G Protein Trafficking

Philip B. Wedegaertner

Department of Biochemistry and Molecular Biology, Thomas Jefferson University, 233 S. 10th St., 839 BLSB, Philadelphia, PA 19107-5541, USA

Abstract

The classical view of heterotrimeric G protein signaling places G proteins at the cytoplasmic surface of the cell's plasma membrane where they are activated by an appropriate G protein-coupled receptor. Once activated, the GTP-bound $G\alpha$ and the free $G\beta\gamma$ are able to regulate plasma membrane-localized effectors, such as adenylyl cyclase, phospholipase C-β, RhoGEFs and ion channels. Hydrolysis of GTP by the $G\alpha$ subunit returns the G protein to the inactive $G\alpha\beta\gamma$ heterotrimer. Although all of these events in the G protein cycle can be restricted to the cytoplasmic surface of the plasma membrane, G protein localization is dynamic. Thus, it has become increasingly clear that G proteins are able to move to diverse subcellular locations where they perform non-canonical signaling functions. This chapter will highlight our current understanding of trafficking pathways that target newly synthesized G proteins to the plasma membrane, activation-induced and reversible translocation of G proteins from the plasma membrane to intracellular locations, and constitutive trafficking of G proteins.

Keywords

Assembly; G protein; Isoprenylation; Myristoylation; Palmitoylation; Trafficking

11.1 Introduction

A major role of heterotrimeric G proteins is to couple activated G protein-coupled receptors at the cell surface to appropriate intracellular signaling pathways (Preininger and Hamm 2004; Dorsam and Gutkind 2007). As the name implies, heterotrimeric G proteins are composed of three subunits, $G\alpha$, $G\beta$ and $G\gamma$, encoded by 16, 5 and 12 genes, respectively, in mammals. Based on sequence identity and functional similarity, $G\alpha$ are typically grouped into four sub-families, termed $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$. $G\beta$ and $G\gamma$ form an irreversible $G\beta\gamma$ dimer, with the exception of the $G\beta_5$ subunit (Sondek and Siderovski 2001), and functional differences between different $G\beta\gamma$ combinations are not well-defined.

G proteins function as molecular switches by cycling between guanine-nucleotide bound states. The G protein cycle is characterized by $G\alpha$ binding to either GDP or GTP and by dissociation and association of $G\alpha$ and $G\beta\gamma$. In the inactive state, the G protein exists as a heterotrimer in which $G\alpha$ binds GDP. When a cell-surface GPCR is activated by binding an

Correspondence to: Philip B. Wedegaertner.

extracellular agonist, it interacts with its cognate heterotrimeric G protein at the cytoplasmic surface of the plasma membrane and catalyzes the release of GDP from Gα. Next, GTP replaces GDP by binding to Gα. GTP binding to Gα induces a conformational change that decreases its affinity for Gβγ and increases its affinity for effector proteins. Consequently, Gα and Gβγ dissociate, at least partially (Lambert 2008), and both GTP-bound Gα and free Gβγ are able to interact with and regulate numerous effector proteins, such as ion channels, adenylyl cyclases and phospholipases, to name a few classical examples. The intrinsic GTP hydrolysis activity of Gα, which is often greatly accelerated through binding to RGS (regulator of G protein signaling) proteins, returns Gα to the GDP-bound state and allows it to interact again with Gβγ, thereby reforming an inactive heterotrimer.

The classical view of heterotrimeric G protein signaling focuses on G proteins being associated with the cytoplasmic surface of the plasma membrane (Fig. 11.1). In this view, G proteins remain tethered to the PM while traversing through the G protein cycle and carrying out their cellular signaling functions. However, it has become clear that G proteins are not always localized at the cytoplasmic surface of the PM. Instead, G proteins dynamically localize to diverse subcellular locations and transit specific trafficking pathways. Thus, superimposed upon the classical view of G protein function, we must consider how trafficking of G proteins provides novel mechanisms for regulating G protein signaling. This chapter will discuss our current knowledge of G protein trafficking to the PM after synthesis, activation-dependent and constitutive internalization and recycling of G proteins, and how trafficking to unique subcellular locations provides novel G protein signaling functions.

11.2 Mechanisms of Membrane Binding by G Proteins

Localization to cellular membranes is required for many, though maybe not all, functions carried out by G proteins. Of major importance, several different lipids are covalently attached to G protein subunits, thereby serving as hydrophobic membrane anchors (Table 11.1). In addition, both Gα and Gβγ subunits appear to contain poly-basic motifs that provide electrostatic interactions with negatively charged regions of membranes. The interplay of these different membrane binding mechanisms is critical for controlling the trafficking of G proteins.

When considering how membrane binding affects trafficking, it is important to consider that often two or more membrane binding mechanisms function together in a protein or protein complex. Whereas a single membrane binding signal, be it an attached lipid, polybasic sequence or other motif, may have weak affinity for a membrane, two or more signals can synergize to provide a high affinity multivalent membrane interaction. This is often referred to as the two-signal hypothesis or kinetic trapping (Dunphy and Linder 1998; Hancock et al. 1990; McLaughlin and Aderem 1995; Morales et al. 1998; Resh 1999). Another key point is that one of the membrane binding signals may direct the protein to a specific membrane or membrane domain. Lastly, changes in one of the membrane binding signals can allow regulation of membrane binding. As will be extensively discussed below, multiple membrane binding signals are found in heterotrimeric G proteins, and thus the combination of signals and the ability of different signals to be regulated can have profound effects on membrane binding by G proteins.

11.2.1 Gα Subunits Are N-Terminally Myristoylated and/or Palmitoylated

All Gα of the $Gα_i$ family are covalently modified by the 14-carbon saturated fatty acid myristate at an extreme N-terminal glycine (Table 11.1). After the initiating methione is removed by a methionyl amino peptidase, a prerequisite for myristoylation to occur, a glycine at position 2 becomes the new N-terminal amino acid. The attachment of myristate through an amide bond to the free amino group of the glycine is catalyzed by an N-myristoyl transferase (NMT), of which there are two isoforms in mammals, NMT1 and NMT2. NMTs recognize a consensus sequence of G_2-X_3-X_4-X_5-$(S/T/C)_6$. The N-terminal glycine is essential; myristate is not attached to any other amino acid. Serine, threonine or cysteine is preferred at position 6, but other amino acids can be allowed. Moreover, other amino acids at positions 3–7 can influence the ability of an N-terminal sequence to be recognized and myristoylated by a NMT. Gα of the other families, $Gα_s$, $Gα_q$ and $Gα_{12}$, are not myristoylated simply because they lack a glycine at position 2 or fail to conform to the N-terminal consensus sequence even though they have a glycine at position, as in the case of $Gα_s$.

Myristoylation typically occurs co-translationally as the nascent $Gα_i$ subunit is emerging from the ribosome. Although long thought to exclusively occur co-translationally, more recently it has become clear that myristoylation can occur post-translationally as well, usually when an internal glycine is exposed and becomes an N-terminal amino acid after cleavage by the apoptotic caspases (Martin et al. 2011). So far, there is no evidence to indicate that $Gα_i$ can be post-translationally myristoylated.

Myristate is not an abundant fatty acid in the cell, and thus the myristoylation process is very selective for myristate as opposed to other more abundant fatty acids (reviewed recently in Martin et al. 2011). However, the retinal-specific $Gα_t$, as well as several other retinal proteins, has been found to be heterogeneously acylated at the N-terminal glycine (Kokame et al. 1992; Johnson et al. 1994; Neubert et al. 1992). Instead of only having myristate (C14:0, in which C14 indicates the carbon chain length, and 0 indicates the number of unsaturated bonds) attached, $Gα_t$ also exists in isoforms with attached 5-cis-tetradecenoic acid (C14:1), 5-cis, 8-cis- tetradecadienoic acid (C14:2) and laurate (C12). This heterogeneous N-acylation appears to occur only in the retinal photoreceptor cells due to unique characteristics of lipid metabolism in the retina compared to other cells in the body (Bereta and Palczewski 2011). The function of the differently modified isoforms of $Gα_t$ remains incompletely understood. Studies have indicated that laurate modified forms bind with lower affinity to Gβγ and have lower steady-state GTPase activity (Hashimoto et al. 2004; Kokame et al. 1992), suggesting that the lipid moiety can influence protein-protein interactions or intramolecular interactions. Alternatively, the functional differences may simply reflect that $Gα_t$ isoforms with less hydrophobic fatty acids attached have lower affinity for membranes and thus decreased function. Regardless, the existence of differently modified forms of $Gα_t$ provides the opportunity for important functional differences, and it remains possible that different isoforms of $Gα_t$ with different affinities for membranes could exhibit differences in trafficking. This speculation remains to be examined.

Lastly, myristoylation appears to be an irreversible modification. Since myristate is added co-translationally and mechanisms for its removal do not seem to exist, myristate appears to

be attached to $G\alpha_i$ subunits throughout their lifetime; thus, myristoylation does not fit the concept of a regulatory modification. However, the relatively weak affinity of myristate for membranes, allows other factors to regulate the reversible membrane binding of myristoylated proteins (Resh 2006). For example, the covalent attachment of another lipid, such as palmitate (discussed below) can synergize with myristate to provide very strong membrane binding, while phosphorylation of a myristoylated protein can shift the protein off of the membrane by providing electrostatic repulsion from acidic membrane lipid headgroups. In this way covalently attached myristate, although irreversible, can affect membrane localization and trafficking of proteins, such as $G\alpha_i$ subunits.

In contrast to myristoylation, palmitoylation occurs post-translationally, is readily reversible, lacks a clear consensus motif and functions as a stronger membrane anchor due to its greater hydrophobicity. With the exception of $G\alpha_t$ and $G\alpha_{gust}$, all Gα subunits can be palmitoylated at one or more cysteines located within the first 20 N-terminal amino acids (Table 11.1). For members of the $G\alpha_i$ family, except for $G\alpha_t$ and $G\alpha_{gust}$ which simply do not have any N-terminal cysteines, palmitoylation occurs at a cysteine at position 3 immediately adjacent to the glycine site of myristoylation. Because palmitoylation is a post-translational modification, co-translational myristoylation obviously happens before palmitoylation, and in fact myristoylation is a prerequisite for palmitoylation of $G\alpha_i$ subunits. Numerous studies have shown that glycine to alanine mutants at position 2 of $G\alpha_i$ subunits not only fail to be myristoylated but also lack palmitoylation (Wilson and Bourne 1995; Mumby et al. 1990; Jones et al. 1990; Hallak et al. 1994; Galbiati et al. 1994). Subunits of the $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$ families all undergo palmitoylation in the absence of myristoylation. No clear sequence motif or position within the N-terminus is obvious for the sites of palmitoylation in the non-myristoylated Gα. In general sites of palmitoylation have not been easy to predict, although recently there have been advances in the computational prediction of palmitoylation sites (Ren et al. 2008; Hu et al. 2011; Li et al. 2011). For Gα subunits the key prerequisite for palmitoylation appears to be the existence of additional membrane targeting signals. These signals can be myristoylation, in the case of $G\alpha_i$ subunits, binding to other proteins, such as the G$\beta\gamma$ subunits, and polybasic membrane targeting motifs. This concept of multiple membrane targeting signals working to fine-tune membrane binding and trafficking is discussed further below.

In palmitoylation, the 16-carbon fatty acid palmitate is linked to a cysteine amino acid through a thioester bond. For many years the cellular machinery of palmitoylation remained elusive. That all changed with the identification of a family of proteins with palmitoyl acyltransferase activity (Lobo et al. 2002; Roth et al. 2002). This family of more than 20 proteins has been termed DHHC proteins for the presence of a conserved catalytic aspartate-histidine-histidine-cysteine motif. A great deal of current research is directed towards trying to understand the specificity of DHHC proteins. Several different DHHC proteins, such as DHHC3 and DHHC7, appear to be capable of mediating the palmitoylation of Gα subunits. Importantly, it was shown that siRNA-mediated depletion of DHHC3 and DHHC7 resulted in decreased palmitoylation of $G\alpha_q$ and a loss of its PM localization (Tsutsumi et al. 2009). Together with numerous studies in which cysteine sites of palmitoylation in various Gα were mutated to non-palmitoylatable serines or alanines (Bhattacharyya and Wedegaertner 2000; Hughes et al. 2001; Pedone and Hepler 2007 ; Wedegaertner et al. 1993, 1996; Wise et

al. 1997), the DHHC knockdown approach establishes palmitoylation as an essential requirement for membrane localization of Gα.

Palmitoylation is a reversible modification, and indeed it has been shown using pulse-chase experimental approaches that the half-life of palmitate attached to Gα is relatively short, on the order of 1 h, compared to the much longer half-life of the Gα protein. More interestingly, the turnover of the palmitate attached to Gα, *i.e.*, depalmitoylation, can be accelerated upon activation (Wedegaertner and Bourne 1994; Chen and Manning 2000; Bhamre et al. 1998; Loisel et al. 1999), suggesting that the palmitoylation status of Gα can be subject to regulation to affect localization and function. The cellular machinery that regulates depalmitoylation of Gα and other signaling proteins is being actively investigated (Zeidman et al. 2009), and acyl protein thioesterase 1 (APT1) is one key enzyme that can depalmitoylate Gα. APT1 in the yeast *Saccharomyces cerevisiae* was shown to be responsible for much of the depalmitoylating activity in yeast, including the depalmitoylation of the yeast Gα, Gpa1 (Duncan and Gilman 2002). Moreover, mammalian APT1 was over-expressed together with $Gα_s$ in cultured cells resulting in a more rapid turnover of palmitate attached to $Gα_s$ (Duncan and Gilman 1998). A more recent study showed that siRNA-mediated depletion of APT1 in cultured cells resulted in an increase in the fraction of $Gα_{13}$ associated with membranes compared to cytoplasm; this result was consistent with the proposal that inhibition of $Gα_{13}$ depalmitoylation leads to more palmitoylated $Gα_{13}$ and thus more membrane bound $Gα_{13}$. This study went on to suggest that APT1 regulation of the palmitoylation status of $Gα_{13}$ is important for dendritic spine morphogenesis in neurons (Siegel et al. 2009). The reversibility of palmitoylation provides an important point of regulation for G protein signaling, and, as will be discussed below, cycles of palmitoylation and depalmitoylation can have important consequences for regulating G protein trafficking.

11.2.2 Gγ Subunits Are C-Terminally Isoprenylated

A variety of cellular peripheral membrane proteins, including all Gγ subunits, are post-translationally modified at their C-termini by an isoprenyl group, either a 15-carbon farnesyl or a 20-carbon geranylgeranyl (Table 11.1) (Lane and Beese 2006; Wright and Philips 2006). Farnesyl and geranylgeranyl transferases have been well characterized. These cytoplasmic enzymes specifically recognize a C-terminal CaaX motif on substrate proteins, in which a cysteine (C) is followed by two aliphatic (a) amino acids and the ultimate C-terminal amino acid (X) can be one of several possibilities. The last amino acid determines whether the substrate protein is farnesylated or geranylgeranylated. $Gγ_1$, $Gγ_9$ and $Gγ_{11}$ are farnesylated due to having a serine in the C-terminal X position, while the other nine Gγ have a leucine in the X position and are geranylgeranylated. In either case, the isoprenyl group is attached via a thioether bond to the cysteine of the CaaX motif. One critical difference between the two isoprenyl modifications is that geranylgeranyl is more hydrophobic than farnesyl, and thus provides stronger membrane binding. Such differences in isoprenylation may contribute to functional differences of Gβγ isoforms. For a discussion of unique functions of Gβγ composed of select Gβ and Gγ subunits, see Chap. 10 in this book. Most proteins that are isoprenylated at the cysteine of the CaaX then undergo further processing consisting of proteolytic removal of the last three –aaX amino acids, thus

providing an isoprenyl cysteine as the new C-terminal amino, followed by methylation of the C-terminal carboxyl group. The role of these subsequent modifications is not altogether clear. As discussed below, the –aaX proteolysis and carboxymethylation may be essential to direct the initial trafficking of nascent G proteins. Additionally, carboxymethylation contributes to an increased hydrophobicity of the Gγ C-terminus; studies suggest that this carboxymethylation is important for stronger membrane binding by farnesylated Gβγ, but not the more hydrophobic geranylgeranylated Gβγ (Michaelson et al. 2002). Our current knowledge is consistent with the following temporal model for Gβγ assembly and lipid modification: (1) Newly synthesized Gβ and Gγ are assembled in the cytoplasm; (2) Gγ, in the context of a Gβγ dimer, is either farnesylated or geranylgeranylated; (3) the C-terminal three (-aaX) amino acids are removed; and (4) the new C-terminus is carboxymethylated (Marrari et al. 2007). Importantly, several chaperone proteins have been identified that facilitate the formation of the Gβγ subunit after synthesis. See Chaps. 8 and 9 in this book for a detailed analysis of these chaperone proteins, and these are also briefly discussed later herein. In summary, the irreversible post-translational isoprenyl modification of Gγ provides an essential membrane anchor for Gβγ, and cooperates with myristate and/or palmitate modifications of Gα to affect the localization, function and trafficking, as discussed further below, of the G protein heterotrimer.

11.2.3 Polybasic Motifs in Gα and Gβγ

In addition to lipid modifications of the G protein subunits, regions of positive charge on the proteins have been shown to contribute to membrane localization. Structural studies of $Gβ_1γ_1$ originally showed that $Gβ_1$ has a surface of positive charge that is predicted to facilitate Gβγ binding to membranes by ionic interactions with acidic membrane phospholipids (Sondek et al. 1996). The positively charged amino acids that comprise the basic region in $Gβ_1$ are conserved in $Gβ_2$, $Gβ_3$ and $Gβ_4$, consistent with an important role for this protein surface (Matsuda et al. 1994; Seitz et al. 1999; Murray et al. 2001). However, the importance of the basic region in Gβ in influencing membrane binding remains to be better defined. Many Gα have a large number of basic amino acids within the N-terminal helical region. For example $Gα_q$ contains 10 basic residues, both arginines and lysines, within amino acids 16–38. Most importantly, helical predictions indicate that most of the positively charged N-terminal amino acids align on one face of the helix, grouped together to form basic patches in position to interact with negatively charged surfaces of cellular membranes (Kosloff et al. 2002; Crouthamel et al. 2008, 2010; Pedone and Hepler 2007). Indeed, replacement of select N-terminal basic amino acids in $Gα_s$ and $Gα_q$ with non-charged alanines or glutamines results in decreased membrane localization of the mutant $Gα_s$ and $Gα_q$ compared to wild type (Crouthamel et al. 2008). Interestingly, these N-terminal polybasic patches are only found in the non-myristoylated Gα, *i.e.*, the $Gα_s$, $Gα_q$ and $Gα_{12/13}$ families; Gα of the myristoylated $Gα_i$ family do not contain pronounced N-terminal polybasic regions (Kosloff et al. 2002). Thus, we can consider that the N-terminal polybasic regions take the place of myristoylation to function, in conjunction with binding to Gβγ, as initial membrane targeting signals required for promotion of palmitoylation and PM localization.

11.3 G Protein Trafficking to the Plasma Membrane After Biosynthesis

Work over the last decade has revealed complex and specific pathways that allow G proteins to move to the plasma membrane after synthesis. G proteins do not simply diffuse from the cytoplasm to the inner surface of the PM after synthesis. Instead, several chaperone proteins mediate proper folding and assembly of the subunits, and distinct trafficking pathways are followed that involve localization at the cytoplasmic surface of intracellular organelles (Fig. 11.2). The assembly of the Gβγ dimer has been well-reviewed recently (Willardson and Howlett 2007; Marrari et al. 2007; Dupre et al. 2009) and is also covered by other chapters in this collection; thus, here will be provided only an overview of the Gβγ assembly process, together with describing recent developments on understanding the assembly of the G protein heterotrimer and the organelles involved in trafficking to the PM.

Our current knowledge indicates that assembly of the Gβγ dimer requires the following steps. Newly synthesized Gβ binds to the chaperonin containing tailless complex polypeptide 1 (CCT) which serves as a chaperone to prevent aggregation of unfolded Gβ (Kubota et al. 2006; Wells et al. 2006). Next, phosducin-like protein 1 (PhLP1) binds to the CCT- Gβ complex to further facilitate proper folding of Gβ (Lukov et al. 2005; Knol et al. 2005). Then phosphorylation of PhLP1 by casein kinase 2 (CK2) releases a complex of PhLP1 and properly folded Gβ from CCT (Lukov et al. 2006; Humrich et al. 2005). This phosphorylation event is particularly intriguing, as it suggests a regulatory mechanism for a cell to rapidly regulate levels of Gβγ (Willardson and Howlett 2007), and indeed different tissues have been shown to contain substantially different amounts of phosphorylated versus non-phosphorylated PhLP1 (Humrich et al. 2003). The upstream cellular signals and pathways that regulate PhLP1 phosphorylation by CK2 remain to be investigated. While nascent Gβ is bound by CCT and PhLP1 to promote its proper folding, one report has demonstrated that a similar chaperone function for nascent Gγ is performed by an ER-localized protein termed dopamine receptor-interacting protein 78 (DRiP78) (Dupre et al. 2007). Then lastly in the assembly process, Gβ and Gγ are released from their chaperones, PhLP1 and DRiP78, respectively, to bind each other and thereby form the properly folded Gβγ dimer. After completion of the Gβγ assembly process, Gγ is modified, as described above, by isoprenylation, C-terminal proteolysis and C-terminal methylation. Although assembled Gβγ has been shown to be cytoplasmic before undergoing lipid modification, the ER localization of DRiP78 suggests that the assembly of Gβγ may actually occur at the cytoplasmic surface of the ER. After isoprenylation of Gβγ, possibly near the ER, the subsequent proteolysis and methylation clearly occurs at the cytoplasmic surface of the ER. Thus, a fully assembled and modified Gβγ initially localizes at the ER.

Recent evidence suggests that specific chaperone proteins may also exist for Gα. In particular, a protein termed Ric-8 stabilizes newly synthesized Gα (Gabay et al. 2011). Ric-8, named for resistance to inhibitors of cholinesterase, was originally identified in a screen in *C. elegans* for activators of neurotransmission. Subsequent studies showed that Ric-8 functioned upstream of Gα and could bind to Gα. Next, it was shown that Ric-8, of which mammals have two forms, Ric-8A and Ric-8B, promoted GDP release from Gα *in vitro* (Tall et al. 2003). These results were exciting because they identified Ric-8 as an intracellular guanine-nucleotide exchange factor (GEF) for Gα; thus, G protein activation

can occur in the absence of a cell-surface localized GPCR. However, studies on the depletion of Ric-8 in organisms or cultured cells revealed the surprising findings that Ric-8 was needed for proper localization and expression of Gα, suggesting that Ric-8 served a role beyond its function as a GEF. In *Drosophila* cells, loss of Ric-8 resulted in diminished amounts of $Gα_i$ and $Gα_o$ and in a failure of the Gα to reside at the PM (David et al. 2005; Hampoelz et al. 2005; Wang et al. 2005). Interestingly, Gβ levels were also reduced and mislocalized. Ric-8 does not directly interact with Gβγ; instead, diminished Gβγ is most likely a consequence of the lack of Gα. As will be discussed below, nascent Gβγ needs to interact with Gα to transit from the ER to the PM. Similar to the observations in *Drosophila*, in cultured mammalian cells depletion of Ric-8B caused a reduction in $Gα_s$ protein. Moreover, consistent with a chaperone function for Ric-8, overexpression of Ric-8A or Ric-8B promoted much higher levels of Gα from all four subfamilies, $Gα_i$, $Gα_s$, $Gα_q$ and $Gα_{12/13}$. Ric-8A binds $Gα_i$, $Gα_q$ and $Gα_{12/13}$, while Ric-8B preferentially interacts with $Gα_s$, suggesting that Ric-8 proteins might have a universal role as a chaperone for all Gα. To further support a role for Ric-8 as a Gα chaperone, a recent report showed *in vitro* that binding to Ric-8A increased the stability of $Gα_i$ (Thomas et al. 2011). A chaperone role for Ric-8 was more firmly established in another recent report. This studied showed that in cultured cells lacking either Ric-8A or Ric-8B the rate of degradation of select Gα was increased (Gabay et al. 2011). In cells lacking Ric-8A, $Gα_i$ and $Gα_q$ were efficiently translated; however, there seemed to be a specific defect in the Gα's ability to initialize localize to membranes (Gabay et al. 2011), thus suggesting that Ric-8 is required for nascent Gα to arrive at a cellular membrane. In summary, accumulated evidence indicates that Ric-8 serves a chaperone function for newly synthesized Gα. Moreover, Ric-8 may help to deliver Gα to the ER for interaction with Gβγ. Further studies should shed even more light on the mechanistic details of how Ric-8 functions as a Gα chaperone.

As alluded to above, a great deal of evidence indicates that G protein heterotrimer formation is crucial for PM localization of Gα and Gβγ. In other words, Gα does not efficiently traffic to the PM unless it binds to Gβγ, while Gβγ does not efficiently traffic to the PM unless it binds to Gα. Our current understanding is that nascent Gα and Gβγ interact to form the heterotrimer at an intracellular location, likely the ER or Golgi, after interaction with the appropriate chaperones and processing of the C-terminus of Gγ as described above. The reciprocal requirement of Gα and Gβγ interaction for efficient PM targeting has been demonstrated in several ways (Marrari et al. 2007). Overexpression studies in cultured cells showed that overexpressed Gβγ localizes to predominantly intracellular membranes, but when various Gα are also overexpressed along with Gβγ both Gα and Gβγ display strong localization at the PM (Takida and Wedegaertner 2003; Michaelson et al. 2002; Evanko et al. 2001). Reciprocally, a number of studies have shown that overexpressed Gα displays both PM and cytoplasmic localization, but co-expression with Gβγ results in strong PM localization of Gα (Hepler et al. 1993). Another line of evidence supporting the importance of heterotrimer formation for PM localization of Gα and Gβγ is the use of mutants deficient in partner interaction. Heterotrimer crystal structures have defined protein surfaces that mediate the association of Gα and Gβγ. Thus, mutation of key Gβγ-contacting amino acids in the N-termini of several Gα results in decreased PM localization to the Gα (Fishburn et al. 2000; Evanko et al. 2000). Likewise, mutation of select Gα-interacting amino acids in

Gβγ prevents its PM localization (Takida and Wedegaertner 2003). Such Gα-binding defective Gβγ localize primarily to ER membranes, where –CaaX processing of the Gγ takes place. Similarly, Gβγ-binding defective Gα localize to intracellular membranes, apparently ER (Fishburn et al. 2000), and cytoplasm (Evanko et al. 2000); intracellular membrane localization is observed for Gβγ-binding defective $Gα_z$, which is co-translationally myristoylated, while cytoplasm localization is the predominant localization for Gβγ-binding defective $Gα_s$ and $Gα_q$, both of which are non-myristoylated subunits (Fishburn et al. 2000; Evanko et al. 2000). Lastly, genetic deletions of specific G protein subunits provide further support for the reciprocal requirement of Gα and Gβγ interaction for PM localization. Because typical mammalian cells contain a number of different Gα and Gβγ subunits, deletion of one or two select subunits may not substantially affect overall Gα/Gβγ stoichiometry and thus may not reveal localization changes. However, model organisms or systems with fewer Gα and Gβγ have proved useful here. Yeast *S. cerevisiae* genetically lacking the Gα Gpa-1, and *C. elegans* embryos depleted of two critical Gα, GOA-1 and GPR-16, using RNAi, show a redistribution of Gβγ from the PM to intracellular sites (Song et al. 1996; Gotta and Ahringer 2001). Conversely, in *Drosophila* photoreceptor cells a loss of the eye-specific Gβ subunits results in a shift of $Gα_q$, the key Gα in photoreceptor signaling, from membrane to cytosolic fractions (Kosloff et al. 2003; Elia et al. 2005). Taken together, a variety of studies indicate that formation of the heterotrimer is an important step in stable localization of both Gα and Gβγ at the PM.

A logical hypothesis to explain the above described reciprocal requirement is that the G protein heterotrimer is formed at an intracellular site before or enroute to trafficking of the heterotrimer to the PM (Marrari et al. 2007). Current evidence supports a model in which heterotrimer formation occurs at the cytoplasmic surface of the ER or Golgi. Moreover, evidence exists for trafficking of nascent G proteins to the PM via either a Golgi-dependent or –independent pathway. Consistent with assembly of the heterotrimer at the ER, mutant Gβγ that fails to bind Gα and wild type Gβγ that is expressed in the absence of Gα localize predominantly at the ER (Michaelson et al. 2002; Takida and Wedegaertner 2003). Because expression and interaction with Gα is required to shift Gβγ from the ER to the PM, the simplest explanation is that Gα must interact with Gβγ at the ER. Moreover, neither treatment of cells with the Golgi disruptor Brefeldin A nor expression of a dominant negative Sar1, which blocks ER to Golgi transport, prevented expressed Gα and Gβγ from localizing at the PM (Takida and Wedegaertner 2004; Fishburn et al. 1999; Gonzalo and Linder 1998). These studies suggested that not only would the heterotrimer form before trafficking to the Golgi, *i.e.*, at the ER, but, in addition, trafficking to the PM would not require the Golgi. On the other hand, some studies have argued that Gα and Gβγ first interact at the Golgi and/or that the Golgi is required for movement of G proteins to the plasma membrane. One study observed apparent Golgi localization of co-expressed Gβγ and a palmitoylation site mutant of $Gα_i$, and thus suggested that heterotrimer interaction and Gα palmitoylation takes place at the Golgi (Michaelson et al. 2002). A recent study showed that DHHC-3 and DHHC-7 are required for palmitoylation of $Gα_i$, $Gα_s$ and $Gα_q$, and that these palmitoyl acyltransferases are localized to the Golgi (Tsutsumi et al. 2009). Moreover, others have argued that the Golgi is the main subcellular site for palmitoylation of all peripheral membrane proteins (Rocks et al. 2010). Although the above studies suggest a

functional Golgi may be required for palmitoylation and trafficking to the PM, they do not address whether or not the heterotrimer would first form at the ER before moving to the Golgi. Some light was shed on the question of the involvement of the Golgi in G protein trafficking to the PM by a report showing that the trafficking pathway depends on the protein complexes being formed. A functional Golgi was not required for PM localization of overexpressed Gα and Gβγ, as shown previously (Takida and Wedegaertner 2004); however, when a GPCR was also overexpressed, the Golgi was indeed required for PM localization of Gα, Gβγ and GPCR, suggesting that a heterotrimer in a complex with a GPCR takes a different pathway than a heterotrimer by itself (Dupre et al. 2006). Virtually all studies of G protein trafficking rely on overexpressed proteins; in the future it will be important to try to follow the route of G proteins that are endogenous or expressed at physiological levels.

In summary, heterotrimeric G proteins follow distinct trafficking pathways to reach the PM after synthesis (Fig. 11.2). Some of the key steps in trafficking routes include (1) localization of Gβγ at the cytoplasmic surface of the ER to undergo C-terminal modifications of Gγ; (2) Gα and Gβγ heterotrimer formation and Gα palmitoylation at an endomembrane site, either the cytoplasmic surface of the ER or Golgi; and (3) trafficking of the heterotrimer to the PM, either in a poorly defined Golgi-independent pathway from the ER to PM or in a Golgi-dependent pathway attached to the cytoplasmic surface of PM-destined vesicles. The different trafficking routes may reflect the intracellular formation of distinct protein complexes. Importantly, all of these novel trafficking steps provide opportunity for G proteins to regulate novel signaling pathways as well as targets for therapeutic intervention.

11.4 Activation-Dependent Internalization and Recycling of G Proteins

Once nascent G proteins reach the PM through specific trafficking pathways, they are able to carry out many of their classical signaling functions through interactions with GPCRs and effector proteins. However, G protein localization continues to be dynamic; they can rapidly and reversibly move from the PM to endomembrane locations. Over the last decade or more, evidence has emerged to support the notion that G proteins move throughout the cell in both constitutive and activation-dependent pathways (Figs. 11.3 and 11.4).

A variety of cell systems and experimental techniques have been used to establish that many G protein subunits translocate from the PM to intracellular locations in response to activation by an appropriate GPCR. One experimental system that has revealed a wealth of information about activation-induced G protein translocation is the mammalian visual system. A large number of studies have shown that activation of the GPCR rhodopsin by light in mammalian retinal photoreceptor cells results in a massive relocation of both the Gα and Gβγ subunits of transducin from the rod outer segment membranes to other subcellular locations (reviewed in Slepak and Hurley 2008; Artemyev 2008; Calvert et al. 2006). This G protein signaling system is somewhat unique due to the high concentration of rhodopsin and transducin and due to the fact that transducin has a weaker hydrophobic lipid attachment to membranes compared to other G proteins. $G\alpha_t$ is not palmitoylated. It is heterogeneously N-acylated by myristate (C14:0) and the less hydrophobic C14:1, C14:2 and C12 fatty acids. The transducin $G\beta_1\gamma_1$ is attached to the membrane by farnesylation of $G\gamma_1$; recall that

farnesylation, like myristoylation, provides a relatively weak membrane anchor. The translocation of transducin off of the rod outer segment membrane is driven by activation-induced dissociation of the subunits. The $\alpha_t\beta_1\gamma_1$ heterotrimer is attached tightly to the membrane due to the presence of two lipids, myristate and farnesyl. However, when light-activated rhodopsin promotes the loss of GDP and binding of GTP by $G\alpha_t$, $G\alpha_t$ and $G\beta_1\gamma_1$ dissociate and now each subunit is able to translocate off the membrane due the weaker attachment by one lipid. Indeed, it has been demonstrated that more than 80% of $G\alpha_t$ and $G\beta_1\gamma_1$ are released from the membrane within minutes of light activation, and this dramatic translocation has the critical physiological role of allowing adaptation of the photoresponse to very bright conditions (Sokolov et al. 2002). After translocation off of rod outer segment membranes, current studies indicate that the transducin subunits spread to the inner segment of the photoreceptor cell, and then during dark adaptation transducin reforms the heterotrimer and returns to the outer segment membranes.

Further mechanistic details of the translocation process have been uncovered recently, and this continues to be a very active area of research. For $G\beta_1\gamma_1$, phosducin binds to it and thereby further decreases the membrane affinity of $G\beta_1\gamma_1$, at least partly by neutralizing electrostatic interactions of $G\beta_1\gamma_1$ with the membrane (Sokolov et al. 2004; Murray et al. 2001). In this way, phosducin promotes the translocation of $G\beta_1\gamma_1$. For $G\alpha_t$, a computational study of its electrostatic potential using solved crystal structures indicated that negatively charged surface regions of $G\alpha_t$ would actually act as electrostatic repulsion to decrease the affinity of $G\alpha_t$ with a negatively charged membrane (Kosloff et al. 2008). Accordingly, once $G\alpha_t$ is dissociated from $G\beta_1\gamma_1$, the electrostatic repulsion would promote $G\alpha_t$ translocation. Additionally, the protein UNC119 was recently identified as a binding partner, with the exciting finding that UNC119 binds to the acylated N-terminus of $G\alpha_t$ in a complex in which the lipid is buried in a hydrophobic cavity of UNC119 (Zhang et al. 2011). The interaction of UNC119 with $G\alpha_t$ appears to promote the return of $G\alpha_t$ to outer segments (Zhang et al. 2011; Gopalakrishna et al. 2011). Another mechanistic detail that has been extensively addressed is the question of whether transducin translocation is driven by active transport or solely by diffusion. This has been a question of much debate and controversy in the field. Although some reports indicate that cytoskeletal elements are involved in transducin translocation and would thus serve as tracks for motor-driven active transport (Peterson et al. 2005), a number of researchers argue for diffusion-mediated transport, based a great deal on theoretical considerations (see these reviews for a in-depth discussion of mechanisms of transducin translocation, including diffusion versus active transport (Slepak and Hurley 2008; Artemyev 2008; Calvert et al. 2006)). Although some of the underlying mechanisms still need to be resolved, light-activated and reversible translocation of transducin clearly provides our most detailed and robust example of activation-induced trafficking of G proteins.

The *Drosophila* visual system has also been used as a model system to study G protein trafficking. Of particular interest, the visual system in flies uses palmitoylated $G\alpha_q$ to signal responses to light rather than the non-palmitoylated $G\alpha_t$ as is used in vertebrate visual signaling. Nonetheless, $G\alpha_q$ has been shown to translocate from membranes to the cytoplasm in response to light activation of photoreceptor cells (Kosloff et al. 2003; Cronin et al. 2004; Frechter et al. 2007). Similar to transducin in vertebrate visual signaling, $G\alpha_q$

translocation occurred in a matter of minutes after light activation. On the other hand, in *Drosophila* visual signaling it is not clear whether the relevant Gβγ traffics from the membrane after light activation, although studies using flies with reduced levels of Gβγ in the eye showed that $G\alpha_q$ needs Gβγ to return to the membrane. Furthermore, the recycling of $G\alpha_q$ required the photoreceptor-specific myosin III NINAC (Cronin et al. 2004). Although this study suggests that active transport would thus drive $G\alpha_q$ back to the membrane after light activation, more studies are needed to address the mechanism. Lastly, and again similar to vertebrate signaling, changes in the membrane content of $G\alpha_q$ allow adaptation to varying levels of light (Frechter et al. 2007). Thus, light-dependent translocation of G proteins appears to be a conserved mechanism for light adaptation.

Activation-induced G protein translocation has also been clearly identified to occur in non-visual signaling. Studies on $G\alpha_s$ provide the most information regarding activation-induced trafficking of G proteins in non-visual systems. A number of reports have demonstrated that $G\alpha_s$ can translocate from the PM to intracellular locations upon activation by a GPCR, such as the β2-adrenergic receptor (β2-AR), by cholera toxin or by introduction of a GTPase-inhibiting mutation (Allen et al. 2005; Ransnäs et al. 1989; Levis and Bourne 1992; Thiyagarajan et al. 2002; Wedegaertner and Bourne 1994; Wedegaertner et al. 1996; Yu and Rasenick 2002; Hynes et al. 2004). Immunofluorescence microscopy and biochemical fractionation of over-expressed and endogenous $G\alpha_s$, as well as live cell imaging of $G\alpha_s$ fused to fluorescent proteins, have been used to document activation-induced translocation of $G\alpha_s$. Agonist stimulation of β2-AR in cultured cells promotes $G\alpha_s$ translocation in a matter of 1–5 min, and indeed this represents a similar timecourse as observed for internalization of β2-AR (Hynes et al. 2004; Allen et al. 2005; Yu and Rasenick 2002 ; Wedegaertner et al. 1996). Some studies using immunofluorescence microscopy of fixed cells have observed β2-AR-activated $G\alpha_s$ to be homogeneously distributed throughout the cytoplasm, suggesting a simple diffusion of activated $G\alpha_s$ from the PM into the cytoplasm (Thiyagarajan et al. 2002; Wedegaertner et al. 1996), while other studies using live cell imaging have observed more punctate cytoplasmic localization of β2-AR-activated $G\alpha_s$, suggesting vesicle-mediated trafficking of $G\alpha_s$ (Hynes et al. 2004; Allen et al. 2005; Yu and Rasenick 2002). The nature of the trafficking pathway utilized by activated $G\alpha_s$ for translocation off of the PM remains to be better understood. As described above for $G\alpha_t$, more studies are needed to understand whether activation-induced translocation of $G\alpha_s$, and other G proteins, is driven by diffusion or mediated by active transport on vesicles.

Although substantial evidence exists for activation-induced trafficking of $G\alpha_s$, similar translocation has been much less documented for other non-visual system Gα. GPCR-induced redistribution of $G\alpha_{q/11}$ has been demonstrated in mammalian cell culture; this is consistent with demonstrations of $G\alpha_q$ translocation in response to light in *Drosophila* photoreceptors, as described above. Accordingly, $G\alpha_{q/11}$ translocation off of the PM was demonstrated in response to agonist activation of two Gq-coupled GPCRs, angiotensin II receptor and thyrotropin-releasing hormone receptor I (Drmota et al. 1998, 1999; Miserey-Lenkei et al. 2001). The temporal and spatial aspects of observed GPCR-induced $G\alpha_{q/11}$ have differed. $G\alpha_{q/11}$ appeared to translocate diffusely into the cytoplasm in 5–20 min in response to angiotensin II activation (Miserey-Lenkei et al. 2001); on the other hand, it took 2 h for $G\alpha_{q/11}$ to translocate off of the PM after stimulation of the thyrotropin-releasing

hormone receptor I, and $G\alpha_{q/11}$ was observed in intracellular puncta, possibly vesicles (Drmota et al. 1998). As with $G\alpha_s$, a great deal more studies with activation-induced trafficking of $G\alpha_{q/11}$ are needed. Somewhat surprisingly, $G\alpha_t$, $G\alpha_s$ and $G\alpha_{q/11}$, both in *Drosophila* vision and non-visual cell culture, are the only $G\alpha$ for which strong evidence has shown activation-induced trafficking. One interesting feature of these $G\alpha$ is that they are either solely myristoylated ($G\alpha_t$) or solely palmitoylated ($G\alpha_s$ and $G\alpha_{q/11}$) but not both myristoylated and palmitoylated as is found in most members of the $G\alpha_i$ family. The inability of certain $G\alpha$, such as the myristoylated plus palmitoylated $G\alpha_i$ family members, to redistribute upon activation may be due to lipid modification differences, such as differences in rates or extents of depalmitoylation, as discussed below, or, alternatively, possibly the necessary imaging techniques have not yet been employed to observe such trafficking of $G\alpha_i$.

Although much remains to be elucidated regarding the mechanistic details of activation-induced G protein trafficking, it is clear that one key step involves depalmitoylation of $G\alpha$ (Marrari et al. 2007). Depalmitoylation dramatically decreases the membrane binding affinity of $G\alpha$, and thus promotes release from the PM. $G\alpha_t$ is the only $G\alpha$ that does not undergo palmitoylation, and it was discussed above how activation decreases its membrane affinity and allow translocation. For other $G\alpha$, removal of attached palmitate is necessary to allow translocation into the cytoplasm. The strongest evidence for activation-induced depalmitoylation has been obtained for $G\alpha_s$, maybe not surprisingly, since $G\alpha_s$ also provides the best non-visual system example of activation-induced translocation. Indeed, a strong correlation has been demonstrated for activation, translocation and depalmitoylation for $G\alpha_s$. As observed for translocation of $G\alpha_s$, mutational activation of $G\alpha_s$ or β_2-AR-mediated activation $G\alpha_s$ promotes more rapid turnover of attached palmitate as revealed by palmitate labeling studies (Wedegaertner and Bourne 1994; Degtyarev et al. 1993; Mumby et al. 1994). Activation of appropriate GPCRs has also been shown to increase palmitate turnover on other $G\alpha$ (Chen and Manning 2000; Stanislaus et al. 1997, 1998; Gurdal et al. 1997; Bhamre et al. 1998; Loisel et al. 1999; Stevens et al. 2001), including $G\alpha_q$ and $G\alpha_i$, thus indicating that activation-induced depalmitoylation may occur in most $G\alpha$.

How does activation of a $G\alpha$ promote its depalmitoylation? Recent results argue that in general depalmitoylation, through the acyl protein thioesterases APT1 and APT2, is a constitutive process, and intrinsic properties of a palmitoylated protein, such as membrane affinity and accessibility of the palmitoyl group to APT1/2, may regulate depalmitoylation (Rocks et al. 2010). Consistent with this idea, several studies have shown that downstream signaling does not affect the palmitate turnover or translocation of $G\alpha$ (Kosloff et al. 2003; Cronin et al. 2004; Degtyarev et al. 1993). Also in line with a model of constitutive depalmitoylation, experiments with purified proteins or cell extracts show that $G\beta\gamma$ decreases the depalmitoylation of $G\alpha_s$, suggesting that association with $G\beta\gamma$ inhibits accessibility of the depalmitoylating enzyme to the palmitoyl group attached to $G\alpha_s$ (Iiri et al. 1996; Wedegaertner and Bourne 1994). Because $G\alpha_s$ has a single attached palmitate, it may be more readily depalmitoylated compared to other $G\alpha$, such as $G\alpha_i$, that are dually myristoylated and palmitoylated, simply because the $G\alpha_s$ palmitate is more accessible to the depalmitoylating enzyme. Taken together, current evidence indicates that a key step in

activation-induced trafficking of palmitoylated $G\alpha$ is an increased rate of depalmitoylation resulting from activation and dissociation from $G\beta\gamma$.

Additional insight into activation-induced trafficking of G proteins has been obtained through comparisons of GPCR and G protein internalization. $G\alpha_s$ translocation off of the PM after activation does not require coincident internalization of a GPCR since $G\alpha_s$ translocation can be promoted by mutation or cholera toxin treatment, conditions under which a GPCR such as β_2-AR remained localized at the PM (Levis and Bourne 1992; Wedegaertner et al. 1996). Moreover, $G\alpha_s$ and $G\alpha_q$ undergo GPCR activation-induced translocation even when GPCR internalization is blocked by mutation of the GPCR, expression of a dynamin dominant-negative mutant or treatment of cells with hypertonic sucrose (Wedegaertner et al. 1996; Hynes et al. 2004; Cronin et al. 2004; Miserey-Lenkei et al. 2001). However, another report showed that dominant negative dynamin blocked β_2-AR-promoted translocation of $G\alpha_s$ in C6 glioma cells (Allen et al. 2005). It is possible that unique mechanisms of G protein internalization are used in different cells or that multiple pathways exist and alternative pathways can be utilized when one pathway is blocked. Dynamin can function in both clathrin-coated pit-mediated internalization as well lipid raft-mediated internalization of membrane proteins. Thus $G\alpha_s$ and other $G\alpha$ may internalize in a lipid raft-dependent manner, at least in some situations, and this has been supported by results showing that $G\alpha_s$ and $G\alpha_q$ are enriched in lipid-raft fractions after activation, translocation of $G\alpha_s$ is inhibited by cholesterol depletion and internalized $G\alpha_s$ co-localizes with a lipid-raft marker (Allen et al. 2005; Hynes et al. 2004; Pesanova et al. 1999). In summary, results from visual and non-visual systems provide strong evidence for a trafficking model whereby activated $G\alpha$ translocate independently of mechanisms responsible for GPCR internalization (Fig. 11.3). Nonetheless, the exact trafficking pathway for G proteins after movement off of the PM remains obscure.

Much of the above discussion has focused on activation-induced translocation of non-visual $G\alpha$ subunits, particularly $G\alpha_s$; however, there is evidence that non-visual $G\beta\gamma$ subunits also redistribute from the PM to intracellular locations, as occurs for $G\beta_1\gamma_1$ in vertebrate photoreceptors. One study used the technique of bimolecular fluorescence complementation (BiFC) to observe the localization of $G\beta_1\gamma_7$ during live cell imaging of transfected cells. It was found that upon agonist-stimulation of β_2-AR $G\beta_1\gamma_7$ translocated from the PM to apparent vesicles, co-localizing with a fluorescent protein fusion of $G\alpha_s$, and both $G\alpha_s$ and $G\beta_1\gamma_7$ displayed a similar time-course of redistribution, on the order of a few minutes (Hynes et al. 2004). This result suggested that activated $G\alpha_s$ and $G\beta_1\gamma_7$ could translocate together upon GPCR activation. Another report showed a translocation of expressed $G\beta_1\gamma_2$ from the PM to large endosomes after LPA stimulation; $G\alpha$ translocation was not addressed (Garcia-Regalado et al. 2008). On the other hand, a series of live cell imaging studies suggested a very different picture (Akgoz et al. 2004, 2006; Azpiazu et al. 2006; Saini et al. 2007, 2009). Different combinations of $G\beta\gamma$ in which either the $G\beta$ or $G\gamma$ subunit was fused to a fluorescent protein were expressed in cultured cells, along with various GPCRs and $G\alpha$. It was found that only certain combinations of $G\beta\gamma$ translocated upon GPCR activation, and the determining factor was the identity of the $G\gamma$ subunit. $G\beta\gamma$ translocation was not determined by whether the $G\gamma$ was geranylgeranylated or farnesylated, but rather depends on $G\gamma$ interactions with a GPCR. In this model, $G\beta\gamma$ combinations that contain a

translocation competent Gγ associate with a particular GPCR via Gγ-mediated interactions, but upon activation the Gγ has a lowered affinity for the GPCR and thus the Gβγ is released from the PM (Saini et al. 2009). Remarkably, this can be a very fast process, with some Gβγ translocating off of the PM and localizing to the Golgi or ER in less that 10 s. Another surprising result of this series of studies is that no activation-induced translocation of Gα was observed, even though several different Gα, including Gα$_s$, were tested, and rapid translocation of Gβγ was dependent upon palmitoylation. Thus, studies from this group have suggested a model whereby Gβγ translocates rapidly via diffusion to the Golgi or ER but Gα remains on the PM after activation of an appropriate GPCR (Akgoz et al. 2004, 2006; Azpiazu et al. 2006; Saini et al. 2007, 2009). Clearly, additional studies will be needed to reconcile different models of activation-induced G protein trafficking. Key questions to resolve include (1) Do both Gα and Gβγ translocate?; (2) If so, what are the rates of translocation, and are they vastly different for Gα and Gβγ?; and (3) Is translocation mediated by diffusion or vesicle-mediate active transport?

Lastly, in terms of activation-induced translocation of G proteins, most studies agree that it is a reversible process. As described above, there has been a substantial amount of research and debate as to whether G proteins that mediate visual signaling recycle to the PM via diffusion or active transport. However, very few studies have addressed the recycling of non-visual G proteins. Studies with Gα$_s$ have shown that after activation-induced translocation off of the PM, it returns to the PM over the course of approximately 1 h (Wedegaertner et al. 1996). This is slower than the initial translocation, which occurs within 10–15 min, yet Gα$_q$ in *Drosophila* photoreceptors recycles with a similar timecourse. Interesting, although Gα$_s$ and β$_2$-AR do not appear to utilize the same mechanisms to translocate off of the PM upon activation, both proteins recycle to the PM at the same time when activation is terminated by the addition of an antagonist (Wedegaertner et al. 1996). Both β$_2$-AR activation-induced translocation of Gα$_s$ and Gβ$_1$γ$_7$ and LPA receptor activation-induced translocation of Gβ$_1$γ$_7$ resulted in localization of the internalized subunits to Rab11 containing vesicles or structures; since Rab11 is associated with recycling endosomes, this colocalization suggests that internalized Gα and Gβγ may recycle via such a endosome-mediated pathway (Garcia-Regalado et al. 2008; Hynes et al. 2004). On the other hand, the series of studies that showed very rapid (<20 s) activation-dependent translocation of select Gβγ, but not Gα, showed a similar fast time course (seconds) for the recycling of Gβγ to the PM (Akgoz et al. 2004). Clearly, much work needs to be done to understand the recycling of G proteins to the PM once they have moved to intracellular locations upon activation.

11.5 Constitutive Trafficking of G Proteins

In addition to the evidence described above for activation-induced trafficking of G proteins, increasing evidence supports the idea that heterotrimeric G proteins, and indeed many peripheral membrane proteins in general, can traffic or shuttle between different membranes within the cell in the absence of an activation input (Fig. 11.4). One recently described constitutive trafficking pathway for peripheral membrane palmitoylated proteins invokes constitutive cycles of palmitoylation and depalmitoylation to direct movement between the PM and Golgi (Rocks et al. 2005, 2010). This model requires several key points, including (1) constitutive depalmitoylation of proteins is constantly occurring at all subcellular

locations; (2) once depalmitoylated, a protein can then diffuse throughout the cell and sample any cellular membrane (PM, Golgi, ER, etc.); (3) the DHHC palmitoyl acyltransferases are concentrated at the Golgi, so that even though proteins are rapidly sampling various membranes, palmitoylation only occurs at the Golgi; (4) once a protein is palmitoylated at the Golgi, it is kinetically trapped there through strong hydrophobic anchoring to the Golgi membrane; and (5) specific transport, probably vesicle mediated, moves palmitoylated proteins from the Golgi to the PM. Most Gα are palmitoylated, and thus this model has relevance for defining and understanding the constitutive movement of G proteins.

Accordingly, recent data has supported rapid G protein shuttling between the PM and Golgi in a palmitoylation-dependent manner. Studies have used imaging techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP) and photoactivation to analyze the movement of G protein subunits fused to fluorescent proteins. Overexpression of all three subunits of a heterotrimer, in this case Gα_o, Gβ_1 and several different Gγ, including Gγ_2, Gγ_3, Gγ_9 and Gγ_{11}, showed substantial localization of the G protein to both the PM and an intracellular location, likely Golgi (Chisari et al. 2007). However, it should be noted that a number of studies overexpressing all three G protein subunits have observed predominant PM localization with little or no intracellular staining. Nonetheless, the above study used various live cell imaging techniques to demonstrate that there was a rapid and constitutive movement of all expressed G subunits between the Golgi and PM in both the anterograde and retrograde direction (Chisari et al. 2007). Based on the identical profile of movement of all three subunits, it appears that the inactive heterotrimer is constitutively trafficking, rather than Gα and G$\beta\gamma$ moving separately. Interestingly, the PM-Golgi shuttling was blocked by treatment with the palmitoylation inhibitor 2-bromopalmitate (2-BP) consistent with the model that a palmitoylation and depalmitoylation cycle mechanistically underlies this constitutive G protein trafficking. Both the retrograde and anterograde movement occurred within seconds and inhibitors of vesicle trafficking failed to block either direction of trafficking, suggesting that constitutive G protein trafficking occurs via diffusion (Chisari et al. 2007; Saini et al. 2009). Another recent study supported the model that G proteins can constitutively move between the PM and Golgi (Tsutsumi et al. 2009). Photoactivation and FRAP were used to follow the trafficking of Gα_q when co-expressed with G$\beta_1\gamma_2$, and indeed movement between the PM and Golgi in both anterograde and retrograde directions was observed. Similar to the studies with the Gα_o-containing heterotrimers (Chisari et al. 2007), the constitutive shuttling of Gα_q-containing heterotrimers was blocked by 2-BP treatment (Tsutsumi et al. 2009). Moreover, the Gα_q study implicated DHHC-3 and DHHC-7 as Golgi-localized palmitoyl acyltransferases responsible for palmitoylation of Gα_q. The movement of Gα_q between the PM and Golgi occurred in approximately 10 min, which was substantially slower than the shuttling of Gα_o-containing heterotrimers which happened in a matter of seconds. The reason for this temporal difference awaits further experimentation.

Based on the above described studies and on extensive experiments with model palmitoylated proteins, such as H- and N-Ras (Rocks et al. 2005, 2010; Goodwin et al. 2005), a model for constitutive movement of heterotrimeric G proteins has been described (Saini et al. 2009). As with Ras and other palmitoylated peripheral membrane proteins, the

model states that an inactive heterotrimer is constitutively depalmitoylated at the PM and then moves rapidly by diffusion to the Golgi, where it is re-palmitoylated and returns by rapid diffusion to the PM. This model should provide the basis for much future investigation to verify and mechanistically expand our knowledge of constitutive G protein trafficking. A number of questions need to be further addressed. First, it is difficult to understand how a heterotrimeric G protein can move from the Golgi to PM by simple diffusion. Once palmitoylation occurs at the Golgi, in this model, the heterotrimer is expected to be bound tightly to the membrane via multiple membrane binding motifs, including palmitate attached to Gα, geranylgeranylation of Gγ, and myristoylation or polybasic N-termini of Gα. Thus, it seems very unlikely that a fully lipid modified G protein heterotrimer could be simply released from a Golgi membrane to diffuse through the cytoplasm to the PM; it would likely require additional proteins that could extract the heterotrimer from the Golgi membrane and sequester the hydrophobic lipids for the trip through the cytoplasm. Likewise, similar questions exist regarding diffusion of the heterotrimer from the PM to the Golgi; however in this case the strength of membrane attachment is decreased by depalmitoylation. Nonetheless, a depalmitoylated heterotrimer could still contain more than one membrane binding motif (*e.g.*, myristoylation of Gα together with prenylation of Gγ). Likely, additional proteins would also be needed to facilitate the diffusion of a depalmitoylated heterotrimer. An important and exciting goal for research in this area will be to identify proteins that bind the heterotrimer and sequester the attached lipids to facilitate diffusion between the cytoplasmic surfaces of different membranes. Another important point to address is that currently it is difficult to reconcile a model in which inactive heterotrimers rapidly and constitutively shuttle between the PM and Golgi and a model which states that in response to GPCR activation select Gβγ but not Gα move rapidly and reversibly from the PM to the Golgi or other intracellular locations (Saini et al. 2009). Future studies should help understand mechanistic differences between constitutive and activation-dependent trafficking of G proteins in a palmitoylation cycle-dependent manner.

No doubt future studies will reveal multiple levels of regulation that promote retention or release of a G protein on the PM or membrane of a particular organelle. In support of this, LRP6, a transmembrane PM-localized protein, was shown to promote PM localization of Gα$_s$ and Gβγ (Wan et al. 2011). In this study, siRNA depletion of LRP6 in cultured cells resulted in a partial redistribution of expressed Gα$_s$ and Gβγ from the PM into poorly defined cytoplasmic locations. Moreover, LRP6 binds inactive Gα$_s$ complexed with Gβγ. Thus, it was proposed the LRP6 enhances PM targeting of a Gα$_s$-containing heterotrimer by helping to tether it to the PM. Obviously, such mechanisms are important to consider when pursuing models of G protein trafficking.

G proteins may additionally be able to constitutively traffic from the PM to intracellular locations in a manner independent of the above PM-Golgi palmitoylation cycle, although such pathways and mechanisms remain obscure. Interestingly, a number of studies have localized G protein subunits to endosomes, suggesting that G proteins may utilize endosome-mediated pathways for constitutive trafficking (Balbis et al. 2007; Scarselli and Donaldson 2009; Van Dyke 2004; Zheng et al. 2004). In most cases, it is uncertain whether endosome localization of G proteins should be interpreted as evidence for constitutive or activation-induced trafficking. As noted in the previous section, increased endosomal

location of some G proteins has been detected upon GPCR activation. Nonetheless, a recent study provided evidence for a novel constitutive endosomal pathway for G proteins (Scarselli and Donaldson 2009). This study examined two GPCRs, β_2-AR and the M3 muscarinic acetylcholine receptor (M3R), and found that they internalized in the absence of agonist, *i.e.*, constitutively, and in a clathrin-independent manner. Surprisingly, $G\alpha_s$ and $G\alpha_q$ co-localized with β_2-AR and M3R, respectively, on tubular recycling endosomes, suggesting that the cognate G protein constitutively traffics along with a GPCR following an endosomal pathway. It was not determined whether $G\beta\gamma$ also constitutively trafficked with the GPCRs; one would predict that indeed the heterotrimer would be trafficking under non-activating conditions. This endosomal pathway provides an intriguing way to constitutively deliver G proteins and GPCRs to endosomes, and possibly other locations. It will be exciting to determine whether the Golgi-mediated constitutive G protein shuttling pathway, described above, is mutually exclusive with this endosomal pathway, and what are the mechanisms that distinguish different pathways of constitutive trafficking.

11.6 Functional Implications of G Protein Trafficking

In conclusion, there is strong evidence that G proteins move within the cell both constitutively and in response to GPCR activation. A major challenge as research moves forward is to better define the underlying mechanisms that control G protein trafficking. Moreover, imaging studies will further resolve the subcellular locations through with G proteins traffic and will determine how G protein trafficking itineraries differ for unique heterotrimers and cell contexts.

The knowledge that G proteins indeed traffic within the cell begs the question of what is the role of such trafficking. One role for activation-induced translocation of G protein subunits is to turn off or decrease signaling by removing the G protein from a PM site of action. Clearly, light-induced translocation of G proteins in both vertebrate and invertebrate visual signaling plays such a role by allowing adaptation to bright light. A similar role has not been well-established for non-visual G protein signaling, yet there are some suggestive results. For example, a mutationally activated $G\alpha_s$ distributes between the PM and cytoplasm, due to more rapid palmitate turnover compared to wild type $G\alpha_s$, but mutants that replace the single N-terminal site of palmitoylation with other signals, such as two lipid modifications, show enhanced PM localization and enhanced activation of its effector adenylyl cyclase (Thiyagarajan et al. 2002). Another study showed a heightened signaling response when a GPCR activated a heterotrimer containing a $G\beta\gamma$ that did not translocate compared to activation of a heterotrimer containing a translocating $G\beta\gamma$ (Chisari et al. 2009).

In contrast to regulating signals emanating from the PM, another important role for G protein trafficking is to deliver G proteins to diverse subcellular locations. It has become increasing clear that G proteins have novel signaling functions at a variety of intracellular locations (reviewed in Hewavitharana and Wedegaertner 2012). Select G protein subunits, $G\alpha$ or $G\beta\gamma$ or both, have been localized to and shown to have functions at endosomes, Golgi, ER, mitochondria and even nuclei. Accordingly, intracellular trafficking pathways can target G proteins to these subcellular localizations to allow G protein regulation of non-canonical signaling, and moreover, regulation of activation-induced or constitutive

trafficking pathways will influence intracellular signaling functions of G proteins. As one example, clear evidence has emerged that Gβγ subunits can localize to the cytoplasmic surface of Golgi membranes, where they regulate a signaling pathway involving a number of Golgi-localized signaling proteins, including PLCβ, PKD, PKCη and PI4-kinase, that ultimately controls the fission at the trans Golgi network of PM-destined vesicles (Diaz Anel and Malhotra 2005; Jamora et al. 1997, 1999; Diaz Anel 2007; Irannejad and Wedegaertner 2010; Saini et al. 2010). Gβγ must arrive at the Golgi through one or more of the trafficking pathways discussed in this review – trafficking of the G protein following biosynthesis, constitutive cycling between the PM and Golgi or activation-induced trafficking from the PM to Golgi. Indeed, one report correlated translocation of select Gβγ isoforms from the PM to Golgi after M1 muscarinic acetylcholine receptor activation with stimulation of insulin secretion (Saini et al. 2010). In the future, it is reasonable to think that a more detailed understanding of G protein trafficking will identify novel therapeutic targets to allow the inhibition or activation of a specific intracellular G protein function without affecting its other signaling functions.

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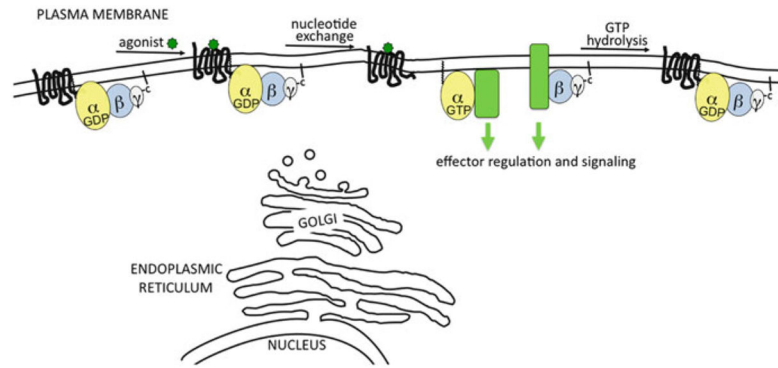
Fig. 11.1. Classical view of G protein signaling
In this view, heterotrimeric G proteins are restricted to the cytoplasmic surface of the PM. However, as discussed in this chapter, G proteins are found at a variety of subcellular locations and they can reversibly move between the PM and intracellular locations
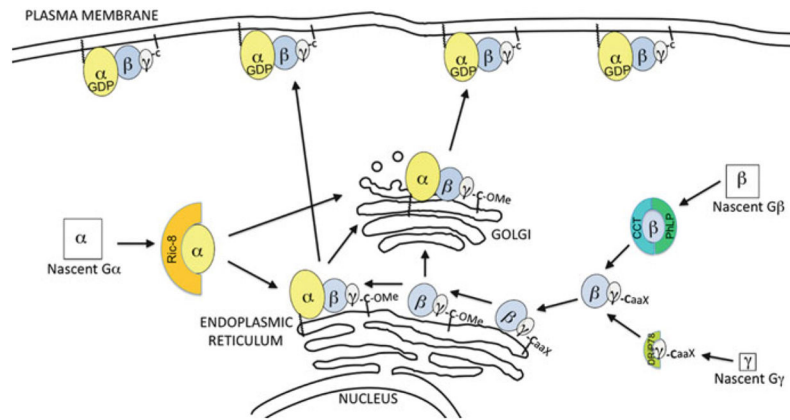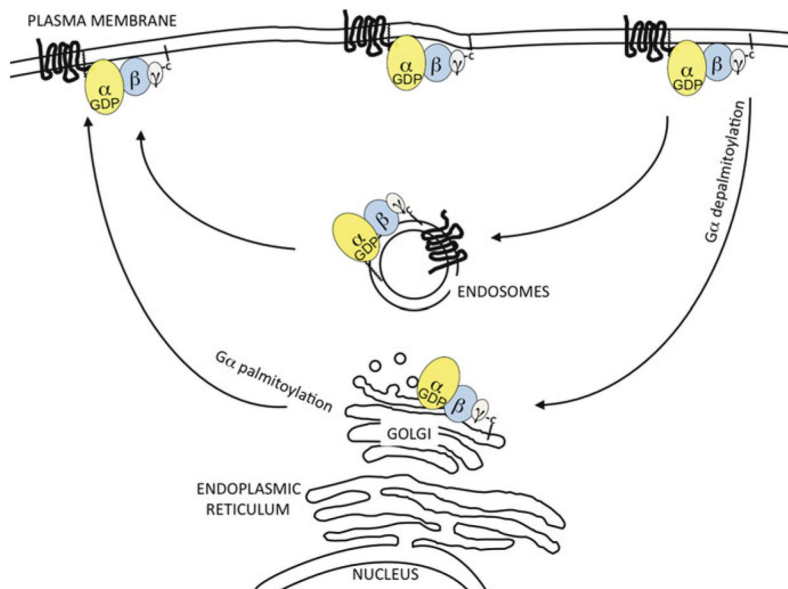
Fig. 11.2. Model of heterotrimeric G protein trafficking to the PM after synthesis
Discussed in detail in the text, the following key steps are known to be involved in trafficking newly synthesized G proteins to the PM. Chaperone proteins have been identified for each G protein subunit, and these chaperones are required for proper folding and/or initial delivery of the subunit to a membrane. After formation of the Gβγ in the cytoplasm, the C-terminal tail (CaaX motif) of Gγ is modified with a farnesyl or geranylgeranyl group, and then Gβγ is targeted to the ER. At the ER, C-terminal proteolysis and carboxymethylation of Gγ occurs. Gα initially binds to Gβγ at either the ER or Golgi and undergoes palmitoylation. The fully lipid modified G protein heterotrimer then traffics to the PM via the Golgi-dependent secretory pathway or through an uncharacterized Golgi-independent process
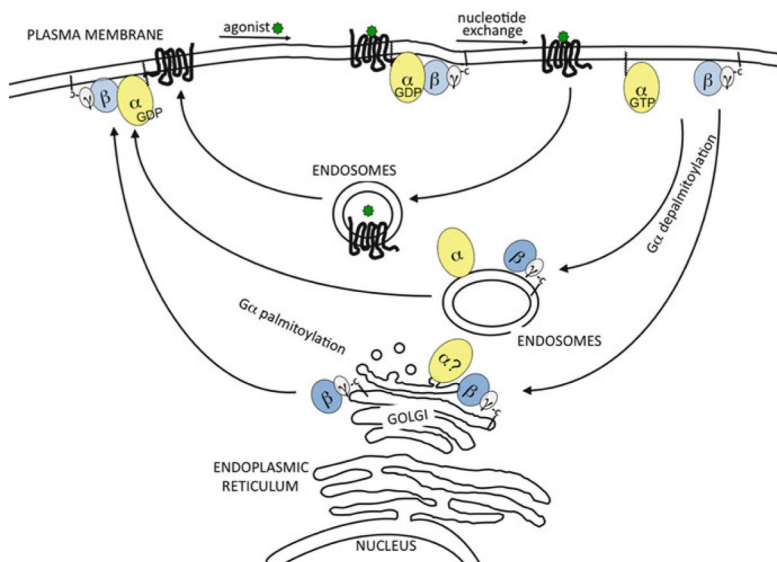
Fig. 11.3. Model of activation-induced G protein trafficking

Upon GPCR-mediated activation of an appropriate heterotrimeric G protein at the cytoplasmic surface of the PM, different, but not necessarily mutually exclusive, pathways of reversible translocation have been described. One series of studies has shown that after GPCR activation select Gβγ can undergo rapid (within seconds) movement from the PM to the Golgi. Then, Gβγ can return from the Golgi to the PM with similar rapidity. This cycle of Gβγ movement has been described as diffusion-mediated. Interestingly, the rapid activation-induced Gβγ PM-Golgi cycle depends upon palmitoylation, and likely by extension depalmitoylation. However, Gβγ appears to undergo this cycle while Gα remains on the PM; thus, Gα at the Golgi during activation-induced rapid translocation is indicated by the question mark. In another pathway, GPCR-mediated activation promotes depalmitoylation of Gα allowing Gα to translocate off of the PM. This pathway is slower (minutes) than the PM-Golgi pathway. Gβγ has also been observed to show such slower movement off of PM along with Gα. Gα may simply be released into the cytoplasm by virtue of its depalmitoylation, or it may follow a vesicle-mediated pathway involving recycling endosomes together with Gβγ. In this slower pathway, Gα and Gβγ would return to the PM even slower (0.5–1 h). The figure also indicates that Gα and Gβγ traffic independently of GPCR internalization. The models in this figure focus on translocation of G proteins in non-visual systems. See the text and accompanying references for more details regarding light-activated translocation of visual G proteins

Fig. 11.4. Model of constitutive trafficking of G proteins

Two pathways of constitutive, *i.e.,* in the absence of GPCR-mediated activation, trafficking of heterotrimeric G proteins have been described. In one pathway, the G protein heterotrimer is constantly moving between the PM and Golgi. This pathway utilizes the palmitoylation cycle, as described for other palmitoylated peripheral membrane proteins, such as H-Ras. In this pathway, Gα in the context of the inactive heterotrimer undergoes continual depalmitoylation at the PM. Next, the depalmitoylated heterotrimer will move by diffusion to the Golgi where palmitoyl acyltransferases can be located. After re-palmitoylation of Gα, the heterotrimer returns by diffusion to the PM. In another described constitutive pathway, inactive Gα internalizes along with a non-activated GPCR. It is likely that Gβγ also follows this pathway, since it would be expected to be bound to inactive Gα. Thus, a G protein heterotrimer would traffic together with a constitutively internalizing GPCR. It is not known whether cycles of Gα depalmitoylation and palmitoylation would regulate this slower GPCR constitutive internalization-dependent pathway. See the text for details of these pathways of constitutive G protein movement

Table 11.1

Sites of G protein lipid modifications

Gα subunits	N-termini of α subunits	Lipid modification
α_{i1}	MG*C*TLSAEDKAAVERSKMID-	Myristoylation, Palmitoylation
α_{o1}	MG*C*TLSAEERAALERSKAIE-	Myristoylation, Palmitoylation
α_Z	MG*C*RQSSEEKEAARRSRRID-	Myristoylation, Palmitoylation
α_t	MGAGASAEEKHSREL-	Myristoylation
α_s	MG*C*LGNSKTEDQRNEEDAQR-	Palmitoylation
α_q	MTLESIMA*CC*LSEEAKEARR-	Palmitoylation
α_{14}	MAG*CCC*LSAEEKESQRISAE-	Palmitoylation
α_{16}	MARSLRWR*CC*PW*C*LTEDEKA-	Palmitoylation
α_{12}	MSGVVRTLSR*C*LLPAEAGAR-	Palmitoylation
α_{13}	MADFLPSRSVLSV*C*FPG*C*VL-	Palmitoylation

Gγ subunits	C-termini of γ subunits	Lipid modification
γ_1	-KGIPEDKNPFKELKGG<u>C</u> $^\downarrow$VIS	Farnesylation
γ_2	-TPVPASENPFREKKFF<u>C</u> $^\downarrow$AIL	Geranylgeranylation

The N-terminal sequences of several Gα and the C-terminal sequences of two Gγ are shown. Myristate links through an amide bond to an N-terminal glycine after removal of the initiating methionine as indicated by <u>G</u>. Palmitate attaches via a thioester bond to cysteine (in bold italics) residues near the N-terminus of Gα. γ_1 and γ_2 are isoprenylated through a thioether bond to a cysteine, indicated by <u>C</u>. After isoprenylation the C-terminal three amino acids are removed ($\downarrow$), and the new C-terminus is carboxyl methylated. This is a representative listing of G protein subunits. In humans, 16 genes encode Gα (plus additional splice variants), 5 genes encode Gβ (Gβ proteins are not known to be lipid-modified), and 12 genes encode Gγ. Reprinted with permission from Marrari et al. (2007). Copyright 2007 American Chemical Society