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The Role of Gβγ Subunits in the Organization, Assembly, and Function of GPCR Signaling Complexes

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Abstract

The role of Gβγ subunits in cellular signaling has become well established in the past 20 years. Not only do they regulate effectors once thought to be the sole targets of Gα subunits, but it has become clear that they also have a unique set of binding partners and regulate signaling pathways that are not always localized to the plasma membrane. However, this may be only the beginning of the story. Gβγ subunits interact with G protein–coupled receptors, Gα subunits, and several different effector molecules during assembly and trafficking of receptor-based signaling complexes and not simply in response to ligand stimulation at sites of receptor cellular activity. Gβγ assembly itself seems to be tightly regulated via the action of molecular chaperones and in turn may serve a similar role in the assembly of specific signaling complexes. We propose that specific Gβγ subunits have a broader role in controlling the architecture, assembly, and activity of cellular signaling pathways.

Keywords

Signaling specificity; G protein heterotrimers; complex assembly; G protein–coupled receptors (GPCRs); scaffolding proteins

INTRODUCTION

In recent years, the roles of heterotrimeric G proteins in cellular signaling have dramatically grown. Gβγ subunits, once thought only to be negative regulators of Gα-dependent signaling have come into their own as mediators of receptor signaling In this article, we discuss the diverse and rapidly expanding roles that Gβγ subunits play in cellular signaling. First, we describe the current view of G protein signaling and the role of Gβγ in modulating classical G protein–coupled effector pathways. We also present information regarding the specificity of different Gβγ pairs with respect to receptor and effector interactions. Next, we discuss more recent studies focusing on novel Gβγ signaling partners and new roles at distinct subcellular

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locations, as well as the assembly of Gβγ subunits and the roles that molecular chaperones may play in orchestrating assembly of specific Gβγ combinations. Finally, we outline a broader role for Gβγ subunits as organizers of subsequent assembly and trafficking of G protein–coupled receptor (GPCR)-based complexes. We hope to convince the reader that targeting individual Gβγ subunits and/or the chaperones involved in their assembly might be a new approach to modulating cellular signaling in a number of diseases.

G PROTEIN–COUPLED RECEPTORS AND G PROTEIN SIGNALING

As the largest family of cell surface receptors, GPCRs recognize and respond to a large array of cellular modulators, including hormones, neurotransmitters, lipids, nucleotides, peptides, ions, and photons (1,2). Information is transmitted by ligands released in intracrine, autocrine, paracrine, and endocrine signaling loops. In addition to being activated by agonists that bind to receptors, GPCR signaling systems also demonstrate spontaneous, ligand-independent activation and certain genetic mutations that can result in constitutive signaling, leading to diseases such as familiar precocious male puberty (3), retinitis pigmentosa (4), and thyroid adenomas (5). GPCRs regulate many critical physiological functions in eukaryotic cells and are major targets for numerous therapeutic drugs (6).

The most common transducers for GPCR signaling are heterotrimeric G proteins that switch between guanosine diphosphate (GDP)-bound and guanosine triphosphate (GTP)-bound states. There are 15 Gα, 5 Gβ, and 11 Gγ subunits, as well as a number of splice variants, in humans (7). Heterotrimeric G proteins can be broadly grouped into four families based on sequence homology and functional similarities of their α subunits: G_s, G_i, G_q/G_{11}, and G_{12} (8,9). In the inactive state, the GDP-bound Gα subunit is associated with the obligate Gβγ dimer, which slows the rate of spontaneous GDP release by Gα acting as a guanine-nucleotide dissociation inhibitor (GDI) (10,11). Agonist-bound or constitutively active receptors act as guanine-nucleotide exchange factors (GEFs), promoting the release of bound GDP by Gα. The nucleotide-free Gα then binds GTP, which is present in molar excess over GDP in cells. When purified, G proteins can be activated by nonhydrolyzable GTP analogs in the presence of detergent and Gα can be dissociated from Gβγ by hyperphysiological concentrations of magnesium. These observations led to the classic model that depicts subunit dissociation accompanying G protein activation in vivo. However, accumulating evidence reviewed in (12), suggests that GTP-mediated activation triggers a conformational change without necessarily causing subunit dissociation. Some studies have indicated that conformational changes within the heterotrimeric G protein (13–16) rather than subunit dissociation per se are sufficient to reveal distinct effector interacting surfaces (reviewed in 12). This model is also supported by chemical (17) and molecular (18) cross-linking studies that showed physically tethered heterotrimers are still functional. Two recent studies appear to support the original hypothesis (19,20), although matters are made more complicated by evidence that both scenarios are operative (21,22). It is conceivable that the outcome of G protein activation depends on the particular receptor, G protein heterotrimer, effectors, and regulatory molecules contained in an individual signaling complex. These latter components might include arrestins, protein kinases, and phosphatases that could regulate the duration and intensity of signal transduction, and change the type of signal or the subcellular localization where different signals might be delivered. In this review, we discuss the roles associated with Gβγ subunits in cellular signaling per se, but we also address the broader role they may serve in organizing the assembly and trafficking of GPCR signaling complexes.

Gβγ DIMERS AND CLASSICAL GPCR SIGNALING PATHWAYS

Gα and Gβγ subunits relay signals to a wide range of downstream effectors, including adenylyl cyclase isoforms, phospholipases, ion channels, protein tyrosine kinases, and MAP kinases,

among others (23–27). Originally, the Gβγ dimer was thought to be necessary mainly for the inactivation of Gα subunits, allowing them to reassociate with the receptor for subsequent rounds of signaling. Thus, Gβγ was viewed as a negative regulator of Gα signaling that increased the signal-to-noise ratio by preventing spontaneous Gα activation in absence of receptor stimulation (reviewed in 28). The first evidence for a direct role of Gβγ dimers in signaling came in 1987 when purified Gβγ subunits from bovine brain were shown to activate a cardiac potassium channel normally activated by muscarinic cholinergic receptor following acetylcholine release (23). We now know that Gβγ subunits can modulate many effectors (Figure 1) via direct interaction that are also regulated by Gα subunits, including the aforementioned Kir3 potassium channels, phospholipase Cβ (29,30), adenylyl cyclase isoforms (31), and voltage-gated calcium channels (32). These Gβγ functions have been the subject of several recent reviews that provide greater detail (8,33,34).

SPECIFICITY OF Gβγ-MEDIATED SIGNALING

The $G\beta_{1–4}$ subunits share 78–88% identity over their approximately 340 amino acid sequences (35). Gβ5 is structurally distinct from the other Gβ subunits (see below), sharing only 51–53% sequence identity with the other Gβ subunits and containing an additional 13 amino acid residues. The crystal structure of Gβγ has been elucidated (36,37). Gβ contains seven distinct β-sheet domains arranged like the blades of a propeller. The N-terminus of the Gβ subunit contains a 25-residue α-helix and a loop (residues 26–45) that connects this helix with the propeller blades. Each propeller blade is a four-stranded antiparallel β sheet. There are also seven WD motifs in Gβ. Gβ and Gγ subunits share nearly 100% amino acid identity among different mammalian species. The Gγ subunits are more structurally diverse than the Gβ subunits. They share between 27 and 76% sequence homology. If divided into subfamilies, the sequence homology among family members is much higher. For example, $G\gamma_1$, $G\gamma_{11}$, and $G\gamma_{13}$ share 62–73% homology (35). Gγ subunits undergo several post-translational modifications, including isoprenylation of an invariant cysteine residue in a conserved CAAX motif at the carboxyl end of the protein, which are important for membrane localization of Gβγ. In most Gγ subunits, the X in the CAAX sequence is a leucine, which allows the addition of a geranylgeranyl group by a thioester linkage, although in some ($G\gamma_1$, $G\gamma_8$, $G\gamma_{11}$ and $G\gamma_{13}$) X is a serine, which permits the addition of a farnesyl group (38–40).

Early studies regarding signaling specificity mainly targeted Gα because it possesses the switch that activates and deactivates signal transduction through guanine nucleotide exchange and hydrolysis, respectively. It has been shown that particular combinations of heterotrimeric G proteins are responsible for coupling receptors to particular effectors (41–51). Evidence has accumulated for an increasing role for Gβγ subunits in a heterotrimeric G protein in direct interactions with receptors and effectors (8). The particular constituents of the G protein heterotrimer affect both specificity and efficiency of signal transduction. Hundreds of receptors are known that interact with G proteins to mediate their function. If all Gβ subunits could interact and randomly form dimers with all Gγ subunits, there would be 60 possible combinations. Most Gβ and Gγ subunits can form dimeric pairs in vitro but some exceptions have been reported. The highly conserved sequences, preserved functions, and specific tissue expression patterns indicate unique or specialized roles in signal transduction pathways. For example, $G\gamma_1$ expression is restricted to retinal rod cells, and the $G\beta\gamma_1$ dimer is highly preferred by rhodopsin compared with other receptors (52). $G\beta_1$ can interact with all Gγ subunits, and $G\beta_2$ can combine functionally with $G\gamma_2$ but not $G\gamma_1$ (53,54). The region of Gγ that defines specificity for the interaction with $G\beta_1$ or $G\beta_2$ subunits has been localized to a 14–amino acid segment (55). Specific Gβγ interactions can be restricted by differential expression in particular cell types, with the extreme examples of the visual system and vasculature (56) as lower and upper limits for diversity. It is likely that unique heterotrimer combinations may be functionally distinct, as well. It is possible that there may also be coordinate regulation of Gβ or Gγ subunit

biosynthesis, spatiotemporal aspects to localization, or formation of particular pairs although this has never been tested directly. What are the functional consequences of this diversity?

The combinatorial association of the different G protein subunits could contribute to the selectivity that is needed to generate the broad range of signals transmitted by G proteins. However, it has been difficult to demonstrate that subunit diversity plays an important role in determining the specificity of signaling. Biochemical approaches have revealed modest differences among the various subunit combinations (8) although genetic approaches have been more successful. As discussed above, an antisense approach was originally used to demonstrate that various GPCRs (somatostatin and muscarinic cholinergic receptors) use different G protein combinations to modulate a calcium channel in pituitary cells (48). Also, a ribozyme approach demonstrated that β–adrenergic and prostaglandin receptors require G proteins of various subunit compositions to stimulate adenylyl cyclase activity (41,42). The first conclusive report that a receptor recognizes a specific set of G protein subunits in an organism came from a gene-targeting approach that showed that the D1 dopamine receptor requires a G protein containing $G\gamma_7$ to stimulate adenylyl cyclase activity in the striatum (44). For most receptors, the G protein subunit combination required to generate specific signaling events in vivo is still unknown. These and other studies are summarized in Table 1.

Gβγ subunits essentially function as a single entity. Specific point mutations in the Gβ1 subunit (S67K, T128F, S98T) alter Gβγ regulation of the Kir3 channel without blocking other Gβγ-mediated functions, such as activation of the PLCβ2 (57). However, it is clear that both Gβ and Gγ are important in effector regulation. The first evidence of a functional role for the Gγ subunit in activation of an effector came in 2003 when it was shown that the C-terminal extremity of the $G\gamma_2$ was needed for the activation of Kir3 channels (58). Gβγ subunits also interact directly with a number of GPCRs, including M2 and M3 muscarinic cholinergic receptors (59,60), β-adrenergic receptors (13), and rhodopsin (61,62). Gβγ subunits are important in recruiting GRKs to activated receptors (59). Further, the ability of Gβγ to interact with receptors is influenced by the isoform of Gγ present in the dimer, for example, $G\beta_1\gamma_1$ dimers can support the binding of $G\alpha_t$ to rhodopsin although $G\beta_1\gamma_2$ cannot (52,63). Following receptor activation, Gβγ dimers can translocate from the plasma membrane to the Golgi apparatus. The rate of this translocation is affected by the Gγ subunit type (i.e., $G\gamma_1$ translocates more rapidly than $G\gamma_5$). Because $G\gamma_1$ is farnesylated and $G\gamma_5$ is geranylated, prenylation-deficient mutants were used, but the results indicated that the type of prenyl group present was not sufficient to explain the differences observed. However, the translocation properties of the $G\beta_1\gamma_1$ and $G\beta_1\gamma_5$ complexes were dramatically altered when their C-termini were mutated (64).

$G\beta_5$, THE BLACK SHEEP OF THE FAMILY

The gene for $G\beta_5$ was cloned in 1994 from a mouse brain library and appears to be enriched in the brain compared with other tissues (65,66). It is able to associate functionally with numerous Gγ subunits (35,67). $G\beta_5$ is associated with plasma membrane but is also present in the soluble fraction (30–50%) in brain extracts in contrast to other Gβ subunits, which are mainly associated with the membrane (68). This indicates that $G\beta_5$ might interact with a number of novel binding partners. A Gγ-like (GGL) domain was shown to foster the interaction of multiple proteins with $G\beta_5$. Indeed, it was shown that RGS11, which possesses a GGL domain, could interact with $G\beta_5$ (69). It was subsequently shown that other RGS proteins of the R7 family could interact with $G\beta_5$ via GGL domains (70). This GGL motif interacts with $G\beta_5$ in a similar fashion as do conventional Gγ subunits (36,70), although other Gβ subunits are unable to interact with RGS6, RGS7, or RGS11. GGL-containing RGS proteins associate with $G\beta_5$ in a similar fashion as do conventional Gγ subunits, but are functionally distinct from conventional Gβγ dimers, although some controversy remains regarding different potential

partners for $G\beta_5$ in signaling. However, $G\beta_5$-R7 dimers increase the steady state GTPase activity of numerous Gα subunits in the context of receptor-coupled heterotrimers reconstituted in proteoliposomes (71). The RGS9-$G\beta_5$L dimer ($G\beta_5$L is a splice variant expressed in the rod outer segments of the retina.) was shown to interact with an anchoring protein, RGS9 anchoring protein (R9AP). In the absence of R9AP, the stability of the RGS9-$G\beta_5$L complex in photoreceptors is severely compromised (72). RGS and its anchoring protein were recently shown to be involved in hereditary abnormalities in photoresponse recovery (73). This is the first report of a human pathology associated with a specific RGS defect.

NOVEL INTERACTING PARTNERS FOR Gβγ SUBUNITS

In addition to the classical signaling paradigms associated with the activation of GPCRs, there are a number of novel pathways that are activated by and/or regulate the activity of either receptors or their G protein partners. This number will certainly enlarge as proteomic techniques become more widely applied to G proteins as they have been for GPCRs (see 73*a*,73*b*, and 73*c* for review). A number of these pathways involve recently identified interacting proteins for Gβγ dimers (Figure 1,Table 2). These include various PI-3 kinase isoforms (74); PDZ proteins (75); guanine exchange factors (GEFs) for small G proteins such as P-Rex1 (76,77), FLJ00018 (78), and p114-RhoGEF (79); as well as protein kinase D (80–82). Receptor for activated C kinase 1 (RACK1) recently was shown to act as a negative regulator of certain aspects of Gβγ signaling, as its binding site on Gβγ overlaps with those of phospholipase Cβ isoforms 2 and 3, adenylyl cyclase II, and PI-3 kinase, but not other effector pathways, such as Gβγ-mediated activation of the extracellular-signal regulated kinase/mitogen-activated protein kinase (ERK MAPK) pathway or Gβγ-mediated chemotaxis (83, 84). RACK1 thus appears to act as a switch that tends to bias Gβγ signaling toward certain effectors. Gβγ also inhibits the opening of voltage-gated calcium channels through its association with syntaxin and other proteins in the soluble NSF attachment protein (SNAP) receptor (SNARE) complex (85). Gβγ and synaptotagmin compete for binding to SNAP-25, syntaxin1A, and the SNARE complex. Gβγ binding therefore serves to impede SNARE-dependent exocytosis, however, this Gβγ-dependent inhibition of neurotransmitter release can be overcome by increases in concentration.

RECEPTOR-INDEPENDENT SIGNALING BY Gβγ

The classical view holds that GPCR signaling is mediated solely via activation of G proteins and their downstream effectors. However, numerous recent reports have revealed that GPCRs can interact with a wide variety of intracellular molecules in addition to G proteins. G protein-independent activation of Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling was demonstrated for the $5\text{-}HT_{2A}$ receptor (86), the angiotensin II AT_1 receptor (87), and the *Dictyostelium* cAR1 cAMP receptor (88). The platelet activating factor receptor (PAFR) interacts with and activates a member of the Janus kinase family (Tyk2) in a G protein–independent fashion (89). These signaling events may also involve trafficking of receptors to endosomes and the recruitment of β-arrestin-dependent complexes (reviewed in detail in 90). Still, given what we discuss below, G proteins may serve roles in organizing and trafficking complexes associated with GPCRs; thus, we prefer to use the term functionally G protein–independent for this class of signaling events. It has become clear that the G proteins may be able to interact with effector molecules in a receptor-independent fashion (see 91 for review).

Ten members of a group of proteins known as nonreceptor activators of G protein signaling (AGS) have been identified to date (92,93). These are now known to work through a variety of mechanisms. Group I AGS proteins are guanine nucleotide exchange factors that promote receptor-independent G protein activation by facilitating GDP dissociation from, and thus GTP

binding to, Gα subunits. Group II AGS proteins (also called GPR or GoLoco proteins), in contrast, inhibit GDP dissociation, but may promote Gβγ signaling by altering the association between Gα and Gβγ. Group III AGS proteins differ from the others in that they do not appear to bind appreciably to Gα subunits but rather they produce their effects by binding directly to Gβγ. This interaction could promote dissociation of the heterotrimer subunits, or simply compete for interaction with Gα. AGS2, also known as TcTex-1, a light chain component of the dynein motor in the cytoplasm, may also be a direct Gβγ effector important for the modulation of neurite outgrowth and other processes required dynamic modulation of the cytoskeleton (94). A recent study indicated that AGS9, a Group III AGS protein, modulated signaling events via interactions with an intact G protein heterotrimer and may in fact form a signaling complex with the G protein heterotrimer and one of the classic Gβγ effectors, phospholipase Cβ (PLCβ (95)). However, the exact function of the Group III AGS proteins remains unclear.

$G\beta_1$ can be phosphorylated on histidine 266 by histidine kinase (96) and this high energy phosphate can be transferred to Gα-GDP, yielding Gα-GTP, by nucleoside diphosphate kinase B (NDPK B) (see 97 for review). This may represent a mechanism for heterotrimeric G protein activation, which does not require a GPCR per se. In rat cardiomyocytes, $G\beta_1$H266L, a mutant that cannot be phosphorylated by histidine kinase showed reduced cAMP stimulation and reduced levels of cardiac contractility. The mutation also resulted in decreased phosphorylation of phospholamban on serine 16 following receptor stimulation by an agonist (98).

CROSS-TALK BETWEEN Gβγ AND OTHER SIGNALING PATHWAYS

Androgens can promote transcription by androgen receptors, but they also induce rapid responses that may be receptor-independent such as the rapid phosphorylation of the Elk-1 transcription factor or c-Raf-1. These rapid actions in response to androgen involve the activation of $PLC\beta_2$ by a $G\beta_4\gamma$ dimer derived from a PTX-sensitive heterotrimer (99). A similar cross-talk phenomenon was noted for estrogen stimulation, as well. This action is mediated by a membrane-localized protein related to the estrogen receptor β and is insensitive to the estradiol antagonist ICI 182,780 (100). A recent study demonstrated that these interactions between the estrogen receptor and Gβγ are likely to be direct (101).

Gβγ SUBUNITS AND EFFECTORS LOCALIZED AT SITES DISTINCT FROM THE PLASMA MEMBRANE

It has become clear that GPCRs can signal from numerous intracellular locations (Figure 2). Further, the notion that all GPCRs are initially trafficked to the plasma membrane has been challenged by data for the . GABA-B_1 receptor subunits, which remain in the endoplasmic reticulum (ER) in the absence of GABA-B_2 subunits (102–105). However, the distribution of GABA-B_1 in the central nervous system is much broader than that of GABA-B_2, which suggests that this receptor may have an independent function intracellularly (106–108). It was demonstrated that a recently deorphanized GPCR, GPR30, targeted exclusively to the ER, is a functional receptor for estrogen (109). Other recent studies demonstrated that receptor-G protein-effector complexes first become associated in the ER, and that their interactions are sensitive to agonist in these compartments, which suggests that these complexes are indeed functional (110–112). On a parallel track, a number of studies demonstrated that heterotrimeric G proteins are localized to ER and Golgi compartments where they can be involved in the regulation of anterograde protein trafficking and Golgi organization (80–82,113–115). The receptors (if they are required) that control these latter events remain unknown, but it is clear that one of the key effectors is protein kinase D. Further, a number of recent studies (reviewed in 116) indicate that GPCRs and their associated signaling machinery are trafficked to the nuclear membrane and, in some cases, the nucleus proper. How GPCRs and G proteins signal

in these compartments is not completely understood, but Gβγ subunits are proving to be signaling molecules in all of these subcellular locations.

Recently, a number of studies have indicated a direct nuclear impact for Gβγ dimers. Gβ1γ2 dimers can interact directly with histone deacetylase 5 (HDAC5) and possibly other HDAC isoforms, as well (117). In the basal state, HDAC5 interacts with the muscle differentiation factor MEF2, resulting in reduced transcriptional activity. Following stimulation of the α_{2A}AR, the activated Gβγ dimers interact with HDAC5, releasing MEF2 and allowing it to stimulate transcriptional activity. Both the $G\alpha_{i/o}$ inhibitor PTX and the Gβγ scavenger βARKct inhibit MEF2 activity (117). It remains uncertain whether cytoplasmic Gβγ dimer sequesters HDAC, or if these events occur exclusively in the nucleus. As mentioned above, the distinctive $G\beta_5$ subunit interacts with a number of RGS proteins. One RGS class, the R7 subfamily, is enriched in the brain and functions as part of a stable RGS-$G\beta_5$ complex, which is localized to both the cytosol and the nucleus (118). The RGS7 binding protein (R7BP) protein interacts with the RGS7-$G\beta_5$ pair and potentiates the capacity of this complex to modulate Kir3 channels in response to M2 muscarinic cholinergic receptor stimulation (119). R7BP is palmitoylated, allowing the anchoring of RGS7-$G\beta_5$ at the plasma membrane to regulate GPCR signaling. However, the addition of palmitate is a transient and tightly regulated process (120). In this case, the loss of the palmitate moiety on R7BP releases the R7BP-RGS7-$G\beta_5$ complex from the plasma membrane and shuttles it to the nucleus. Other RGS proteins also localize to the nucleus and include RGS6, which can regulate transcription in mammalian cells (121). The precise role of these proteins in the nucleus remains to be determined. The authors of this latter study proposed this as a novel mechanism for transmitting neurotransmitter signals from receptor at the plasma membrane directly to the nucleus (see 122 for review). Interestingly, mutant $G\beta_5$ subunits unable to form a complex with RGS7 but still capable of interacting with $G\gamma_2$ are not found in the nucleus of either HEK 293 cells or PC12 cells, which suggests the importance of the RGS protein in nuclear localization of $G\beta_5$ (123). It has been shown that Gβγ subunits containing the other Gβ isoforms can interact with the transcriptional repressor known as the adipocyte enhancer-binding protein (AEBP1) (124). AEBP1 specifically forms a complex with Gβγ subunits containing $G\gamma_5$ in the nucleus of 3T3-L1 cells, but interestingly not in NIH 3T3 cells. The $G\beta\gamma_5$-AEBP1 interaction attenuates AEBP1 transcriptional repression activity.

Another newly recognized effector of the Gβγ dimer is the glucocorticoid receptor (GR). These receptors localize in the cytoplasm and translocate to the nucleus in response to ligand binding, thereby transcriptionally regulating several target genes. Both $G\beta_1$ and $G\beta_2$ subunits can directly interact with the GR and can be translocated with it to the nucleus in the presence of the glucocorticoid dexamethasone (125,126). The interaction of Gβ with the receptor is dependent on the presence of the Gγ subunit and suppresses GR transcriptional activity, most likely by associating with transcriptional complexes formed on GR-responsive promoters. Indeed, $G\beta_2$ mutants unable to bind Gγ are not able to suppress GR transcriptional activity.

Results from the studies mentioned above highlight the central role of Gβγ subunits in numerous subcellular compartments, directly regulating fundamental processes such as transcription and the trafficking of proteins through the ER and Golgi apparatus. Thus, we can now develop our central theme: that Gβγ subunits are more than simple signaling molecules responsive to GPCR stimulation.

ASSEMBLY OF Gβγ DIMERS

While functional Gα subunits can be synthesized in almost any expression system, Gβγ synthesis seems more tightly regulated. This is not simply owing to differential posttranslational modification as both Gα and Gγ subunits are modified by the addition of lipid

moieties that facilitate G protein association with lipid bilayers. For example, Gβγ can be synthesized in vitro in rabbit reticulocyte lysates. However, either cotranslationally or by subsequent attempts at assembly in vitro, formation of functional Gβγ dimer is inefficient; only 30 to 50% of the synthesized Gβ and Gγ subunits can form functional Gβγ dimers. Interestingly, Gβ subunits can be synthesized separately from Gγ subunits in rabbit reticulocytes and wheat germ extract, but these will not interact efficiently with Gγ subunits. By contrast, Gγ subunits can be synthesized in rabbit reticulocyte lysates, wheat germ extracts, and bacteria, and will efficiently associate with Gβ subunits (127,128). This specificity suggests that cellular cofactors such as chaperones are necessary for the proper folding of Gβ and subsequent assembly into a Gβγ dimer (128). Recent studies have indicated that there are preferential associations for different Gβγ subunits in living cells (129). One early hint in this regard was that the 90kDa-heat shock protein (hsp90) could interact with Gβγ (130), mainly with the non-dimerized form of Gβ rather than with the native Gβγ dimer (67). More recently, specific mechanisms used for the assembly of the Gβγ dimer have been revisited.

Members of the phosducin family were originally proposed to act as inhibitors of G protein signaling via sequestration of the Gβγ subunits from Gα and effector molecules (131,132, reviewed in 133). Members of the phosducin family (phosducin-like proteins, or PhLP 1–3) have been shown to serve as co-chaperones with the cytosolic chaperonin complex (CCT) to assist in folding a variety of nascent proteins (134–136). CCT is an essential chaperone required for protein folding in the cytosol of eukaryotic cells (137). Nascent polypeptides associate with the ring structure formed by a stack of two groups of eight CCT subunits, and interactions with residues in the ring diminish the activation energy required to form the three-dimensional structure of the native protein (138). Among the known substrates of CCT are Gα and multiple proteins with β-propeller WD40 structures similar to Gβ (139–141). PhLP1 is not a substrate of CCT because it interacts with CCT in its native form. This suggests a regulatory role for PhLP1 in CCT-dependent folding processes (135). In fact, PhLP acts as a co-chaperone by binding above the CCT cavity and occluding the cavity to stabilize folding processes until the native protein formation occurs. PhLP1 may act as a co-chaperone for the folding of the Gβ subunit until Gβγ reaches its native stable state. This idea is consistent with observations that when PhLP1 is deleted in *Dictyostelium*, Gβ does not co-localize with Gγ at the plasma membrane but is expressed in the cytosol, as if the Gγ interaction was inhibited (142). To facilitate Gβγ dimer formation, PhLP1 must be phosphorylated on serine residues by casein kinase 2 (CK2). A mutant of PhLP1 that cannot be phosphorylated (S18-20A) inhibits both Gβ release from CCT and subsequent Gβγ assembly (143,144). The mechanism for Gβ release from CCT may involve steric repulsion, thereby triggering release of a PhLP1-Gβ complex intermediate. Here, Gβγ subunits are not yet in their native form because the intermediate complex of PhLP1-Gβ does not contain Gγ subunits (145,146). Interestingly, Gγ was not found to interact with CCT either directly or in a complex with Gβ. A separate chaperone has recently been identified for Gγ subunits. Dopamine receptor interacting protein 78 (DRiP78) is an ER membrane–bound HSP40 co-chaperone that regulates receptor transport to the plasma membrane via an F*XXX*F*XXX*L motif found in various GPCRs such as β_2-adrenergic (β_2AR), dopamine D1, M2 muscarinic cholinergic, and angiotensin II AT1 receptors (147–149). Gγ subunits and DRiP78 initially colocalize in the ER, presumably facing the cytosolic compartment where they can interact with Gβ. DRiP78 competes with Gβ for the interaction with Gγ and this may facilitate its release from the chaperone, as well. shRNA knockdown of endogenous DRiP78 reduced formation of Gβγ and resulted in a more rapid decline in $G\gamma_2$ levels when de novo synthesis was blocked. This suggests that DRiP78 maintains the stability of nascent Gγ in the absence of its heterotrimeric partners. Further, DRiP78 can also interact directly with PhLP1 (149), suggesting that PhLP-Gβ complex might interact with DRiP78-Gγ complex, thus participating in the assembly of the native Gβγ dimer. DRiP78 can also, therefore, be considered as a co-chaperone for Gβγ assembly, protecting Gγ from degradation until both subunits can be assembled into their native form. Subsequently, association of Gα

with Gβγ would release PhLP and potentially DRiP78 for additional rounds of Gβγ dimmer assembly.

As discussed above, Gβ subunits, with the exception of $G\beta_5$, are quite homologous. This suggests that the same set of chaperones might function for them all. However, it remains to be seen whether there are specific chaperones for the more divergent Gγ subunits, and whether different chaperones facilitate the assembly of specific Gβγ pairs, specific heterotrimers, or specific receptor-based complexes. There are numerous other DnaJ family members, including DRiP78, that may serve these roles. Although it is clear that DRiP78 does not interact directly with any of the Gβ subunits, its effects are more pronounced when $G\beta_1$ is involved. This may suggest that the DRiP78-PhLP complex is also important in selective formation of different Gβγ pairs. Moreover, expression of the Gα subunit may alter this pattern even though we demonstrated that DRiP78 did not interact with Gα subunits (149). Proteins that may be considered chaperones for Gα subunits include the J domain–containing cysteine string protein (CSP) (150), Gα-interacting vesicle-associated protein (GIV, also known as girdin), Daple, and FLJ000354 (151). These proteins may be involved in assembling G protein heterotrimers and may also represent potential interacting proteins for PhLP-1 and/or DRiP78. The fact that certain chaperones such as CSP and DRiP78 can also interact with effector molecules [e.g., voltage-gated calcium channels (152–154)] or GPCRs [e.g., dopamine D1, M2 muscarinic cholinergic and angiotensin II AT_1 receptors (147,148,155), and the β_2AR (149)] hints that these chaperones may also be involved in the formation or trafficking of larger, or perhaps specific complexes of receptor, G protein heterotrimer and effector.

RECEPTOR-BASED SIGNALING COMPLEXES

Phototransduction in mammalian rod outer segments is extremely sensitive, i.e., photoreceptor cells are capable of detecting single photons. This level of sensitivity requires signal amplification, part of which occurs at the second step of the transduction process i.e., when activated receptors (rhodopsin) interact with the G protein (transducin). Every active rhodopsin molecule can potentially interact with hundreds of transducin molecules via random collisions (156). Although this collision coupling almost certainly contributes to the sensitivity of phototransduction, it is by no means certain that a similar mechanism operates in other G protein signal transduction pathways, even in the case of other sensory systems that are highly sensitive to external stimuli (157).

Although transient interactions between rhodopsin and transducin, and then between transducin and cGMP phosphodiesterase are required for signal amplification in the mammalian visual system, other organizational paradigms operate in other cell types that express multiple receptor, heterotrimeric G proteins, and effector molecules (reviewed in 158). The possibility that receptors (R) and G proteins (G) might be associated prior to receptor activation has been incorporated into models of G protein signaling for some time (159,160), but experimental evidence that precoupled R-G complexes exist in living cells has been obtained only recently (13,14,16).

The notion that GPCRs stably interact with their G protein and effector partners has been proposed as a mechanism to assure rapid and specific signaling (reviewed in 158). Thus, one might consider the receptor itself as a scaffolding protein for the formation of specific signaling complexes. A large number of studies have demonstrated co-purification or co-immunoprecipitation of receptors with G proteins (reviewed in 158). It has been shown, for example, that the β_2AR remains associated with the Gs heterotrimer regardless of its state of activation [i.e., the interaction is stable under basal, activated, and desensitized conditions of the receptor (160)]. More recent studies using imaging techniques such as bioluminescence resonance energy transfer (BRET) (13,161) and fluorescence resonance energy transfer

(FRET) (162) have validated these findings and highlighted the constitutive nature of the interactions between receptors and G proteins. These techniques have been the subject of a number of recent reviews (163–167) and are not discussed further here.

OTHER G PROTEIN SIGNALING COMPONENTS THAT PARTICIPATE IN STABLE COMPLEXES

A number of effectors are stably associated with receptor-G protein complexes, including adenylyl cyclase isoforms, L-type calcium channels, calcium-activated potassium channels, and inwardly-rectifying potassium channels (30,168–174). Perhaps more surprisingly, some of these effectors directly associate with receptor molecules. For example, it was demonstrated using BRET that the β_2AR was associated with both Kir3 ion channels and adenylyl cyclase (112,173), and that D4 dopamine receptors associate with Kir3 ion channels (173). Protein-protein interaction assay were used to show that opioid-like receptor 1 physically associates with voltage-gated N-type calcium channels (175,176). The existence of stable interactions, independent of receptor activation between G proteins and their effector molecules, including Kir3 ion channels and adenylyl cyclase, have also been shown (110,177). Several recent studies elegantly demonstrate that G proteins remain associated with Kir3 channels throughout the signaling event (178–180). Other key regulatory molecules such as RGS proteins interact constitutively with receptors, G proteins, and effector molecules (181–183). There are also numerous scaffolding proteins that interact with GPCRs to create an even greater diversity of signaling arrays and signaling outcomes (reviewed in 184,185).

Gβγ SUBUNITS AS AN ORGANIZING DETERMINANT FOR SIGNALING COMPLEX ASSEMBLY

Because receptors, G proteins, effectors and various scaffolding or chaperone proteins have been observed as parts of multimeric complexes at the plasma membrane, new questions have arisen about the formation of these complexes, the trafficking of the different partners, and the assembly sites of these complexes. If signaling partners interact before receptor activation, the question of where these proteins first interact and what facilitates their assembly into specific complexes becomes critical. Recent studies have painted a complicated picture regarding trafficking of individual components of GPCR signaling complexes. It is known that receptor oligomers are assembled in the ER (reviewed in 186). One study has shown that fully processed Gβγ subunits form heterotrimers with Gα on the cytosolic face of the Golgi apparatus (187). However, another study demonstrates Golgi-independent trafficking of Gβγ (188). In any case, assembly of the heterotrimer precedes acylation of the Gα subunit, which is necessary for delivery of the heterotrimer to the plasma membrane (187,189, reviewed in 190). Trafficking of Kir3 channels is extremely complicated, and the ultimate destination depends on which channel subunits are present (191).

Constitutive trafficking of some GPCR-regulated effectors, such as adenylyl cyclase isoforms and various ion channels, demonstrates that components of these signaling pathways can transit to the membrane independently of the receptor or G protein. However, there is now substantial evidence that, like GPCR dimers, these complexes are formed during or shortly after biosynthesis. A number of studies have also demonstrated that receptors can directly interact with Gβγ, as well as Gα, subunits (59,60,192). Recent studies have shown that many of these proteins interact initially in the ER, including monomeric receptor equivalents in receptor oligomers (193), receptor and Gβγ subunits (194), and effectors such as Kir3 channels and adenylyl cyclase with nascent Gβγ (110,112). The interactions between adenylyl cyclase or β_2AR and Gβγ, and between receptor monomers in the β_2AR homodimer were insensitive to dominant-negative Rab 1 or Sar 1 constructs (112,194), which regulate receptor trafficking

(177, reviewed in 195). However, these latter studies highlight the fact that the Gα subunit is assembled with nascent receptor-Gβγ-effector complexes either at ER exit sites or in the Golgi, as these interactions were blocked by dominant negative Sar 1 and Rab 1 (112,194). If these complexes are preformed during protein biosynthesis and maturation, they would need to be trafficked inside the cell as a complex and not necessarily as individual proteins (Figure 3). The individual preformed complexes may be distinct for receptor monomers, homodimers, and heterodimers, leading to a unique signaling output for each receptor complex (Figure 2). What is the role for Gβγ in organizing the assembly and or trafficking of these complexes?

On the basis of the data described above, it is clear that both GPCRs and their effector molecules interact with G protein subunits before targeting to the plasma membrane. We propose that Gβγ subunits may play an organizing role for assembly of GPCR-based signaling complexes as they interact with all of the relevant components–: receptors, Gα, subunits and effectors before each of them is trafficked to the cell membrane (or other subcellular destination). Specific Gβγ combinations may in fact act as chaperones in this regard. Many studies have demonstrated that GPCR dimers are formed constitutively, often before they reach the cell surface (see 186 for review).

Given that most cells express multiple receptor subtypes that could, in principle, assemble into heterodimers, what mechanisms does the cell use to assemble specific receptor complexes at any one time? There are a number of indications that these sorting and assembly decisions must occur in the ER, but the molecular basis for these decisions has so far proven elusive. One simple and relatively unsatisfying answer is that the timing of receptor synthesis or coordinate regulation of particular subtypes may have something to do with this. A thorough examination of upstream and downstream regulatory sequences of GPCR genes may be informative in this regard. Another potential mechanism may rely on precocious interactions (i.e., interactions that occur during biosynthesis) with G protein subunits, which may be involved in controlling receptor assembly in the ER (177,194). One tantalizing observation that indicates a precocious role for Gβγ in assembling receptor dimers is that when Gβγ function in membranes is inhibited by using a membrane-localized version of the carboxy terminus of G protein-coupled receptor kinase 2 (GRK2, βARK-CT) construct, formation of Kir3.1-Gβγ complexes in the ER is blocked (e.g., M. Robitaille and T.E. Hébert, unpublished results) and, more surprisingly, we also reduce the formation of β_2AR homodimers (Figure 4). These preliminary observations need to be validated using other approaches such as siRNAs that target specific Gβγ combinations. Interestingly, a recent study using siRNA to knock down various Gα and Gβγ subunits in HeLa cells showed not only alterations in cellular signaling events, but also coordinate loss of expression (or in some cases upregulation) of other signaling partners (194*a*). For example, knockdown of $G\beta_1$ lead to a coordinate knockdown of $G\alpha_s$ and $G\alpha_{i3}$ while knockdown of $G\beta_4$ lead to a coordinate knockdown of $G\alpha_q$ and $G\alpha_o$. Conversely, knockdown of $G\beta_2$ lead to an upregulation of $G\beta_4$. Coordinate changes in signaling partner expression were also detected in other studies where individual Gγ subunits were knocked down or knocked out (see Table 1). The roles that individual Gβγ subunits play in the expression and assembly of signaling complexes, and where in the cell these events are controlled, remain to be determined.

It is also clear that a number of effectors [e.g., homo- and heterotetrameric Kir3 channels and putatively dimeric AC isoforms (see, for example, 111)] possess multiple sites of interaction for heterotrimeric G proteins. In the context of a large signaling complex with many potential sites for interactions with G proteins, it becomes critical to determine the interactions that are precocious (i.e., relevant to assembly and trafficking) as compared to those important for signaling per se. Further, it will be critical to determine the relative specificity for precocious versus signaling interactions and to characterize the relevant interaction sites at the structural level.

The trafficking itinerary of GPCRs following prolonged or repetitive agonist stimulation is well characterized (see 196,197 for review). There is also some evidence that trafficking itineraries for receptor and Gα may diverge upon long-term agonist stimulation (198, reviewed in 199). These findings need to be reassessed using imaging approaches in living cells to determine where signaling complexes dissociate. What happens to the G protein and effector signaling partners when receptors are targeted for degradation? Do some portions of signaling complexes containing G proteins and effectors remain intact and reassociate with recycled receptors? These remain open questions as little is known about the internalization of effector molecules. Do Gβγ subunits play an analogous role in the reassembly of signaling complexes when receptors are recycled? These pathways may be more difficult to dissect than the relatively straightforward de novo synthesis pathway. More attention will need to be paid to the assembly and trafficking of GPCR-based signaling complexes in both a tissue-specific context and with respect to subcellular localizations within a single cell. Modulation of subtype-specific Gβγ-dependent events with regard to assembly or subsequent signaling using siRNA (200,201) will perhaps identify targets for small- molecule peptidomimetics (202,203). Use of proteomic and imaging-based techniques, followed by mutagenic and structural analyses of interaction domains, should allow identification of the sites of interaction among Gβγ, specific chaperones, and different members of the various signaling complexes. It should also, in principle, be possible to create peptidic or peptidomimetic compounds based on these interaction domains that will allow individual signaling pathways linked to a particular receptor or class of receptors to be targeted. For specific targeting, a partner or chaperone that is unique to a particular complex or class of complexes would need to be identified. Of course, the converse may be useful as well in certain cases where a signaling pathway or multiple pathways that share components might be targeted. Here, we may wish to target common interaction partners. However, it may be difficult to design peptidomimetics that target a specific interaction if the interaction surface is large or contains many different shifting contact points. This might be expected for a stable receptor-based complex that occupies multiple conformations even during its basic activation/deactivation cycle. Further complexity is added when we consider recruited proteins, receptor desensitization, and internalization. Targeting specific molecular chaperones may be a way around some of these difficulties. Evidently, much work remains before this approach becomes viable as a therapeutic strategy.

SUMMARY POINTS

1. Gβγ subunits play broad roles in cellular signaling with respect to effector modulation as well as during “precocious” interactions in internal subcellular compartments prior to receptor activation.
2. Molecular chaperones play key roles in the assembly of Gβγ dimers. These chaperones may also be important for assembly of larger GPCR-based complexes directed by Gβγ.
3. Gβγ subunits initially interact with receptors and certain effector molecules in the ER, and with Gα subunits at ER exit sites.
4. Interference with Gβγ function in internal compartments blocks these precocious interactions and may in.uence the formation of receptor dimers.

FUTURE ISSUES

What determines whether particular Gα and Gβγ subunits dissociate when regulating cellular signalling?

What are the roles of multiple Gβγ binding sites on effector molecules (such as Kir3 channels) and GPCR homo- and heterodimers?

Is there a specificity for particular combinations of Gβγ that can be attributed to classes of GPCRs or effectors?

What other specific molecular chaperones can be identified for the assembly of different Gβγ pairs, G protein heterotrimers, and larger complexes involving receptors and effectors? How and where in the cell do they act? Can these chaperones be developed as potential drug targets?

What are the precise roles of individual Gβγ subunits in fostering the assembly of GPCR signaling complexes? Can these be targeted to interfere with assembly of specific signaling complexes?

What is the role (if any) of Gβγ in the assembly of GPCR dimers?

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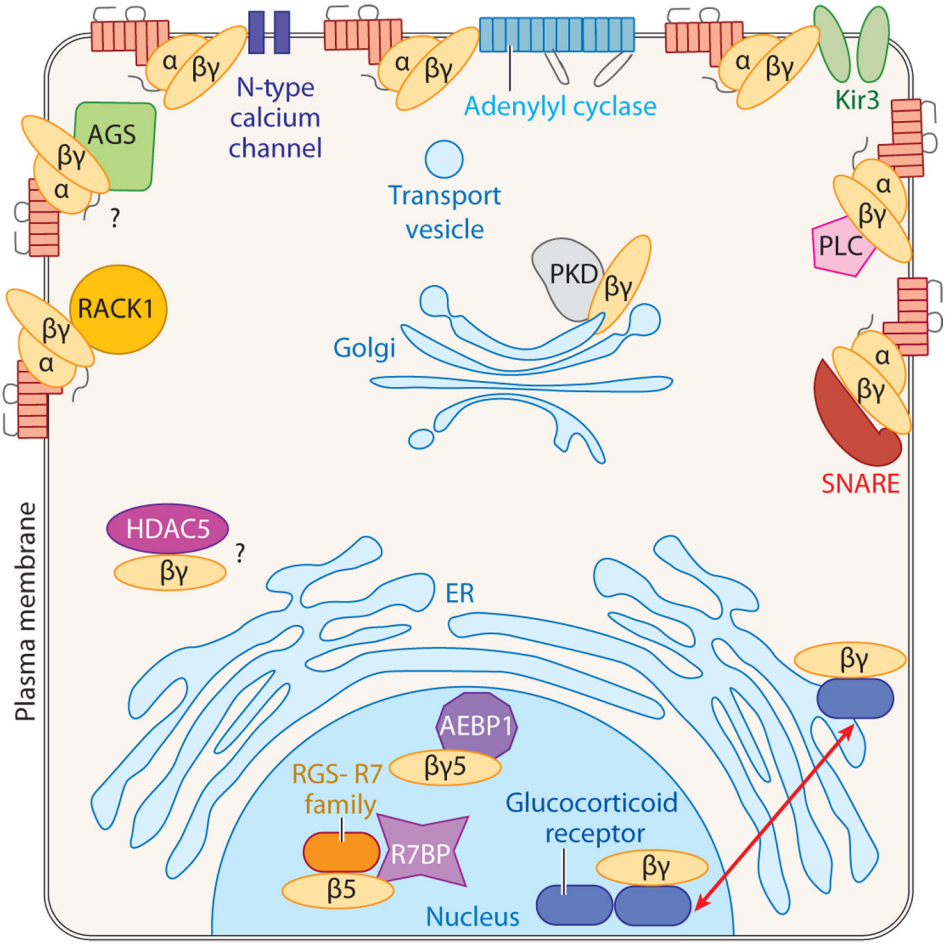

Figure 1.

Established and more recently identified effectors that are regulated by Gβγ subunits. Gβγ subunits regulate a number of effectors at the cell surface, including adenylyl cyclase isoforms, Kir3 and voltage-gated calcium channels, and phospholipase Cβ isoforms, among others. More recently, a number of novel interacting proteins have been identified that transduce Gβγ-dependent signals in other subcellular compartments such as the Golgi apparatus [protein kinase D (PKD)], cytosol [histone deacetylase 5 (HDAC5)], and nucleus [RGS7 binding protein (R7BP), adipocyte enhancer-binding protein (AEPB1)], glucocorticoid receptor (GR), and possibly HDAC5). Whether all of these intracellular events require GPCRs or Gα subunits remains to be determined. The examples presented here are representative and do not include all of the either classical or novel effectors. For simplicity, GPCRs are shown as monomers even though they can be dimeric and may even be multimeric. See the text for more details.

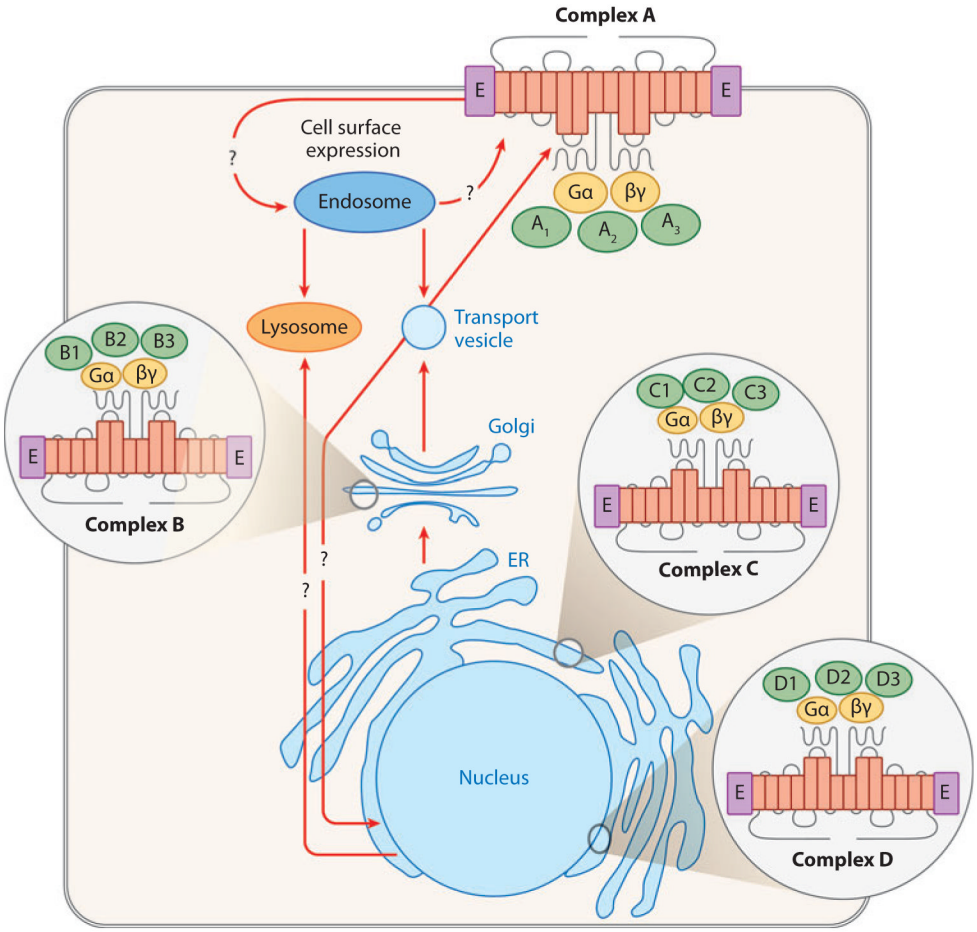

Figure 2.

GPCRs signal from distinct subcellular compartments. GPCRs have also demonstrated to signal from a number of different intracellular locations. In addition to ligand-induced signals activated at the cell surface, GPCRs have been identified with functional effects in internalizing endosomes, in the nucleus (perhaps as cleaved fragments), on the nuclear membrane, and in the ER (reviewed in 116). These receptors can be associated with unique sets of signaling partners at each of these sites as denoted by the different possible complexes. This is made even more complicated when the combinatorial power of receptor heterodimerization is taken into account. The red arrows denote potential trafficking itineraries, which could lead to the presence of particular receptors and their associated signaling machinery at distinct intracellular locations. See the text for more details.

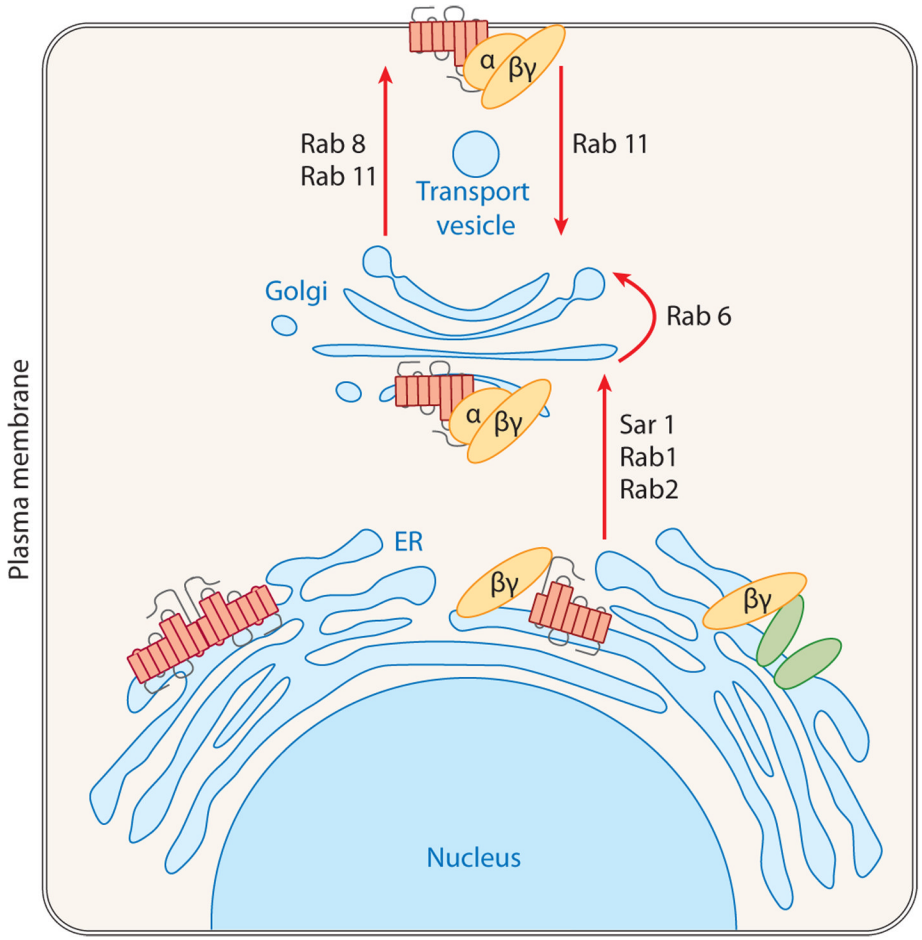

Figure 3.
GPCRs initially interact with their signaling partners before they leave the biosynthetic machinery. Receptor dimers or oligomers, receptor-Gβγ complexes and effector-Gβγ complexes can form in the ER even when antegrade protein trafficking from ER to Golgi is blocked with dominant negative versions of GTPases such as Sar 1 and Rab 1. The Gα subunit becomes associated with the nascent Gβγ-based complexes last, probably at ER exit sites, the ER/Golgi intermediate complex (ERGIC), or perhaps the *cis*-Golgi. Gβγ subunits play a central role in early interactions in the assembly of GPCR signaling complexes.

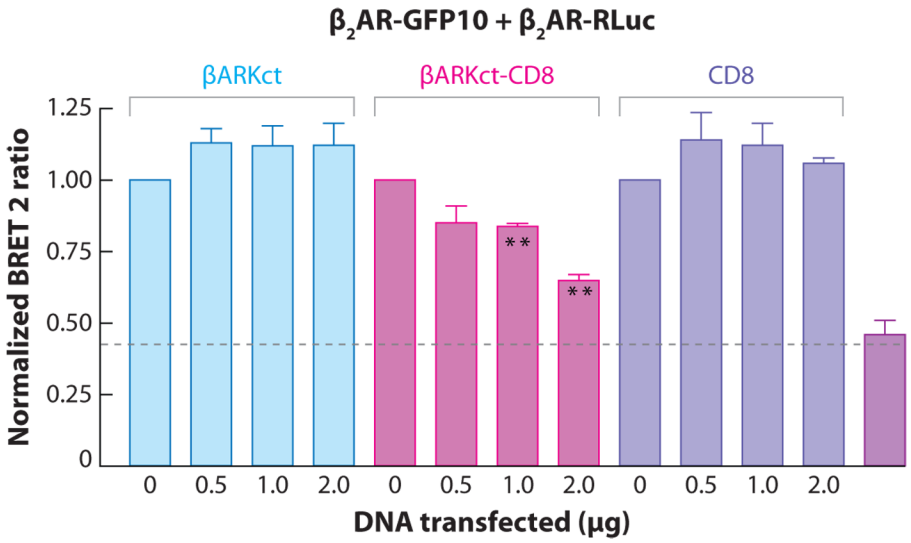

Figure 4.

A role for Gβγ subunits in the assembly of GPCR dimers? An experiment using bioluminescence resonance energy transfer (BRET) to demonstrate that increasing amounts of the membrane-localized Gβγ scavenger, CD8-βARK-CT, but not soluble βARK-CT or CD8, can inhibit β_2AR homodimer formation in a concentration-dependent manner. HEK 293 cells were transfected with recombinant plasmids to express β_2AR tagged with *Renilla* luciferase (β_2AR-RLuc), and β_2AR tagged with green fluorescent protein (β_2AR-GFP10). BRET was measured as described (194). Data are expressed as mean ± SEM of at least 3 different experiments and normalized by comparing with β_2AR-Rluc and β_2AR-GFP expressed alone. Total cDNA levels were equalized for transfection using pcDNA3. * indicates $p < 0.05$ compared with controls (Rluc- and GFP-tagged donor and acceptor alone) using a one-tailed Student's t-test. The final bar represents a negative control in which CD4-Rluc is used as the BRET donor.

Table 1

G protein signalling specificity demonstrated by selective knockdown or knockout

Target	Method	Effects	Ref.
α_{o1}	Antisense oligonucleotides	Coupling between muscarinic receptors and voltage-dependent Ca^{2+} channels	(46)
α_{o2}	Antisense oligonucleotides	Coupling between somatostatin receptors and voltage-dependent Ca^{2+} channels	(46)
β_1	Antisense oligonucleotides	Coupling between muscarinic M4 receptor and voltage-dependent Ca^{2+} channels	(47)
β_2	Antisense oligonucleotides	Reduced inhibition of Ca^{2+} channel current by galanin receptors	(49)
	shRNA	Decreased chemotactic response to C5a	(203*a*)
β_3	Antisense oligonucleotides	Reduced inhibition of Ca^{2+} channel current by galanin receptors	(49)
γ_2	Antisense oligonucleotides	Reduced inhibition of Ca^{2+} channel current by galanin receptors	(49)
γ_3	Gene knockout	Reduced $G\beta_2$ and $G\alpha_{i3}$ expression	(203*b*)
	Antisense oligonucleotides	Coupling between somatostatin receptors and voltage-dependent gated calcium channels	(48)
γ_4	Antisense oligonucleotides	Coupling between muscarinic receptor and voltage-dependent gated calcium Channels	(48)
	Antisense oligonucleotides	Reduced inhibition of Ca^{2+} channel current by galanin receptors	(49)
γ_7	Gene knockout	Decreased $G\alpha_{olf}$ expression and activity of adenylyl cyclase in striatum	(44)
	Ribozyme	Decreased Gβ expression	(41)
		Decreased AC activity stimulated by isoproterenol but not by prostaglandin E1	(41)
		Decreased SKF81297 stimulated AC in cells expressing D1- but not D5 dopamine receptors	(43)

Knockdown and knockdown approaches that address signaling specificity and/or coordinate regulation of signaling partners.

Table 2

Gβγ interactors old and new

		Subcellular location (site of action)	References
Classical Effectors	Adenylyl cyclase isoforms	Plasma membrane	(25)
	Kir3 potassium channels	Plasma membrane	(23)
	N-type calcium channels	Plasma membrane	(32)
	Phospholipase Cβ	Plasma membrane	(29)
	MAPK		(27)
Novel effectors	PKD	Golgi	(80)
	RGS-R7 Family	Nucleus	(118), (119)
	AEBP1	Nucleus	(124)
	RACK1	Plasma membrane	(83)
	Glucocorticoid Receptor	Cytosol/nucleus	(125), (126)
	HDAC5	Cytosol/nucleus	(117)
	SNARE	Plasma membrane	(85)
	AGS	Plasma membrane	(92), (95)
	Tctex-1	Plasma membrane	(94)
	NDPK-B	Plasma membrane	(96), (97)
Gβ chaperones	PhLP1	Cytosol	(155)
Gγ chaperones	DRiP78	ER	(149)

Gβγ effectors old and new. Here we summarize classic and more recently identified Gβγ effectors as well as novel chaperones identified for Gβ or Gγ. We also note the variety of intracellular sites where these interactions occur.

Abbreviations are as described in Figure 1 and in the text: mitogen-activated protein kinase (MAPK), protein kinase D (PKD), RGS7 binding protein (R7BP), histone deacetylase 5 (HDAC5), adipocyte enhancer-binding protein (AEPB1), receptor for activated C kinase (RACK1), glucocorticoid receptor (GR), SNAP receptor (SNARE), activators for G protein signaling (AGS), nucleoside diphosphate kinase B (NDPK-B), phosducin-like protein 1 (PhLP1 and dopamine receptor-interacting protein 78 (DRiP78). As in Figure 1, the examples presented here are representative and do not include all of the either classical or novel effectors. See the text for more details.