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Review

Membrane interactions of G proteins and other related proteins

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ABSTRACT

Guanine nucleotide-binding proteins, G proteins, propagate incoming messages from receptors to effector proteins. They switch from an inactive to active state by exchanging a GDP molecule for GTP, and they return to the inactive form by hydrolyzing GTP to GDP. Small monomeric G proteins, such as Ras, are involved in controlling cell proliferation, differentiation and apoptosis, and they interact with membranes through isoprenyl moieties, fatty acyl moieties, and electrostatic interactions. This protein–lipid binding facilitates productive encounters of Ras and Raf proteins in defined membrane regions, so that signals can subsequently proceed through MEK and ERK kinases, which constitute the canonical MAP kinase signaling cassette. On the other hand, heterotrimeric G proteins undergo co/post-translational modifications in the alpha (myristic and/or palmitic acid) and the gamma (farnesol or geranylgeraniol) subunits. These modifications not only assist the G protein to localize to the membrane but they also help distribute the heterotrimer (Gαβγ) and the subunits generated upon activation (Gα and Gβγ) to appropriate membrane microdomains. These proteins transduce messages from ubiquitous serpentine receptors, which control important functions such as taste, vision, blood pressure, body weight, cell proliferation, mood, etc. Moreover, the exchange of GDP by GTP is triggered by nucleotide exchange factors. Membrane receptors that activate G proteins can be considered as such, but other cytosolic, membranal or amphitropic proteins can accelerate the rate of G protein exchange or even activate this process in the absence of receptor-mediated activation. These and other protein–protein interactions of G proteins with other signaling proteins are regulated by their lipid preferences. Thus, G protein–lipid interactions control the features of messages and cell physiology.

Contents

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1. Introduction

The cell membrane provides a structure that functions as a platform for the assembly of many signal transduction pathways, and that provides an additional level of regulation in cell signaling networks. The complex dynamic structure of the plasma membrane permits lipid–lipid and lipid–protein interactions, as well as the interaction of lipid–protein complexes with the submembrane cytoskeleton. The existence of membrane microdomains adds further complexity to such interactions, as well as the messages propagated in cells through G proteins and other non-permanent (extrinsic) membrane proteins.

Here, we aim to review the current understanding of the role of membrane interactions in the activity of G proteins and their relevant partners. These abundant membrane-bound receptor-coupled amphitropic proteins constitute the first elements, following receptor activation, in various key intracellular signaling pathways. Both their interactions with membrane lipids and their lipid co/post-translational modifications permit the regulation of protein function in a highly versatile manner.

Over and above the direct involvement of the alpha subunits of heterotrimeric G proteins in signal transduction, a large number of different GTP/GDP-binding proteins are present in eukaryotic cells. At least five distinct families have been recognized and they also include elongation factors active in protein biosynthesis, subunits of the signal recognition particle (SRP) and its receptor, the ADP-ribosylation factor (ARF) family, and the products of the Ras gene family [1]. The first part of this review will mainly focus on the Ras family of proteins as small monomeric G proteins, their lipid modifications and interactions with membranes, and their relevance in malignant transformation. The second part summarizes our current knowledge of the interactions between heterotrimeric G proteins and membranes as well as the role of G protein modulators on these interactions and G protein activity. This review highlights the relevant roles of membrane lipids and lipid structures on the localization and activity of G proteins. These interactions are relevant in the context of the physiology of cells, and their alteration may lead to pathologies whose treatment could be addressed by modifying membrane lipid composition and structure through so-called membrane-lipid therapy.

2. Small monomeric G protein structure and their interaction with the plasma membrane

Ras and Ras-like proteins are abundant small monomeric GTPases found mainly at the inner surface of the plasma membrane, and are key elements in complex and crucial cell signaling events. Members of this family include Ras, Rap1, Rap2, R-Ras, Ral, Rheb, M-Ras, and TC21, which all display distinct signaling properties [2]. At the plasma-membrane, these proteins function as molecular switches that initiate and regulate multiple signal transduction pathways. Their fundamental role is in coupling external stimuli with cytoplasmic and nuclear targets, and their activation depends on their association with growth factor receptors. In this way, they can regulate cell proliferation, differentiation, and apoptosis [3].

Mammalian Ras proteins have a molecular weight of 21 kDa and, except for the K-Ras isoforms with 188 amino acids, they all contain 189 amino acids. Mammalian cells contain three very similar Ras genes: H-ras, N-ras, and K-ras (with splice variants A and B). In this review, we will refer to Ras as the products of these three genes, even though one or the other may be predominant in different cell types. At the protein level Ras is present in all cells, although they were prominent in proliferating cells.

Their structure can be divided into three domains [4]:

The G domain (amino acids 1–166) that is 95% conserved among the different isoforms. This region of the protein binds guanine nucleotides, contains the switch 1 and switch 2 loops, and it undergoes major conformational changes on GTP–GDP exchange. It also contains the binding surfaces for effectors, exchange factors, and GAPs (GTPase-activating proteins).

The region containing the C-terminal 24–25 amino acids forms a poorly conserved HVR domain (hypervariable region). This amino acid sequence undergoes direct post-translational processing. And it is involved in plasma membrane anchoring, as well as the trafficking of newly synthesized and processed Ras from the cytosolic surface of the endoplasmic reticulum to the inner surface of the plasma membrane.

The C-terminal CAAX motif is a region that is post-translationally processed to generate an *S*-farnesyl cysteine carboxymethyl ester. The membrane anchor is completed by one (N-Ras, R-Ras, and K-Ras4B) or two (H-Ras) proximal *S*-palmitoylable cysteine residues or a polybasic domain of six lysine residues in K-Ras4B [[5–7], Silvius, 2002 #372]. The other isoform (R-Ras4B) is very likely palmitoylated [8]. The minimal membrane anchors tN, tH, and tK (t = targeting motif) are sufficient to traffic and anchor heterologous proteins to the plasma membrane.

The linker region of the HVR connects the anchor sequence with the N-terminal G-domain. As with the gamma subunit of heterotrimeric G proteins (see below), Ras proteins have the CAAX (C = cysteine, A = aliphatic, X = amino acid) isoprenylation motif at the carboxy terminus. This post-translational modification favors the anchoring of proteins to membranes, which modifies the lipid structure of the latter, creating non-lamellar prone regions. This increased H_{II}-phase propensity favors the binding of isoprenyl moieties and induces segregation of membrane microdomains enriched in isoprenoids. Several Ras and Ras-like proteins are also palmitoylated near the farnesylated carboxy-terminus. The main divergence among Ras isoforms lies in the 24 C-terminal amino acid domain, where sequence identity falls to less than 15% [9]. This diversity provides the different isoforms of the Ras proteins with different propensities to interact with diverse plasma membrane lipid structures.

2.1. Lipid modification of Ras proteins and targeting to the plasma membrane

The correct targeting of Ras to the inner surface of the plasma membrane requires a series of post-translational modifications at the protein's C-terminus. These reactions include attachment of the isoprenoid farnesyl by farnesyl transferase to a cysteine residue (in the case of H-, K-, and N-Ras) or geranylgeranylation by geranylgeranyl transferase (for N- and K-Ras). Farnesylation and geranylgeranylation both occur on the same cysteine residue (Cys-186), located four residues from the C terminus. If the CAAX box´s last residue is Ser or Met, then the farnesyl moiety is attached and if instead it consists of a Leu, then geranylgeranyl is attached. All Ras isoforms preferentially undergo farnesylation, although in the presence of farnesylation inhibitors N-Ras and K-Ras can undergo geranylgeranylation [10]. If, in the CAAX box, the last residue is Ser or Met, then a farnesyl moiety is attached. If it is Leu, then a geranylgeranyl moiety is attached. H-Ras and N-Ras can also undergo palmitoylation and, while H-Ras has two palmitoylation sites (Cys-181 and Cys-184) and N-Ras has only one (Cys 181), K-Ras4B appears to lack a palmitoylation site. Both farnesylation and geranylgeranylation are important to target Ras to the cell membrane. Likewise, palmitoylation may also localize Ras to the plasma membrane, as well as participating in its specific micro-localization at the plasma membrane [7].

The consequences of farnesyl attachment are particularly important because prenylation is the first and indispensable step in the processing of Ras proteins. This processing includes the proteolytic removal of the last 3 amino acids followed by subsequent lipidic modifications that promote the association of Ras to intracellular membranes. In fact, Ras remains soluble without this initial modification. Besides initiating membrane attachment the farnesyl group seems to bind to specific membrane proteins [11], possibly enhancing Ras-membrane interactions or participating in the activation of Ras effector proteins. Experiments with mutant constructs of H-Ras with C-terminal palmitates but no isoprenoid, demonstrated that palmitates can nevertheless support substantial farnesyl-independent activity [12]. Therefore, palmitate seems to be more than just a strong form of membrane tether, and it may have unique and dynamic roles of its own.

While classic studies indicate that all RAS proteins rely on farnesylation and either palmitoylation or a stretch of polybasic amino acids for stable binding to membranes, it has been recently shown for N-RAS and K-Ras4A that mono-palmitoylation and farnesylation are not sufficient to direct stable cell-surface localization and that altered localization is achieved without changes in palmitoylation status [13]. A third motif that is present within the linker domain of all palmitoylated RAS HVRs is necessary for stabilizing localization to the plasma membrane. This motif comprises acidic residues that stabilize palmitoylation and basic amino acids likely to interact electrostatically with acidic phospholipids enriched at the cell surface.

The different Ras protein isoforms interact in different ways with the plasma membrane. On the one hand, H-Ras interacts transiently with lipid rafts when bound to GDP and it aggregates in cholesterol-insensitive, galectin-1-dependent, non-raft domains. On the other hand, K-Ras is clustered in cholesterol-insensitive, non-raft domains that differ from the activated H-Ras microdomains [14,15]. Electron microscopy and biochemical studies suggest a new model for the microorganization of Ras at the plasma membrane, whereby Ras promotes the molecular assembly of signaling microdomains. Recent NMR and neutron-diffraction spectroscopy studies of the biophysics of Ras membrane anchors, as well as those involving Molecular Dynamics simulations, confirmed that insertion of the lipid chains into the cell membrane is driven by hydrophobic interactions. However, once in place, the backbone peptide that is inserted into the lipid bilayer contributes to the stability of the complex. Moreover, there is emerging evidence that the palmitate anchors of Ras proteins may help drive the formation of signaling clusters by a combination of lipid–lipid and protein–lipid interactions with the plasma membrane [4].

2.2. Ras signaling from platforms other than the plasma membrane

Ras proteins are captured by endosomal compartments from where a signal output can also be generated. Ectopically expressed mutant Ras proteins with defective anchoring sequences accumulate in Golgi and endoplasmic reticulum membranes, from where they are competent to generate a signal output. The physiological significance of these platforms is not known, and it is unclear whether they constitute important signaling platforms for endogenous Ras [4]. Nevertheless, there is evidence that lipid modifications such as isoprenylation and *N*- and *S*-acylation also play an important roles as specific recognition elements for protein–protein interactions, and as hydrophobic switches. As such, they permit temporal regulation of the docking of Ras to subcompartments like lipid rafts, caveolae, and other cellular localizations. Unlike farnesylation, palmitoylation of Ras-like proteins is reversible and it therefore constitutes a key regulatory element.

2.3. Interaction of Ras with membranes

The relevance of this issue originates from various sources. The anchors in the hypervariable region may target the Ras isoforms to specific microdomains of the plasma membrane with different lipid and protein content. However, it is fundamental to determine whether these different micro-environments influence Ras signaling. Selective microdomain localization based on the structural differences in membrane targeting domains may account for the extensive biological differences between Ras isoforms. Therefore, insights from Ras may extend to the microlocalization and regulation of other lipid-modified signaling proteins. Fluorescent-labeled probes with Ras C-terminal anchors are increasingly being used as tools for SPT (single particle tracking) and FRET (fluorescence resonance energy transfer) to explore the dynamics of micro- and nano-compartmentalization in the plasma membrane. A recent study illustrates the use of these techniques to find a new switch region that regulates H-ras membrane orientation and signal output [16]. This example also illustrates how the plasma membrane acts as a semi-neutral interaction partner by spatially constraining signal conformations.

Interestingly, another recent study shows that none of the 121 genes found to be regulated uniquely by Ras signals emanating from plasma membrane microdomains is specifically controlled by lipid raft-anchored Ras [17].

2.4. Ras signaling

The plasma membrane provides a platform for the assembly of proteins that belong to many signal transduction pathways and therefore, it has the capacity to regulate cell signaling networks. An initial step in the transduction of signals through Ras involves the interaction of these G proteins with growth factor receptors at the cell surface. The next steps involve interactions with exchange factors and specific proteins that will transduce the signals in a cascade of events that leads to the activation/inhibition of transcription factors and hence, the regulation of different cellular processes. Ras proteins activate several signal transduction pathways that control gene expression and that are involved in the regulation of critical cell processes (such as cell proliferation, differentiation and apoptosis [3]) and cytoskeleton organization. Indeed, Ras is the common upstream molecule in several signaling pathways including the Raf/MEK/Erk cascade (also known as the MAP Kinase or MAPK pathway), PI3K/Akt, Ral/MEKK1/JNK, and PLC/DAG/PKC [18]. While the three highly homologous Ras isoforms can each activate these signal transduction pathways, they display different affinities for each of them [19]. The best known of these signaling mechanisms is the MAPK (mitogen activated protein kinase) pathway, which involves a series of cytoplasmic serine/threonine kinases. The cascade is activated by ligand binding to receptors that provoke the post-translational modification of Ras (through palmitoylation) and its translocation to the membrane. Following the binding of cytokines, growth factors, or mitogens to their appropriate cell membrane receptors, activation of the Shc/Grb2/SOS coupling complex occurs, causing the inactive Ras to exchange GDP for GTP, to undergo a conformational change, and to become active. The GTP-bound active Ras can then recruit Raf kinase to the cell membrane and this localization of Ras at the membrane is crucial for its biological activity. Thus, the membrane binding of Ras enables it to act as a GTP-dependent membrane-localized docking site for Raf, among other downstream effectors [20].

2.5. The role of protein–lipid interactions and malignant mutations in Ras

The role of each specific lipid modification of Ras has mainly been inferred from mutant Ras proteins that entirely lack one or all three of these lipids. The decreased membrane binding observed with these mutants indicates that their primary role is in membrane binding. It is certainly of great interest that Ras protein–lipid interactions control cell proliferation/differentiation pathways, as key elements in signal transduction processes. The *ras* genes were initially discovered due to their highly oncogenic potential in retroviruses or human oncogenes. Variants of the different isoforms were independently characterized

as the transforming genes present in many human and animal tumors. Different mutation frequencies have been observed between the *ras* genes in human cancer, in which K-Ras is the most frequently mutated Ras isoform. Given the abundance of Ras as a membrane-associated protein and its key role in membrane receptor signaling events, it is hardly surprising that mutations which affect the Ras proteins have been detected in a number of cancers including pancreatic, breast, colon, and non-small cell lung cancer. While tumorigenesis requires multiple hits, carcinogenesis involving Ras requires little more than an increase in Ras levels or mutations that lock Ras into its activated state. Nevertheless, there is increasing evidence that structural changes which transform Ras into an oncogenic protein are not sufficient to induce cancer [21].

The signal transduction activity of Ras proteins can be altered by both cell membrane lipids and by their own lipidic modifications. Post-translational modifications of Ras such as farnesylation, geranylgeranylation, and palmitoylation are important as they represent sites for therapeutic intervention. Indeed, mutant Ras proteins that bind poorly to the membrane have a low transforming potential [7,22]. These facts impelled the development of inhibitors of farnesyltransferase as potential antitumor drugs in the 90s. Although some such compounds showed some activity against cancer, most failed to have any real effect because the inhibition of farnesyltransferase provokes the modification of Ras with gerenylgeraniol, a closely related isoprenoid that also enables membrane binding [10]. In addition, it was also found that a non-farnesylated H-Ras protein can be palmitoylated and can trigger potent differentiation and transformation [12]. However, myristoylation of both wild-type and activated Ras is sufficient to produce a protein with transforming potential [23], again lending support to the view that membrane localization is a key element in transformation by Ras.

3. Heterotrimeric G protein–lipid interactions

As peripheral membrane proteins, G proteins interact with the inner side of the plasma membrane and form part of the signaling cascade activated by G protein-coupled receptors (GPCR). They are comprised of three subunits Gα, Gβ and Gγ, which are often closely associated with the intracellular domains of GPCRs. The binding of an extracellular agonist activates GPCRs, inducing a conformational change in its protein structure that leads to the activation of the corresponding heterotrimeric G protein. As a consequence, the Gα-subunit of the heterotrimeric complex exchanges bound GDP for GTP and subsequently dissociates from the Gβγ-dimer, enabling both molecular entities to regulate the activity of their specific effectors. During this process G proteins can be affected by lipid–protein interactions in several ways. First, like all membrane-associated proteins, the localization and function of G proteins is influenced by the biophysical properties of the membrane with which they interact. Second, the close interaction of G proteins with the membrane enables them to also modulate their immediate lipid environment, thereby modifying plasma membrane lipid structure and organization which in turn modulates binding of G proteins to membranes. Third, in order to ensure tight membrane attachment, the different subunits of G proteins undergo distinct co- or post-translational lipid modifications. The nature of the lipid moiety attached in this process dictates the specific requirements of the optimal membrane lipid environment necessary for its correct localization and activity. This section of the review focuses on the variety of interactions between membrane lipids and proteins reflecting the complexity of their relationship and the multitude of possible regulatory processes involved in G protein-mediated signal transduction.

3.1. GPCR activation of heterotrimeric G proteins

A large number of hormones, neurotransmitters, chemokines, and sensory stimuli exert their effects on cells by binding to GPCRs. Heterotrimeric G proteins located at the cytoplasmic face of the plasma membrane are the intracellular partners of these receptors in the generation of the cellular responses to extracellular signals. When a ligand binds to a GPCR the receptor acts as a guanine nucleotide exchange factor (GEF), activating a Gα subunit and promoting the release of GDP and the binding of GTP. These modifications cause conformational changes in Gα that induce its dissociation from the Gβγ dimer. As a result, both the GTP-bound Gα subunit and the Gβγ dimer independently modulate dowstream effectors. The Gα subunit has intrinsic GTPase activity that can hydrolyze GTP to GDP, which induces the re-association of the inactive Gα subunit with a Gβγ dimer, terminating the signal. Recently, as will be discussed in section 3.6, other proteins known as activators of G protein signaling (AGS) regulate G protein-mediated signals in the absence of receptor activation. Regulator of G protein signaling (RGS) proteins help accelerate the GTPase activity of Gα and the re-association of the inactive heterotrimer (Fig. 2). Thus, Gβγ acts as a guanine-nucleotide dissociation inhibitor (GDI), since when bound to Gα it slows the spontaneous rate of GDP release [24]. The assembly of Gα to the Gβγ subunit in its GDP-bound form allows Gα to associate with the plasma membrane and has an important role in its functional coupling to GPCRs. Lipid modifications of Gα subunits are important for their membrane localization. Indeed, it is known that the localization of the G protein heterotrimers to the plasma membrane involves multiple processes such as post-translational modifications, subunit assembly, and protein–membrane interactions [25–31]. As indicated below, Gα subunits can be *N*-myristoylated and/or *N*-palmitoylated, which helps define the localization of these proteins, although the presence of a fatty acyl moiety (e.g., myristic acid) is not sufficient to translocate Gα subunits from the cytosolic to membrane fractions [32–34]. Myristoylation and/or palmitoylation of Gα subunits not only regulate their targeting to specific cell membrane regions but also, they regulate their interaction with other signaling proteins [35–38]. Nevertheless, all Gγ subunits are C-terminally prenylated [39] and this lipid modification is also important for the proper membrane localization of the Gβγ dimer.

3.2. Lipid modifications of G proteins

Lipidation of heterotrimeric G proteins (Gαβγ) involves the covalent binding of a hydrophobic molecule to the signaling protein. Three different types of lipid modification have been described in the case of heterotrimeric G proteins, namely myristoylation, palmitoylation and isoprenylation. These moieties participate in G protein–lipid interactions and they are involved in the translocation of G proteins to membranes and their further mobilization to different membrane microdomains.

3.2.1. Myristoylation of G proteins

Myristoylation is a co-translational modification that involves the covalent attachment of the saturated fatty acid myristate to a protein via an amide bond (*N*-acylation) [40]. In contrast to *S*-acylation and palmitoylation that are characterized by a labile thioester linkage, the amide bond represents a comparatively stable chemical structure and it is therefore considered to be irreversible. Myristoylation is catalyzed by the enzyme *N*-myristoyltransferase and it is specific to the N-terminal glycine in proteins. The absolute requirement for a free glycine residue at the N-terminus of the substrate protein implies that the initiating methionine must be eliminated by a methionyl amino peptidase prior to lipidation. However, a free N-terminal glycine residue alone is not sufficient to guarantee myristoylation, because substrate recognition also depends on the subsequent amino acid sequence. In particular, the nature of the sixth amino acid (considering methionine as the first) seems to be important in this context, and myristoylated proteins usually contain a serine or threonine residue at this position [41]. Consequently, subunits of the G_i family including $G\alpha_{i1-i3}$, $G\alpha_o$, $G\alpha_z$, that contain a Gly^2/Ser^6 motif, are potential substrates

for *N*-myristoylation, while the subunits of the G_s, G_q and G_{12} families are not. Although *N*-myristoyltransferase almost exclusively catalyzes the attachment of myristate (C14:0), it has also been found to transfer other acyl fatty acids under certain circumstances, such as lauryl [C12:0], (*cis*-delta 5)-tetradecaenoyl [C14:1(Δ5)] and (*cis*,*cis*-delta 5, delta 8)-tetradecadienoyl [C14:2(Δ5,8)] [42]. This relaxation in specificity is confirmed by the different lipid modifications other than myristoylation observed at the N-terminal glycine of $G\alpha_t$ (transducin). However, this exceptional diversity of lipid modifications on Gly_2 appears to be specific to retinal rod cells [43,44].

Lipid modifications assist peripheral proteins in their docking to membranes by inserting a hydrophobic moiety that serves as a membrane anchor. Indeed, mutations in Gα subunits that prevent *N*-myristoylation ($Gly_2 \rightarrow Ala_2$) prevent protein binding to membranes [45,46]. Nevertheless, the presence of a myristoyl moiety is not sufficient for stable membrane attachment [47], and moreover the majority of Gα subunits are not myristoylated. Therefore, it is clear that this sort of lipid modification must also have another function besides simply enhancing the membrane affinity of a protein. For the G_i family myristoylation is a precondition for palmitoylation [32,48], the secondary lipid modification found on Gα subunits. In this context, the role of the myristoyl group is to facilitate an initial contact with the membrane and to direct the Gα subunit to the palmitoylation compartment. At least in the case of $G\alpha_t$, another important function of myristoylation could be to maintain the N-terminal structure of the corresponding Gα subunit, as recently proposed [49].

3.2.2. Palmitoylation of G proteins

Palmitoylation is a post-translational modification that consists in the covalent attachment of the saturated fatty acid palmitate to a protein via a thioester bond (*S*-acylation). In contrast to other covalent lipid modifications, palmitoylation is a very dynamic modification, because this bond is chemically labile and has been shown to be rapidly turned over *in vivo* [50,51]. Besides the palmitoylation of Ras proteins or GPCRs [52], this lipid modification also occurs on one or several cysteine residues within the first 20 amino acids of the N-terminal region of the Gα subunit, but the specific amino acid sequence required to facilitate palmitoylation has not yet been defined in detail. As an exception to the rule it was recently found that native $G\alpha_s$ can also be palmitoylated at the N-terminal glycine (Gly_2), and that this lipid modification occurs via *N*-acylation [53]. The apparent dual Gly_2/Cys_3 palmitoylation of $G\alpha_s$ is similar to the myristoylation/palmitoylation motif of the $G\alpha_i$ family and it has functional consequences on signal transduction [54]. Generally, with the exception of α_t and α_{gust} all Gα subunits undergo palmitolylation, but neither the specific palmitoyltransferase that catalyzes the lipidation of mammalian Gα subunits nor the exact cellular site where this occurs have so far been discovered. Palmitoylation of membrane proteins has in fact been detected in several subcellular localizations and while palmitoylation of caveolin-1 [55] and transferrin receptor [56] appears to take place at the plasma membrane, that of viral polypeptides and glycoproteins seems to occur in the Golgi [57,58]. Other proteins are palmitoylated even earlier in their biosynthetic pathway (i.e. in the endoplasmatic reticulum [59]) and it is difficult to discern whether all these compartments are true sites of palmitoylation or whether they simply reflect the rapid intracellular transition through the vesicular system. Point mutations of $G\alpha_s$ and $G\alpha_q$ aimed at impairing binding to Gβγ dimers not only inhibited trafficking to the plasma membrane but also their palmitoylation [60]. Conversely, restoring the plasma membrane localization of these mutants also restored palmitoylation. These results imply that Gα and Gβγ most likely interact before Gα reaches the plasma membrane and that heterotrimer formation is a necessary part of the plasma membrane targeting signal for both entities. Disruption of the Golgi does not alter the plasma membrane localization or palmitoylation of Gα subunits [61,62], indicating that heterotrimer formation may take place in the endoplasmic reticulum.

3.2.3. Isoprenylation of G proteins

Isoprenylation is a multi-step post-translational process that involves covalent attachment via a chemically stable thioether bond of either a farnesyl group, consisting of 3 isoprene repeats with a total of 15 carbon atoms, or a geranylgeranyl group of 4 isoprenes and a total of 20 carbon atoms. Isoprenylation is an irreversible lipid modification that has only been found on the Gγ subunit of heterotrimeric G proteins. In this context, farnesylation occurs in $G\gamma_1$, $G\gamma_9$, and $G\gamma_{11}$, whereas the remaining 9 Gγ subunits are all geranylgeranylated [63].

Isoprenylation depends on the existence of a rather simple sequence at the C-terminus of the protein, the so-called CAAX box, which determines the substrate specificity for a farnesyl or geranylgeranyl transferase. This motif contains the cysteine to be modified (C), followed by two aliphatic amino acids (AA) and an amino acid at the C terminus (X), controlling the recognition by one of the two enzymes. If the last residue of the CAAX box is serine or methionin, then the preceeding cysteine is farnesylated, whereas in the case of leucine a geranylgeranyl moiety is attached. Isoprenylation takes place in the cytosol and the Gβγ dimer then translocates to the endoplasmatic reticulum for further processing. In this cellular compartment, proteolysis of the three terminal -AAX amino acids is catalyzed by the endoprotease Ras-converting enzyme (Rce) [64], and the newly formed C-terminal cysteine with the isoprenyl modification becomes a substrate for isoprenylcysteine carboxyl methyltransferase (Icmt) [65]. The exact reason for this proteolysis and methylation is unknown (especially as this methylation is generally reversible), but it seems that carboxy methylation of the last amino acid further increases its hydrophobicity, thereby enhancing the membrane anchorage properties of the C-terminus. This might still make some sense in case of farnesylated Gγ subunits, but the greater hydrophobicity of the geranylgeranyl moiety compared to the farnesyl group already makes it a strong membrane anchor that would not be expected to need further enhancement to bind tightly to the membrane [27]. Nevertheless, the tight association to intracellular membranes is not sufficient to target the Gβγ dimer to the plasma membrane, and isoprenylated proteins need additional targeting signals to shuttle from the endoplasmatic reticulum to the plasma membrane. In this context, it has been demonstrated that the subcellular localization of the Gβγ dimer is regulated by the presence of the Gα subunit and its trafficking pathway [66].

3.3. Biophysical properties of membranes on heterotrimeric G protein signaling

Membrane microviscosity, or its reciprocal parameter fluidity, was one of the first parameters used to demonstrate the relationship between the biophysical properties of the lipid bilayer and the activity of associated proteins [67]. Membrane fluidity depends on the lipid composition of the membrane, especially on the type of fatty acid moieties in the membrane phospholipids and the amount of cholesterol. In general, as the degree of unsaturated fatty acid moieties increases in membrane phospholipids, so does the fluidity of the membrane [68]. It has been shown that metabolic disorders or nutritional interventions can alter the lipid composition of the membrane and as a consequence, its fluidity [69,70]. In fact, diseases in which G protein-mediated signaling plays an important role, such as hypertension and Alzheimer disease, have been associated with altered membrane fluidity [71,72]. Although changes in membrane fluidity are obviously related to certain illnesses, this parameter is not very helpful in carrying out a profound characterization of the biophysical properties of the membrane. This is in part because it still considers the plasma membrane as a homogeneous structure, as depicted by the traditional Singer–Nicolson model.

While the lipid–protein interactions between G proteins and lamellar-prone membranes have been studied intensely [73], little attention has been paid to the potential interactions with nonlamellar-

prone membranes. Lipids with a propensity to form nonlamellar phases affect the physical properties of biomembranes by inducing alterations in the intrinsic curvature of the monolayer, in the lateral surface pressure, and in the hydration of membranes [74]. Cell membranes are usually rich in phosphatidylethanolamine (PE), a nonlamellar prone, inverted hexagonal (H_{II}) phase inducing lipid that is mainly found at the inner face of the plasma membrane [75], and thus determines the properties of the normal site for G protein binding. The physiological amount of PE in natural membranes is sufficiently high to induce negative curvature strain *in vivo*, which might be stabilized by the presence of certain membrane proteins [76]. It has been demonstrated that the greater the hexagonal-phase propensity, the higher the affinity of heterotrimeric α_i proteins for the membrane [25]. Consequently, membrane domains with enhanced negative curvature strain could act as a platform for the interaction of these peripheral proteins with other signaling proteins. This idea is in agreement with the observed loss of G proteins from plasma membranes after disruption of nonlamellar H_{II} structures by daunorubicin [26].

As G protein-mediated signaling operates as a signal amplification cascade where every agonist-bound GPCR activates numerous G proteins, efficiency would be enhanced if a large number of inactive heterotrimeric G protein molecules were readily available in the proximity of the receptor. Indeed, an early study of signal transduction kinetics in human platelet membranes suggests that about one third of the α_2-adrenergic receptors seem to be coupled to $G\alpha_i$ prior to agonist binding [77]. In general, transmembrane peptides with α-helical structures, such as the membrane-spanning regions of GPCRs, can promote the formation of hexagonal phases [78], and other data indicate that GPCRs seem to display similar lipid phase preferences as heterotrimeric G proteins. It was proposed that lipids with a negative spontaneous curvature favor the elongation of the G protein-coupled photoreceptor rhodopsin during the activation process. This was explained by the fact that they facilitated the conformational change of rhodopsin to its activated state in model membranes [79]. Moreover, measuring the interaction of this receptor and its corresponding G protein transducin (α_t) in lipid bilayers demonstrated that PE markedly increased receptor affinity for α_t upon light activation, while the affinity of α_t for dark-adapted rhodopsin remained unchanged [80]. By contrast, in pure lamellar PC bilayers the affinity of α_t for light-activated rhodopsin is substantially lower. Retinal rod outer segment membranes, where rhodopsin is found, contain about 50% docosahexaenoic acid (DHA), which is a fatty acid with a strong propensity to induce curved structures [81]. Nevertheless, the observation that rhodopsin activation can also be supported by other non-lamellar lipids, such as the hexagonal-prone PE, indicates that the physical curvature stress induced by these lipids is more likely to facilitate the conformational change of this GPCR rather than a direct lipid-specific reaction between DHA and rhodopsin. In fact, a pronounced restructuring of membrane lipids during photoactivation of rhodopsin occurs, which provides strong evidence for its dynamic interaction with the lipid phase of the disc membranes [82]. It might be possible that GPCRs reorganize the lipid environment around them during agonist activation, and that the presence of nonlamellar-prone membrane lipids is indispensable for them to produce this conformational change. Thus, hexagonal phase-forming membrane lipids seem to fulfill a dual role in signal transduction, by inducing the co-localization of the first signaling components, and by participating in GPCR activation.

On the other hand, GPCRs and heterotrimeric G proteins have also been found in membrane domains with strong lamellar membrane organization, such as lipid rafts and caveolae. Lipid rafts are defined membrane regions in a liquid-ordered (l_o) state and caveolae are a subtype of these microdomains, whose flask-shaped membrane invaginations are stabilized by a matrix of caveolin molecules that serve as scaffolds for a variety of different proteins of the GPCR signaling cascade [83]. For example, in cardiomyocytes the G protein-coupled β_2-adrenoreceptor (β_2-AR) and $G\alpha_i$ were found in caveolae, although the β_1-AR and $G\alpha_s$ were predominantly present in non-caveolae regions of the plasma membrane of these cells [84]. Furthermore, the correct localization of the β_2-AR in caveolae was essential for efficient signaling of this receptor subtype [85]. In the case of the β_3-AR differential membrane localization might be even true for the two splice variants of this receptor, because there is evidence that the β_{3A}-AR but not the β_{3B}-AR resides in caveolae [86]. As a consequence, in CHO cells the β_{3A}-AR exclusively couples to $G\alpha_s$, whereas the β_{3B}-AR couples to both $G\alpha_s$ and $G\alpha_i$ [87], a coupling preference which can be easily eliminated by disruption of raft-like microdomains with filipin. Although G_q has also been found enriched in detergent-resistant membrane areas of human platelets, this was only observed after activation of the corresponding signaling pathway by thrombin [88]. A similar activation-dependent translocation was also true for $G\alpha_t$, which only translocated to lipid rafts upon activation [89]. Together, these results indicate that membrane compartmentalization of GPCR signaling is obviously complex and influenced by several parameters, such as the type of GPCR or G protein, the downstream effectors, and maybe also the specific cell type. Consequently, lipid rafts and caveolae may enhance or attenuate interaction of specific signaling molecules by promoting or hindering their spatial proximity. In addition, interpretation of scientific literature is often difficult, because a considerable amount of published articles only discriminate these microdomains by their general physicochemical property in terms of “detergent-resistant” membrane areas or by experimental cholesterol depletion with β-cyclodextrin, which in fact would apply to both lipid-rafts and caveolae.

Biophysical studies demonstrated, that in contrast to G protein heterotrimers, the activated monomeric α_i-subunit showed a marked preference for pure lamellar structures [25], which would provoke the rapid exit of activated α_i-monomers from a receptor environment where the hexagonal-phase propensity is increased. Mobilization of the Gα protein subunit far from the receptor environment may facilitate its interaction with effector proteins (e.g. adenylyl cyclase) located in other membrane areas. This idea is further strengthened when considering that a large number of effector proteins are localized to lipid rafts. Both $G\alpha_i$ and $G\alpha_s$ have been found in lipid rafts [90] and some G protein subunits indeed partially co-localized with adenylyl cyclase in membrane structures containing caveolin [91]. However, most G proteins resided in irregular structures within the plasma membrane that have not been morphologically defined. When considered in conjunction with the finding that certain Gα subunits only translocate to lipid rafts upon activation, lipid rafts appear to represent platforms whose specific biophysical properties are able to enhance the effectiveness of the second step of the signaling cascade, trapping activated Gα-subunits and augmenting the spatial proximity between them and their effectors.

On the other hand, dissociated Gβγ-dimers still maintain a high affinity for membranes with a hexagonal propensity [25], which may also influence their distribution in native membranes. The general observation that prenylated proteins are normally not localized to lipid rafts [92] has also been confirmed for Gβγ-subunits, which are excluded from synthetic lipid rafts [35]. This indicates that the Gβγ-dimer most probably determines the lipid preference of heterotrimeric G proteins. In other words, the Gβγ-dimer determines the preference of complete Gαβγ heterotrimers for the hexagonal-phase, thereby masking the lamellar membrane affinity of the Gα-subunit. Therefore, one of the functions of the Gβγ-dimer could be to transport Gα-subunits to the vicinity of the receptor, making them available for immediate activation (Fig. 1).

3.4. Effects of G proteins on biophysical membrane properties

The fact that the interaction between membrane lipids and proteins is not unilateral but that it occurs in both directions is often overlooked. It is obvious that the association of G proteins with the plasma membrane makes them susceptible to their lipid environment,

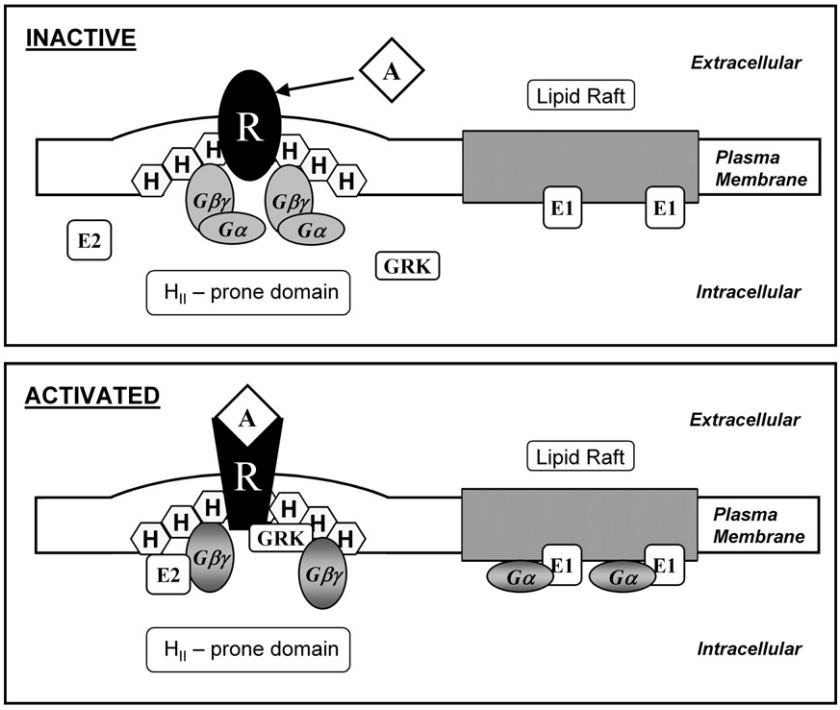

Fig. 1. Membrane structure and GPCR-associated signaling. (Upper panel) GPCRs (R) induce the formation of hexagonal-phases (H) in their vicinity and these nonlamellar membrane regions attract heterotrimeric (inactive) G proteins, driven by the Gβγ-subunit. (Lower panel) Upon agonist (A) binding, several heterotrimeric G protein molecules are activated by one GPCR (R) molecule. Thus, Gα-subunits dissociate from the Gβγ-dimers and they are targeted to special regions of the plasma membrane, such as lipid rafts, due to their greater affinity for bilayers with highly lamellar organized lipids. There, they may activate their corresponding effector proteins (E1). Gβγ-dimers remain in nonlamellar-prone regions, where they can interact with their specific effectors (E2) and guide GRKs directly to the receptors. The latter would promote GPCR phosphorylation leading to receptor inactivation.

such that lipid–protein interactions are able to influence protein function. In turn, G proteins also seem to be capable of modulating lipid structure and the organization of the membrane with which they interact. An interesting property of the lipid moieties of Gγ subunits was recently discovered given that Gγ subunits are post-translationally lipidated with the isoprenoids farnesol or geranylgeraniol. These isoprenyl moieties alone have the propensity to segregate in a multidomain system formed by a lamellar crystalline (Lc) phase, and lipid structures enriched in isoprenoid form when added to model membranes containing PE [93]. In addition, they promote temperature-dependent growth of the hexagonal phase that can coexist within the lamellar Lc phase. Similar behavior could be observed in experiments with these isoprenyl moieties bound to the carboxy-terminal peptide of the $G\gamma_2$ subunit [30]. Therefore, it is likely that not only does the Gβγ-dimer display a preference for areas enriched in nonlamellar lipids *in vivo*, but that it is also actively involved in the formation or stabilization of microdomains with intrinsic negative curvature. It is tempting to speculate that these biophysical characteristics of the Gβγ-dimer could also be important for GPCR desensitization following agonist-mediated activation. Dissociated Gβγ-dimers can bind to the GPCR kinases (GRKs) that phosphorylate and inactivate GPCRs. The preference of the Gβγ-dimer for the nonlamellar membrane environment, where membrane receptors are also located, could direct these kinases to their target, provoking the termination of the signal initiated by agonist binding to the receptor (Fig. 1). This hypothesis is strongly supported by the involvement of membrane lipids other than PC in the regulation of GPCR phosphorylation and desensitization. Indeed, with the exception of PC, other membrane lipids that either provide nonlamellar propensity or a net charge to the bilayer can actually regulate GRK activity [94]. Notably, retinal GRK1 also has a farnesyl moiety covalently attached to its carboxy terminal domain, like the Gβγ-dimer, and the presence of this isoprenoid group is essential for light-dependent membrane association of GRK1 [95]. A mutant unfarnesylated form of the kinase remains in the soluble fraction following light exposure and displays a reduced capacity to phosphorylate rhodopsin. In contrast, a mutant kinase bearing a more hydrophobic geranylgeranyl (C20) isoprenoid moiety was constitutively associated with the membrane, although it phosphorylated rhodopsin at a rate comparable to wild-type (farnesylated) GRK1 [95]. Thus, a specific in vivo modification (farnesylation) ensures that membrane association of GRK1 only occurs in the presence of its activated receptor substrate. This lipid modification of GRK, common to Ras proteins and the gamma subunit of G proteins, is reflected in the amino acid homology found in the carboxy-terminal region of all these proteins (Table 1). However, GRK 4 and 6 are palmitoylated rather than farnesylated [96,97], making it possible that these kinases are regulated in a different way by other membrane lipids.

In general, a palmitate moiety favors the localization of proteins in membrane areas with a highly ordered lamellar state (l_o). Therefore, GRK 4 and 6 may have a stronger affinity for membrane domains with this biophysical property, in agreement with their localization in membrane regions of this kind, i.e. caveolae. However, targeting of GRKs to caveolae seems to depend rather on a direct interaction with caveolins than on the type of lipid modification. In fact, Carman et al.

Table 1
C-terminal region of various subunits of GRK, Gγ and Ras proteins

Protein	C-terminal sequence
GRK1	EL-----NVWRSDGQMPDD---------MKGISGGS----SSSSKSG--------MCLVS
$G\gamma_7$	DL-----MSYCEQHARNDP--------LLVGVPASE----NPFKDKKP--------CIIL
$G\gamma_8$	EL-----LAFCETHAKDDP--------LVTPVPAAE----NPFRDKRLF-------CVLL
$G\gamma_9$	EI-----KEYVEAQAGNDP--------FLKGIPEDK----NPFKEKGG--------CLIS
H-RAS	DLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKLNPPDESGPGCMS--CKCVLS
R-RAS	AFGASHHVAYFEASAKLRLNVDEAFEQLVRAVRKYQEQELPPSPPSAPRKKGGGCPCVLL
	: : . : : . . . *::

Consensus key: * single, fully conserved residue (isoprenylation site); : conservation of strong groups; · conservation of weak groups; x homology for members of more than one protein family; x no consensus.

demonstrated that GRKs are able to bind to the scaffolding protein caveolin by a consensus caveolin-binding motif present in the pleckstrin homology (PH) domain and in the N-terminus of GRKs [98]. Interaction between GRKs and caveolin via this motif, being present in all GRKs, results in potent inhibition of GRK activity and may be necessary to suppress basal GRK activity. In this scenario, palmitoylation of GRKs could contribute to co-localization of these kinases and caveolins, which would facilitate their further protein–protein interaction.

3.5. Subcellular localization of G proteins

Heterotrimeric G proteins are signaling molecules involved in the propagation of extracellular stimuli to the interior of a cell. Therefore, G proteins are targeted to the plasma membrane after transcription and lipidation in order to exert this function. Nevertheless, there is substantial evidence that these signaling molecules are additionally able to translocate to intracellular compartments upon agonist stimulation. The best characterized subunit of heterotrimeric G proteins in this sense is $G\alpha_s$, for which a movement from the plasma membrane to the cytosol after agonist stimulation has been demonstrated [99,100]. This internalization is not mediated by clathrin-coated vesicles, which form part of the typical internalization mechanism for most G protein-coupled receptors, including the β_2-adrenoceptor, although the time-course of this process is similar to that of GPCRs [101]. A study carried out in living cells indicated that $G\alpha_s$ may be internalized via lipid rafts [102]. Furthermore, it has been shown that agonist-activated trafficking of $G\alpha_s$ to the cytoplasm is reversible, so that the internalized G proteins are able to return to the plasma membrane when receptor activation is terminated [103]. Interestingly, a recent work provided evidence that shuttling of heterotrimeric G proteins between the plasma membrane and intracellular membranes can also take place without activation in a vesicle-independent way [104]. However, the exact intracellular trafficking pathways are still poorly understood, and there is still a debate about whether internalized $G\alpha_s$ is truly cytosolic or remains bound to membrane vesicles within the cytosol. Apart from $G\alpha_s$, also $G\alpha_t$ and $G\alpha_q$ are able to translocate to the cell interior after stimulation. Rapid internalization of photoreceptor-specific G protein, transducin, and its redistribution from rod outer segments to other cellular compartments after activation is an important physiological adaptation mechanism to light exposure, allowing these cells to maintain their function in a much wider range of light intensity [105]. Similarly, upon activation of the angiotensin II receptor, a considerable fraction of $G\alpha_{q/11}$ appeared in the cytosolic fraction of the cell within a few minutes [106].

Palmitoylation is the only lipid modification of most Gα subunits (with exception of the G_i protein family, whose members are additionally myristoylated, and G_t, being only myristoylated) and at the same time the main membrane anchor for these peripheral membrane proteins. These and the fact that palmitoylation is a very dynamic lipid modification has strengthened the hypothesis that palmitoylation turnover is a key mechanism in the observed movement of Gα subunits to the cytosol, because the loss of this hydrophobic membrane anchor through depalmitoylation could easily explain the observed translocation. Actually, it has been demonstrated that activation of Gα by the appropriate GPCR increases palmitate turnover [32]. Irrespective of whether depalmitoylation is responsible for cytosolic translocation, the extent of $G\alpha_s$ depalmitoylation upon β_2-adrenergic receptor activation was inversely correlated with the efficacy in stimulating adenylyl cyclase, underlining the functional importance of this cellular process for signal transduction [107].

Much less information has been gathered about the activation-dependent redistribution of the Gβγ dimer from the plasma membrane. One study demonstrated co-localization of the Gβγ dimer with $G\alpha_s$ in small vesicular structures [101], whereas others found it associated to the Golgi complex [108] after stimulation. The extent and time course of βγ-dimer internalization varies widely, which is partly attributed to the kind of lipid modification on the gamma subunit, but also depends on its interaction with the receptor and the type of the Gα subunit found in the heterotrimeric complex [109,110]. In this context, a recent, very comprehensive study in living cells including all known gamma subunits, revealed that not all of these subunits are able to internalize upon agonist stimulation [111]. The subunits $G\gamma_1$, $G\gamma_9$ and $G\gamma_{11}$ were able to rapidly translocate to the Golgi complex, whereas $G\gamma_5$ and $G\gamma_{10}$ moved slowly to this membrane complex. On the other hand, $G\gamma_{13}$ translocated rapidly to the endoplasmatic reticulum. In contrast, the subunits $G\gamma_{2-4}$, $G\gamma_{7,8}$ and $G\gamma_{12}$ did not traffic to intracellular sites after agonist stimulation.

A final conclusion concerning the physiological function of G protein internalization in cell biology would surely be premature, but most of the data gathered point to an important role in the desensitization process following GPCR activation. Besides, basal cycling of inactive heterotrimeric G proteins between cell and intracellular membranes may be essential to maintain an intracellular pool of these signaling molecules and to prepare (sensitize) the cytoplasmatic surface of cell membranes for activation. Whether subcellular localization of G proteins has more yet undiscovered functions remains to be investigated.

3.6. Receptor-independent activators of G protein signaling

During recent years, alternative forms of regulating signaling through heterotrimeric G proteins have been studied, forcing us to broaden our perspective regarding the role of G proteins as "signaling switches" [112]. In this regard, several accessory proteins that can interact with G proteins independently of receptor activation have been characterized through different approaches. A direct activator of G proteins was partially purified from NG108-15 cells, which acts on both hetrotrimeric brain G protein and free Gα in a manner distinct to that of receptors [113]. Yeast-based screens have also identified receptor-independent activators of heterotrimeric G protein signaling [114], including the so-called activators of G protein signaling (AGS). These proteins have been classified into different groups depending on their role in signal transduction. AGS1, previously identified as a dexamethasone-inducible Ras family GTPase [115], is the only member of the first group and has been characterized as a putative guanine nucleotide exchange factor (GEF) for $G\alpha_i$ subunits [116] (later renamed RASD1 by the Human Genome Organization [HUGO] Gene Nomenclature Committee). This protein contains a Ras-like domain with extensions in the N- and C-terminal regions containing consensus farnesylation motifs with similar characteristics to those of G protein farnesylation [112]. Like the G protein activator partially purified from NG108-15 cells, AGS1 increases GTPγS binding to $G\alpha_{i1}$ and $G\alpha_{i2}$ monomers as well as to G protein heterotrimers purified from the brain (Fig. 2). Recently, another protein called Resistant to Inhibitors of Cholinesterase 8A (Ric8A) was identified as another receptor-independent GEF that exclusively acts on free Gq/i/o α-subunits [117].

The second group of AGS proteins is also called GoLoco or GPR motif-containing proteins because they contain at least one GPR or GoLoco domain. These GPR motifs have been discovered within several proteins of diverse nature, such as *C. elegans* GPR 1/2 [118–120], *Drosophila* PINS [121,122] and the mammalian Purkinje cell protein-2 (Pcp2/L7) [123,124], Rap1GAPII [125,126], AGS5 (LGN or mPINS) [127–130], AGS3 [130–132], AGS4 [133,134], Wave [135], AGS6 (RGS12) and RGS14 [136,137]. AGS3-5 have been renamed by the HUGO Gene Nomenclature Committee as G protein signaling modulator (GPSM) 1–3, respectively, and PCP2 is now called GPSM4 [24]. The GPR motif is generally selective for $G\alpha_i$ subunits in the GDP-bound form, and the interaction slows the spontaneous GDP release by Gα and it interferes with Gβγ binding to $G\alpha_i$ [138] (Fig. 2). Thus, these proteins can behave as guanine nucleotide dissociation inhibitor (GDI) for Gα and can accelerate the rate of heterotrimer dissociation Gαβγ.

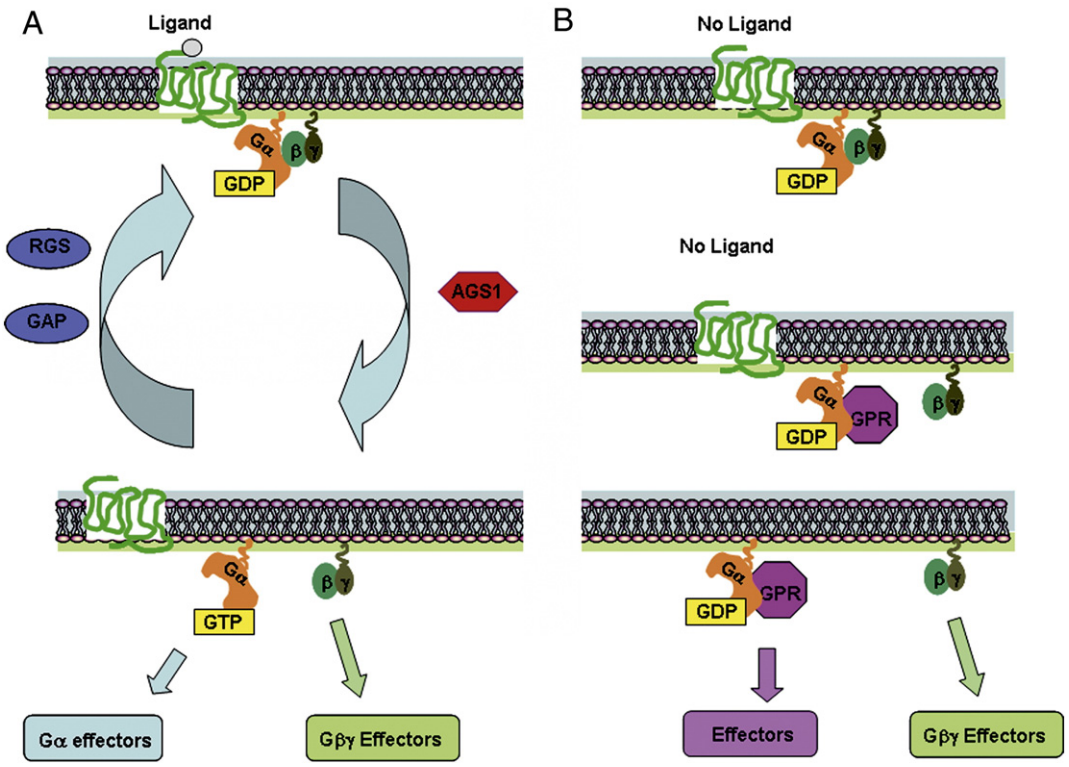

Fig. 2. G protein activation by receptors and AGS proteins. (A) G protein heterotrimers (Gαβγ) can be activated by GPCRs (green), inducing the exchange of GDP by GTP. Moreover, some activators of G protein signaling (AGS1) can promote the exchange process in the abscence of receptor activation, whereas regulators of G protein signaling (RGS) and GTPase activating proteins (GAP) increase the rate of GTP hydrolization. (B) Alternatively, other G protein regulators can also activate G proteins signaling in the absence of receptor-mediated activity and without promoting nucleotide exchange.

3.7. Subcellular localization of GPR-containing proteins and membrane interactions

The interactions between proteins containing GPR-motifs and $G\alpha_i$ subunits can be regulated by restricting the subcellular distribution of these proteins to specific microenvironments. In this sense, both AGS3 and AGS5 (LGN) contain a tetracopeptide (TPR) motif, a highly degenerate sequence found in a large number of proteins that serves different functions [112]. Thus, TPR motifs contribute to target the protein to different microdomains within the cell via protein–protein interactions [139]. TPR-interacting proteins can influence the subcellular localization of AGS3 and 5 and/or their interaction with $G\alpha_i$ subunits. Neither AGS3 nor LGN have any apparent hydrophobic domains or consensus sequences for acylation that would mediate their membrane association [140]. However, the TPR regions of the *Drosophila* AGS3 homologue PINS play a key role in its localization to the cell membrane. Indeed, these domains are required for translocation of the protein to a specific membrane region of the neuroblast, which is achieved by the binding of the protein INSCUTEABLE to the TPR domains in PINS [121,122]. It has been shown that the correct targeting of the complex PINS-Gαi to the apical cortex of *Drosophila* neuroblasts is essential to induce asymmetric cell division, a mechanism to achieve cellular diversity during development. In this context, a proper positioning of the mitotic spindle is an essential step for asymmetric division. Although the molecular bases of this mechanism are not fully understood, their spatial and temporal activation is controlled by heterotrimeric G protein signaling as well as proteins containing the GPR motif. In *C. elegans*, the interaction of GPR1/2 and Gα at the posterior cortex is critical for proper asymmetric positioning of the mitotic spindle. Furthermore, the role of the mammalian GPR proteins LGN and AGS3, G-proteins and possibly other accessory proteins, in cell division and polarity in higher organisms is still under investigation. Recent reports indicate important roles for these proteins in asymmetric cell division of neuronal precursors, retinal progenitors and epidermal cells, and in cell divisions that occur in the absence of polarity cues [112].

AGS3 is one of the best AGS proteins studied to date and two isoforms of this protein have been identified: the AGS3-long form and a truncated version named AGS3-short. The major difference between them is that AGS3-short lacks the seven TPR motifs of the amino-terminal region of AGS3-long, which affects its cellular distribution. As such, confocal microscopy studies indicate that while AGS3-short is diffusely distributed across the cytoplasm of the cell, AGS3-long adopts a punctuate distribution that reflects its localization to defined membrane microdomains. Therefore, the TPR motifs play a key role in the localization of AGS3 as further reflected by the segregation of AGS-short mostly to the cytosolic fraction following subcellular fractionation, while AGS3-long appears in both the crude membrane and cytosolic fractions. Interestingly, interaction of AGS3-short with G proteins also inhibits guanine nucleotide exchange and the translocation of AGS3-short to membranes [139].

Hence, most cellular AGS3 is cytosolic although a small subpopulation is found in membrane fractions [138,140,141]. It is not known how AGS3 associates with membranes, and while the TPR domains may be implicated as mentioned above, other possible binding partners may also participate in this process. Membrane expressed AGS3 does not appear to interfere with receptor-G_i protein coupling [142] although cytosolic AGS3 can interact with membrane-associated $G\alpha_{i1}$ subunits to remove them from their native membrane environment. Thus, cytosolic AGS3 interferes with receptor-Gi protein coupling by sequestering $G\alpha_i$ and blocking its membrane association during reconstitution. Furthermore, cytosolic AGS3 blocks the membrane association of $G\alpha_{i1}$ subunits, but has no effect on the membrane association of Gβγ subunits. However, when cytosolic AGS3 associates with the membrane it loses the capacity to interfere with receptor-G protein coupling. Several studies indicate that assembly with Gβγ

drives the localization of Gα to the plasma membrane or to specific membrane domains [25,27,28,31]. Recently, a model was described in which all three G protein subunits are synthesized in the cytosol and that Gα and Gβγ bind to each other before they translocate to the plasma membrane [27]. Since AGS3 can compete with Gβγ subunits for $G\alpha_i$, cytosolic AGS3 may disturb the assembly of Gβγ with Gα subunits and thereby decrease the amount of Gα protein at the plasma membrane, which may influence signaling pathways regulated by Gβγ.

The interaction of AGS3 with G proteins can be also regulated by phosphorylation of the GPR motif by the serine-threonine kinase LKB1, blocking its interaction with $G\alpha_i$ [143]. Similarly, phosphorylation close to the GPR motif of RGS14 may influence its interaction with $G\alpha_i$ [144]. RGS14 interacts with proteins of the heterotrimeric and monomeric G protein pathways through its multiple GPR domains [145]. In addition to its N-terminal RGS domain, RGS14 also contains a C-terminal GPR motif that acts as a GDI for $G\alpha_{i1}$ and $G\alpha_{i3}$ [146,147]. However, when $G\alpha_i$ and Gβγ are appropriately modified with lipids, the heterotrimer can not be disrupted by RGS14, indicating that only free $G\alpha_i$ is a substrate for the GPR domain of RGS14. Alternatively, RGS14 might require another protein to exert its effects on heterotrimeric G proteins. The resistance of the heterotrimer to the GDI activity of RGS14 depends on the presence of lipid modifications on both the $G\alpha_i$ and Gβγ subunits. However, myristoylation of Gα and prenylation of Gγ are required for the high affinity interaction of Gα and Gβγ [148,149]. In transducin ($G\alpha_t$), the Gα acyl chain appears to bind co-operatively with the Gγ farnesyl moiety to model membrane lipids [150]. Thus, lipid–lipid interactions also participate in the association of the subunits of oligomeric proteins by regulating their orientation, providing productive protein interaction surfaces for their final binding. Thus, in the absence of lipid–lipid interactions, the affinity of Gα for Gβγ is reduced, enabling RGS14 to compete with Gβγ. This is supported by other studies, indicating that GPR peptides are able to physically disrupt the heterotrimeric complex when G proteins lack some lipid modifications. Indeed, this occurs with a peptide derived from AGS3 that interferes with the association of the G protein heterotrimer when the $G\alpha_i$-subunit is not myristoylated [138]. Moreover, it has been shown that a peptide corresponding to the RGS14-GPR motif prevents the formation of heterodimers containing non-lipidated G protein subunits [151]. In other studies, using lipidated G protein subunits, a peptide derived from AGS3 has been shown to induce heterotrimeric subunit dissociation, but the excess of peptide over the G proteins used in these experiments probably does not reflect a physiological situation [152]. Overall, it seems that G protein lipid modifications also play an important role determining the effect of the GPR-containing proteins in these signaling pathways.

4. Conclusions

The purpose of this review was to highlight how protein–lipid interactions of monomeric and trimeric G proteins with membranes influence their activity. We also described the role of lipid modifications and membrane lipid–protein interactions in the induction of canonical and alternative G protein signaling pathways, independent of receptor activation. During recent years, the interactions between G proteins and membranes have been shown to be involved in controlling cell signaling. These interactions also regulate the way G proteins propagate signals and add versatility to the messages received by cells. In addition, signal processing via heterotrimeric G proteins has been challenged with alternative and unexpected regulatory mechanisms based on the identification of new proteins. The functional roles associated with these new proteins identify novel areas of research that mostly remain to be addressed. GPCRs and associated proteins are important in cell signaling and human physiology, controlling important functions and influencing the etiology of some diseases. Therefore, it is not surprising that over a half of all drugs currently under development are targeted to these receptors. In this context, membrane lipid composition and structure regulates GPCR-associated signaling and therefore, membrane lipid regulation can be used to define new therapeutic approaches such as membrane–lipid therapy [153].

Acknowledgements

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