested a role in regulation of cAMP levels [135]. In these studies a $G\beta_1$ His 266 Leu mutant was transduced into neonatal or adult cardiac myocytes where the mutant is functionally incorporated into endogenous heterotrimers replacing the endogenous subunits. Basal cAMP levels were reduced in both neonatal and adult cardiac myocytes in cells transduced with $G\beta_1$ His 266 L compared to cells transduced with wild type $G\beta_1$. Interestingly, baseline contractility was reduced by this mutant in adult myocytes without any affect on stimulation by a β -adrenergic receptor agonist. These data suggest that, in a physiological system, this receptor-independent signaling mechanism that relies on transient phosphorylation of $G\beta$, regulates baseline cAMP levels and contractility in the heart.

Physiological significance of $G\beta\gamma$ activation

G protein $\beta\gamma$ subunit-mediated activation of effectors has diverse roles in regulating of cell physiology. In excitable cells, $G\beta\gamma$ subunits released from G_i modulate membrane potential through activation of K^+ channels and inhibition of voltage gated Ca^{2+} channels. In neurons this suppresses excitability and inhibits neurotransmitter release. In atrial myocytes vagal release of acetylcholine suppresses heart rate through $G\beta\gamma$ -dependent activation of K^+ channels [74]. In migrating immune cells, chemokine receptors, such as the IL-8 receptor or CXCR4, are coupled to the release of $G\beta\gamma$ subunits from G_i [136, 137] that is critical for mediating directional chemotaxis as well as release of superoxide and other inflammatory mediators. Several mouse knockout studies implicate $G\beta\gamma$ -regulated effectors in various physiological functions; For example, in mice lacking $G\beta\gamma$ -regulated PLC β 3, morphine acting at G_i linked opioid receptors produced painkilling effects at much lower doses [138]. Genetic deletion of $G\beta\gamma$ -regulated PI3K γ resulted in decreased neutrophil migration and a reduction in inflammation [139, 140].

Activation of multiple G_i and G_q -coupled receptors, including thrombin, lysophosphatidic acid (LPA), and acetylcholine receptors, results in a mitogenic response in several cell types. MAP kinases are critical components in the growth-promoting pathways regulated by these receptors. $G\beta\gamma$ subunits indirectly activate MAP kinase, suggesting that $G\beta\gamma$ subunits may mediate the growth-promoting effects of many G protein-coupled receptors [141, 142]. Sequestering $G\beta\gamma$ in smooth muscle cells inhibits serum stimulated growth and vascular restenosis [143].

G Protein $\beta\gamma$ subunits as a target for therapeutic development

The diverse functionality of $G\beta\gamma$ signaling in cellular physiology suggests that manipulating $G\beta\gamma$ function could have significant therapeutic potential. On the other hand $G\beta\gamma$ is known to be required for G protein activation by all G protein coupled receptors, so blocking **all** $G\beta\gamma$ functions would be predicted to have side effects. The potential therapeutic usefulness of targeting $G\beta\gamma$ signaling has been investigated extensively using the carboxy terminus of GRK2 (GRK2ct) [143–147] and, to a lesser extent, with other $G\beta\gamma$ binding peptides such as QEHA [148]. GRK2ct, despite binding at the $G\alpha/\beta\gamma$ “hot spot” interface, interferes with $G\beta\gamma$ signaling to downstream targets without disrupting GPCR dependent G protein activation in general. The basis for this selectivity is unclear. This has strong implications for small mole-

cule development, indicating that a strategy that targets the $G\alpha/\beta\gamma$ interface “hot spot” could successfully block downstream $G\beta\gamma$ signaling without disrupting G protein signaling in general.

G $\beta\gamma$ and heart failure. One well studied example where GRK2ct has been used to demonstrate the therapeutic potential of targeting $G\beta\gamma$ is in cardiac function and failure. One of the characteristics of heart failure is the loss of β -adrenergic receptor (β AR)-dependent cardiac reserve. A prominent hypothesis is that the underlying mechanism involves an increase in the activity of GRK2, a kinase that phosphorylates and desensitizes the β AR as well as other GPCRs. During progression to heart failure, chronically elevated catecholamine levels lead to chronic stimulation of β AR resulting in chronic desensitization of the receptor by GRK2. GRK2 activity is controlled by $G\beta\gamma$ which, upon GPCR activation, is released and recruits GRK2 to the receptor, leading to its phosphorylation and desensitization. GRK2ct blocks this recruitment and enhances β AR function. A seminal study indicating successful application of this strategy was the demonstration that transgenic cardiac over-expression of GRK2ct in mice increased cardiac performance in response to β AR stimulation [147]. Later, it was demonstrated that cardiac over-expression of GRK2ct in murine models of heart failure dramatically rescued cardiac function [146] and expression of GRK2ct in cardiac myocytes isolated from biopsies of human heart failure patients significantly improved contractile function [149]. These and a plethora of other studies have shown the value of blocking $G\beta\gamma$ signaling function in improving cardiac functions in disease [145].

G $\beta\gamma$ and inflammation. Chemokines and chemokine receptors have been the subject of anti-inflammatory pharmaceutical development [150–156]. A potential problem is the overwhelming complexity of these signaling molecules (multiple chemokines, chemokine receptors, and redundancy) making it difficult to know which specific receptors to target for conditions such as arthritis. Polychemokine [157] or combinations of different chemokine [158] antagonists have been suggested, but there may be chemokines that act as an agonist at one receptor and an antagonist at another [159]. Of recent interest is the demonstration that deletion of PI3K γ in mice inhibits neutrophil migration in response to chemoattractants and inhibits inflammation. PI3K γ activity is directly regulated by $G\beta\gamma$ released from chemokine and chemotactic peptide receptors and is relatively selectively expressed in monocytic cells, suggesting that blocking

G $\beta\gamma$ -regulation of PI3K γ could be an effective strategy for treating inflammatory diseases that may overcome the necessity to target multiple chemokine receptors [139]. In a related study it was demonstrated that deletion of PI3K γ protected apoE^{-/-} mice from development of atherosclerosis, potentially through disabling macrophage migration and inflammatory functions [160]. An alternate approach that is currently being investigated is specific pharmacological targeting of PI3K catalytic activity with inhibitors that are relatively selective for PI3K γ relative to other PI3K isoforms [161]. In this approach blocking PI3K γ would circumvent the problems associated with chemokine receptor redundancy by blocking a common signaling target of chemokines. An alternate approach may be to inhibit G $\beta\gamma$ -dependent activation of PI3K γ , which would selectively block PI3K γ relative to other PI3K isoforms since these isoforms are not regulated primarily by G $\beta\gamma$.

These are just two of multiple examples where G $\beta\gamma$ binding proteins or peptides have been used to demonstrate the involvement of G $\beta\gamma$ in pathology and disease and where inhibition of G $\beta\gamma$ with these agents has ameliorated the pathology. Other examples include vascular restenosis [143], drug addiction [162] and prostate cancer [144].

Small Molecule Targeting of G $\beta\gamma$

Screening the NCI diversity library against the “hot spot”

Given that G $\beta\gamma$ may be a suitable target for therapeutic development, our laboratory screened for small molecules that could be used *in vivo* to inhibit G $\beta\gamma$ signaling. The “hot spot” was targeted because this is a major site of protein-protein interactions and our studies with peptides suggested that differential modulation of G protein signaling functions could be accomplished by binding to this site. In this screen a number of molecules that bound to the “hot spot” were identified based on the ability to compete with SIGK binding and bound with IC₅₀ values ranging from 0.2 to 50 μ M [107]. More recently direct binding of M119 and a related molecule, gallein, to G $\beta\gamma$ was examined by surface plasmon resonance [163]. In the SPR assay, gallein bound to immobilized G $\beta\gamma$ with an apparent K_d that was similar to the IC₅₀ value obtained for M119- or gallein-dependent inhibition of SIGK binding. Structurally related molecules that did not compete for SIGK peptide binding did not bind in the SPR assay, confirming the specificity of the SPR assay for active compound binding.

Protein-protein interactions

While the compounds identified in the screen inhibited interactions between G $\beta_1\gamma_2$ and the peptide SIGK, it is thought to be relatively difficult for small compounds to disrupt true protein-protein interactions. Thus, selected compounds were tested for their ability to disrupt protein interactions with *bona fide* G $\beta\gamma$ binding partners: G α_{i1} and effectors. The overall G α_{i1} - $\beta\gamma$ interaction surface spans 1800 Å² [11, 12] and the dissociation constant (K_d) for G α_{i1} binding to G $\beta\gamma$ is approximately 1 nM [164]. One compound, M119, potentially inhibited G α_{i1} binding to G $\beta_1\gamma_2$. M119 and other compounds inhibited binding of effector molecules to G $\beta\gamma$ both in direct binding assays and in functional reconstitution experiments.

Based on the selectivity of phage displayed peptides that bound to the “hot spot”, and the idea that each target has a unique “foot print” on the G $\beta\gamma$ surface, it was predicted that different small molecules, binding in different ways to the “hot spot”, would have distinct effects on individual G $\beta\gamma$ -target interactions. Initial support for this idea came from comparative analysis of M119 and M201 with respect to target interactions. While both compounds were able to compete for G $\beta\gamma$ -GRK2 interactions with similar potency, M119, and not M201, blocked G $\beta\gamma$ -dependent activation of PLC β_2 *in vitro*. This indicates that both compounds can bind to G $\beta\gamma$ but have differential effects on G $\beta\gamma$ protein-protein interactions. Other compounds also have similar selectivity characteristics (unpublished data).

Analysis of compound efficacy and selectivity in intact cells

Based on the biochemical selectivities described in the previous section, it would be predicted that the compounds should be able to differentially modulate G $\beta\gamma$ -dependent signaling processes downstream of GPCRs. This was tested in neutrophils where G $\beta\gamma$ mediates signaling responses to chemoattractants and chemokines that are responsible for directing chemotactic migration and superoxide production involved in inflammatory responses. The pathways regulated in these cells include activation of PI3-kinase γ , PLC activation, ERK1/2 activation and GRK2 regulation. Compounds that inhibited G $\beta\gamma$ -dependent PLC and PI3-kinase activation *in vitro* were able to inhibit these pathways in neutrophils in response to chemoattractants. These compounds did not block activation of ERK1/2 by fMLP, indicating that GPCR signaling was intact and demonstrating a level of selectivity of the compounds for G protein $\beta\gamma$ subunit signaling in intact cells. Compounds shown to be selective for particular pathways *in vitro* displayed similar characteristics in cells. M201, for example, blocked GRK2 recruitment

but did not affect $G\beta\gamma$ -dependent PLC activation, while M119 was able to inhibit both, consistent with their *in vitro* properties.

***In vivo* evaluation of small molecules**

Based on the discussion of therapeutic relevance it would be predicted that compounds that inhibit $G\beta\gamma$ signaling would have predictable and potentially beneficial effects *in vivo*. Some areas with clear potential include heart failure and inflammation. As discussed earlier, knockout of PLC β 3 leads to increased potency of morphine-dependent analgesia. Since PLC β 3 is regulated by $G\beta\gamma$, one would predict that $G\beta\gamma$ -blocking compounds, if introduced into analgesic centers in the brain, would have similar effects.

PLC β 3 and Opioid-dependent antinociception: Co-administration of M119 with morphine intracerebroventricularly (i.c.v) resulted in an 11-fold increase in the analgesic potency of morphine, whereas administration of M119 alone had no effect on antinociception. Importantly, M119 also had no effect on morphine-dependent antinociception in PLC β 3^{-/-} mice. $G\beta\gamma$ may block interactions with PLC β 3 but not $G\alpha$ or other effectors such as K^+ or Ca^{2+} channels critical for the actions of opioid agonists [165]. If M119 were globally blocking $G\beta\gamma$ subunit functions, morphine-induced antinociception would have been attenuated rather than potentiated with M119 co-administration. These data highlight the specificity of M119 actions and the selective nature of M119 both *in vitro* and *in vivo*.

Neutrophil Chemotaxis and inflammation: As discussed, $G\beta\gamma$ -dependent activation of PI3K γ in neutrophils is important in directing neutrophil migration in response to chemoattractants. Activation of this receptor system leads to a gradient of PIP₃ production with enhanced accumulation at the leading edge of the cell that is important for polarizing the cells in the direction of the chemo-attractant [166, 167]. In animal models of neutrophil chemotaxis, deletion of PI3K γ results in defects in neutrophil accumulation and reduced inflammation [139, 140]. Since PI3K γ and other molecules important for chemoattractant-dependent chemotaxis are activated by $G\beta\gamma$, M119 and the related molecule, gallein were tested for their ability to inhibit chemoattractant-dependent neutrophil migration [163]. M119 and gallein significantly blunted fMLP-, but not $G\beta\gamma$ -independent GM-CSF-dependent, neutrophil migration, supporting the idea that blocking $G\beta\gamma$ -dependent signaling in neutrophils inhibits migration. Consistent with this data, gallein inhibited inflammation in a whole animal model of

inflammatory processes. In a carrageenan-induced footpad inflammation assay, intraperitoneal and oral administration of gallein inhibited inflammatory responses with a potency similar to a cyclo-oxygenase inhibitor, indomethacin. Thus, inhibiting $G\beta\gamma$ signaling with small molecules could be a novel approach to treat inflammation.

Basis for $G\beta\gamma$ targeting and selectivity by small molecules

Molecules were found that bound to $G\beta\gamma$ and selectively inhibited $G\beta\gamma$ protein-protein interactions in a limited screen of a small set of organic molecules. Two readily apparent questions that arise are: 1) What is the molecular basis for small molecule selectivity and, 2) what are the properties of the $G\beta\gamma$ “hot spot” that allow it to bind to small molecules with relatively high affinity?

With regard to the first question, one hypothesis is that small molecule selectivity is based on differential spatial occupancy of the “hot spot”. The basic premise as discussed in section 7 is that different $G\beta\gamma$ targets interact with the “hot spot” utilizing different subsets of amino acids on $G\beta$ for binding. If the small molecules occupy different spatial regions of the hot spot and the basis for their effects is steric occlusion of effector interactions, the prediction is that the compounds would have distinct effector inhibition profiles based on where they bound in the “hot spot”. An alternate hypothesis is that the chemistries of the compounds, rather than steric effects, alter target binding. For example, a compound containing a carboxylic acid moiety could introduce a negative charge at the surface that could differentially alter effector binding. Currently, direct evidence in support of either of these hypotheses is lacking, but identification of the binding modes for each compound, either by mutagenesis or structural methods, should provide some illumination.

A prevalent idea is that finding small molecules that bind at protein interaction surfaces to disrupt protein-protein interactions is difficult. In contrast either to active sites of enzymes or cell surface receptors, protein-protein interaction surfaces have been thought to be generally flat and may not have a clearly defined three dimensional binding pocket that can support the multiple interactions in three dimensions that are likely required for high affinity binding of a small molecule to a protein [168, 169]. A second issue is that protein interfaces are generally large, often greater than 1500 Å², suggesting that occupation of a small portion of this surface with a small molecule might not disrupt enough of the binding energy to disrupt the interaction. Increasingly, however, examples of small molecules that bind to crevasses in

protein interaction surfaces and disrupt protein-protein interactions are emerging [170]. In the case of $G\beta\gamma$, because of the hole in the middle of the β -propeller, the protein-protein interaction surface is concave rather than flat, providing 3-dimensionality to the surface that may provide more binding interactions for small molecules. This interaction surface is also a “hot spot”, as previously discussed, that contributes a large portion of the binding energy for $G\beta\gamma$ -target interactions. Thus, binding of small molecules to this surface would be predicted to disrupt this critical binding site and inhibit interactions between $G\beta\gamma$ and its effectors. This combination of having a good binding site for small molecules overlapping with a critical protein interaction surface may not be coincidental and could reflect the inherent “binding” capability of this site.

Concluding remarks

G protein $\beta\gamma$ subunits are central participants in G protein signaling, scaffolding receptors, G protein α subunits, and effectors. As investigations of this protein continue to move forward, its importance in a myriad of physiological functions is increasingly appreciated. Despite years of investigations by many investigators, novel and interesting properties, mechanisms and functions for these proteins continue to emerge, and this will likely continue. Some of the major questions still remaining concern how signaling specificity is maintained with such a promiscuous signaling protein and what is the molecular significance of the very large isoform diversity of these $G\beta\gamma$ combinations. Given the biological potential of these proteins as therapeutic targets, answering these questions could contribute significantly to development of novel pharmacologic approaches to therapeutics for a number of important diseases.

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