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The Self-Scaffold Model of G Protein Signaling

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Jingting Wang

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Abstract of the Dissertation

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Heterotrimeric G proteins are composed of three subunits, α, β and γ. They are activated via specific seven transmembrane receptors, G protein coupled receptor (GPCR), which catalyze the exchange of GTP for GDP on the Gα subunit, and allow the Gα(GTP) and Gβγ subunits to effect specific intracellular enzymes and change their catalytic activity. Previously, it was believed that Gα(GTP) dissociates from Gβγ after activation, but this model is contrasted by more recent studies, including those in my thesis project.

In this thesis, I will present a model in which G proteins Gα, Gβγ and an effector, phospholipase C β_2 (PLCβ_2) remain associated through the G protein activation and Gα_i, Gβγ deactivation circle. These studies were done using fluorescence resonance energy transfer (FRET) studies, and cross-linking experiments using purified proteins. Using a series of single Cys Gα_i mutants, we have assessed the changes in subunit

conformation that occur upon activation. I also verified these interactions in live cells using FRET. These measurement offer clues of how $G\alpha_i$ and $G\beta\gamma$ can remain associated even in the activated state. Taken together, my studies suggest a model of the orientation changes between G protein subunits that occur upon activation that allow the G proteins to remain complexed in cells.

My studies also revealed the surprising result $G\alpha_i$ subunits contain a second binding site for $G\beta\gamma$ subunits, resulting in the formation of a $G\beta\gamma$-$G\alpha$-$G\beta\gamma$ complex. This second site has a 10 fold lower affinity and may be related to the binding site between activated $G\alpha_i$ and $G\beta\gamma$ subunits. We have carried out a series of biophysical measurements that have allowed us to identify this second binding site.

This work is dedicated to my dear parents,
and my loving boyfriend, Ti.

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Chapter I: General Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of three subunits: an α subunit that binds and hydrolyzes GTP, a β subunit, and a γ subunit (1). With few exceptions, Gγ binds tightly to subunit Gβ to form a constitutive dimer that is known to function only as a complex. Heterotrimeric G proteins are peripheral membrane proteins found on the cytoplasmic side of the plasma membrane. They transduce extracellular signals by coupling the activation of heptahelical receptors—G protein coupled receptors (GPCRs) on the cell surface to an array of intracellular effectors(2). One of these is mammalian phospholipase Cβ (PLCβ), a member of PLC family.

Gβγ subunits and a family of Gα isoforms can activate the intracellular enzyme, phospholipase Cβ. PLCs catalyze the cleavage of the head group of phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) generating two intracellular messengers, diacylglycerol (DAG), which is responsible for protein kinase C (PKC) activation, and inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] which diffuses through the cytoplasm to the endoplasmic reticulum where it binds to its receptor causing the release of Ca^{2+} from intracellular stores(3-6).

The activation and deactivation of PLCβ enzymes are highly regulated processes. To date, the crystal structures for activated (GTP-bound), unactivated (GDP-bound) (7) and transition state ($GDP\text{-}ALF_4^-$) $G\alpha_t$ and $G\alpha_i$ have been solved (8). The crystal structure

of the entire PLCβ enzyme is still not available. A partial structure of PLCβ has been solved. These structures provide the framework for our understanding of the biomechanics of G-protein signaling(9).

The G protein family

G proteins are highly conserved protein families. According to current knowledge, compared to large family of GPCRs (around 900 different known heptahelical recepors), G protein subunits have relatively few members. To date, there are 21 α subunits encoded by 16 genes, 6 β subunits encoded by 5 genes and 12 γ subunits encoded by 12 genes in mammalian cells (10).

Classically, G proteins are currently grouped into four families based on amino acid sequence similarity of the α subunits: $G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$, and $G\alpha_{s}$ (Figure 1.1) (11). Gα subunits range in size from 39 to 52 kDa, and share between 35%-95% sequence identity(10). Each family has a distinct role in the cell, and each shows specificity to effectors. For instance, $G\alpha_{i/o}$ inhibits adenylyl cyclases to reduce cAMP, whereas $G\alpha_{s}$ activates most isoforms of adenylyl cyclase resulting in an increase in the levels of cAMP. $G\alpha_{q/11}$ activates phospholipase Cβ and $G\alpha_{s}$ activates cGMP phosphodiesterases in retinal rods and cones. Gα subunits are intrinsic GTPases, hydrolyzing the terminal phosphate of GTP to restore GDP to the nucleotide-binding pocket (12).

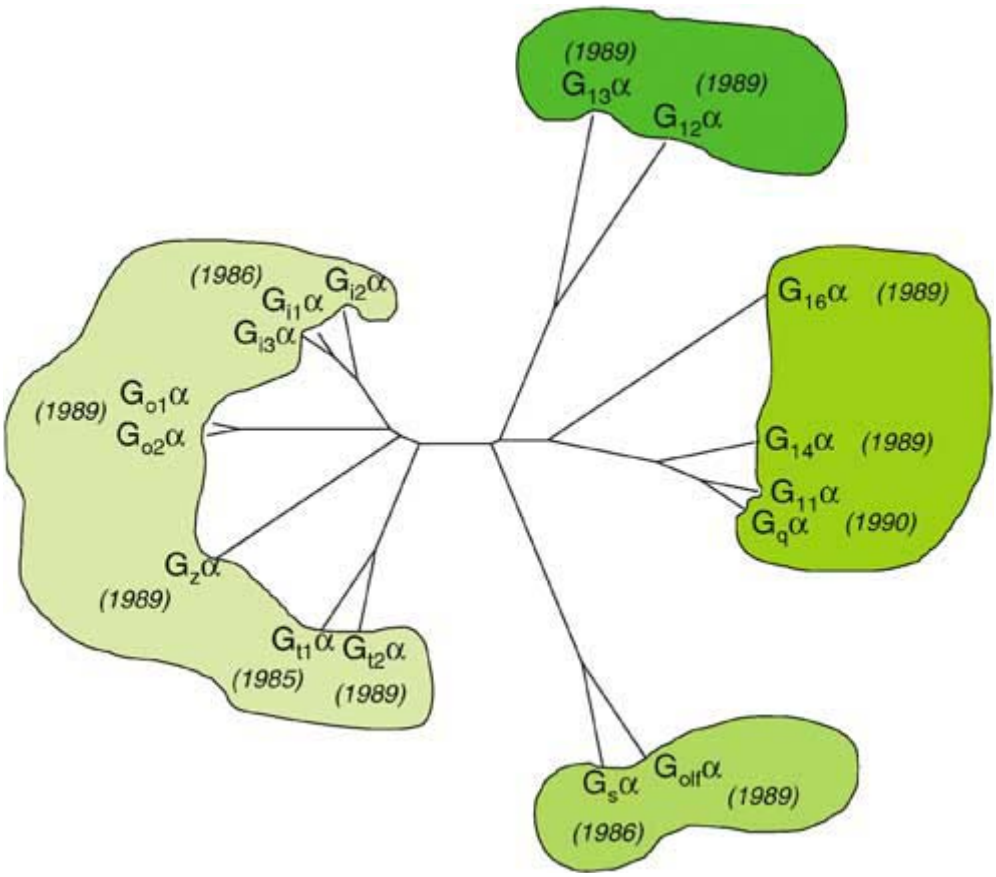

Figure1.1: Family map of mammalian Gα subunits. Adapted from Milligan & Kotenis (2006)(12)

The 6 Gβ subtypes are approximately 36 kDa in size, and are grouped into two families based on amino acid homology, $G\beta_{1\text{-}4}$ and two variants of $G\beta_5$. Long and short splice variants of $G\beta_5$ have been shown to have unique properties among Gβ isoforms, suggesting that these subunits may play a different functional role in the cell. $G\beta_5$ can exit in solution as a monomer and is able to form complexes with proteins that contain a Gγ-like domain, such as regulator of G protein signaling (RGS) protein. $G\beta_5$ can also form dimers and functional heterotrimers(13-17).

The 12 Gγ subunits identified so far are all 7-8 kDa in size. Based on homology in its C-terminal domain and overall sequence similarity, the Gγ subunits are divided into four groups (10).

The numerous Gβ and Gγ families and subtypes make the numbers of potential Gβγ combinations very large, although not all the forms are possible (18). With a few exceptions, almost all subtypes of Gβ and Gγ form dimers that bind to each family of Gα subunits (19). Therefore, Gβγ appears to have little specificity to extracellular signaling agonist and effectors they regulate (20).

Initially, Gβγ was considered simply as a regulatory binding partner for Gα subunits to suppress signaling and to provide a membrane anchor for Gα subunit. Today, this belief has changed and Gβγ is known to interact with and activate a variety of downstream effectors such as ion channels, PLCβs, adenylyl cyclases, and phosphotidylinositol-3-kinase (21). Certain Gβγ combinations regulate particular effectors, which plays an important role in determining the specificity and temporal characteristics of the cellular responses to a diverse array of extracellular stimuli.

Lipid modification of G proteins may help direct the localization of the heterotrimer to the membrane and protein-protein interactions (22-25). All the Gα family members, except $G\alpha_t$, are modified with the saturated 16-carbon fatty acid palmitate by a reversible thioester bond to cysteines near the N-terminus, and some are palmitoylated at multiple sites. $G\alpha_i$ family members undergo both myristoylation and palmitoylation at their N-termini (26,27). Recent data shows that myristoylation also helps the N-ternimus to form α–helical structure (28). All Gγ subunits are geranylgeranylated at their C-

termini, with a few exceptions such as γ_1, γ_8 and γ_{11}, which undergo post-translational isoprenylation with a 15-carbon farnesyl.

G proteins - phospholipase C β signaling system

In the classic model of G protein-phospholipase C β signaling system, a G-protein coupled receptor (GPCR) is stimulated by extracellular chemical or physical signals, such as photons, odorants, neurotransmitters, hormones, glycoproteins, and chemokines. The activated GPCR then catalyzes the exchange of GTP for GDP on the Gα subunit. When the GDP-bound α subunit is an unactivated state, it associates with βγ subunits to form an inactive heterotrimer. In contrast to Gα(GDP), Gα(GTP) has a much weaker affinity for Gβγ subunits which may allow the dissociation of Gα(GTP) from Gβγ subunits and the receptor. To date, it is now accepted that both Gα(GTP) and Gβγ are free to activate downstream target effectors leading to cellular events such as differentiation, proliferation, development and survival. Specially, Gβγ can activate PLCβ_2 and β_3, as is discussed in the following section.

All isoforms of Gα have the intrinsic GTPase activity, which hydrolyzes GTP to GDP. Once GTP is hydrolyzed, Gα(GDP) reassociates with Gβγ and receptor to return to the basal state ready to be activated again.

Our lab has focused on inositiol–specific phospholipase C-β (PLCβ), which is a soluble enzyme. PLCβs are isozymes of the five known mammalian subfamilies (β, γ, δ, ε and ξ) of PLCs which differ in their structural organization and regulation (Figure 1.4). PLCs catalyze the hydrolysis of the signaling lipid, phosphatidylinositol 4,5 bisphosphate [PI(4,5)P_2] to generate two intracellular messengers, DAG and Ins(1,4,5)P_3. These

messengers then promote the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores, respectively. Only PLCβ can be strongly activated by G proteins. All PLCβs are activated by $G\alpha_q$. So far, there are four known mammalian types of PLCβs ($PLC\beta_1$- $PLC\beta_4$) (29-31). Additionally, $PLC\beta_2$ and $PLC\beta_3$ can be activated by Gβγ (32).

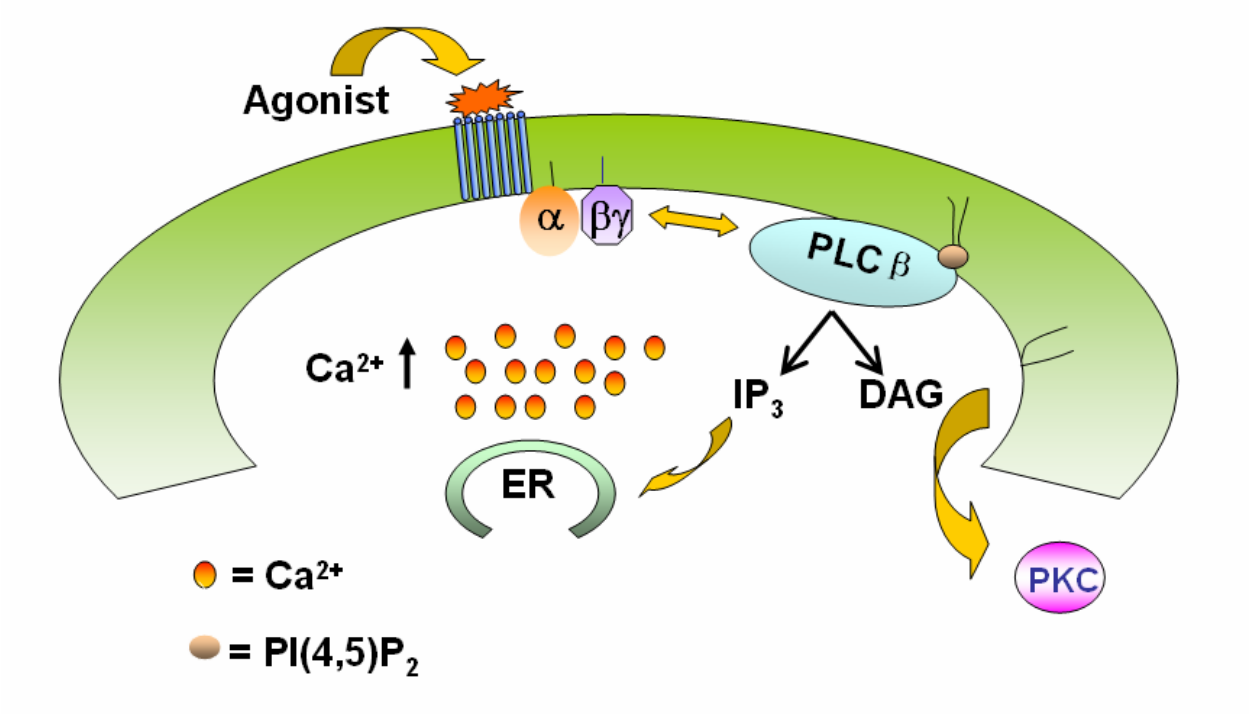

Figure1.2: Receptor mediated G protein - phospholipase β signaling system. G proteins transduce extracellular signals from a seven transmembrane GPCRs to the inside of the cell. The exchange of GDP for GTP is favored on the Gα subunits. After the activation, Gβγ goes on to activate its effector PLCβ, which hydrolyze PIP_2 into two secondary messengers, IP_3 and DAG. Cessation of the signaling occurs when GTP is hydrolyzed to GDP on the Gα subunit which has intrinsic GTPase ability.

The structural basis of PLCβ and G proteins

Crystal structures reveal that Gα subunits consist of a N-terminus, followed by a GTPase domain that composed of a six-stranded β-sheet surrounded by five α-helices, and a helical domain that is composed of six α-helices to form a lid over the nucleotide binding site (33,34) (Figure 1.3). The GTPase domain is the region where GTP is hydrolyzed to GDP and this domain also interacts with Gβγ and effector proteins. Besides forming a lid structure to help bury nucleotides in the core of Gα subunit, the helical domain helps to increase the affinity of Gα for guanine nucleotides and increase the GTP hydrolytic activity. GTPase domains are structurally homologous, whereas helical domain is highly divergent among Gα subfamilies, which indicates that helical domain might be relevant to the specificity between the association of Gα to its effectors and receptors (35).

Gβ subunits have an N-terminal helix, followed by a seven-blade β-propeller structure formed from seven WD40 sequence repeats (36). The N-terminal extended helix is essential for interaction with Gγ and Gγ like motifs.

Gγ subunit consists of 2 α-helices connected by a loop. The N-terminal α-helix binds to an extended hydrophobic groove on Gβ subunit forming a rigid coiled coil structure, while the C-terminal of Gγ appears to be involved in receptor binding (37).

In two separate crystal structures of heterotrimeric complex, $G\alpha_{t/i}\beta_1\gamma_1$ and $G\alpha_{i1}\beta_1\gamma_2$ (7), there are two interaction sites between Gα and Gβγ (Figure 1.3) (33). The first interaction surface is between GTPase domain of Gα and the β-propeller of Gβ, stabilized by hydrogen bonds and two ion pairs. The second site is between the N-

terminal helix of Gα and side surface of the β-propeller of Gβ. To date, there is no evidence of interaction between Gα and Gγ.

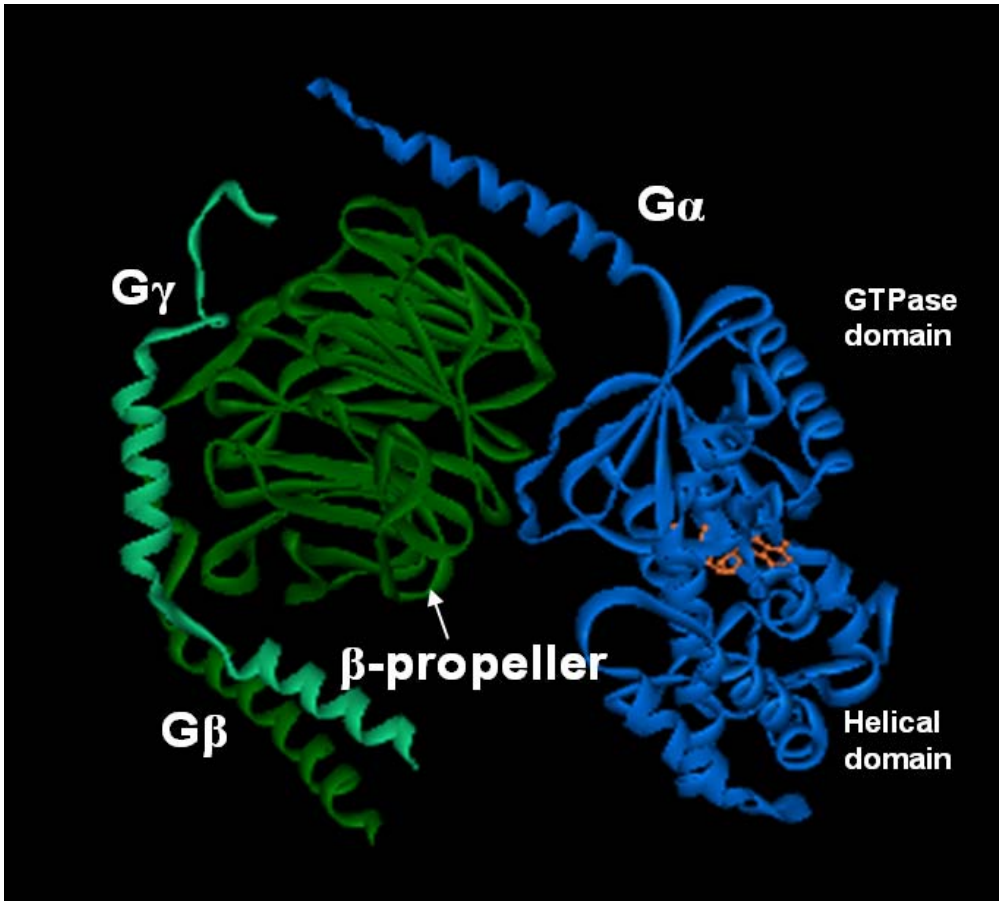

Figure1.3: Crystal structure of Gαβγ, with Gα in blue, Gβ in green and Gγ in cyan (7).

The phospholipase C family (PLC) is currently made up of five family members (38). There are also recent reports of a sixth family member, PLCη, which has a similar domain organization to the PLCβ family) (39,40). All PLCs, including PLCβ, have a multidomain structure consisting of a catalytic domain with several regulatory domains (Figure 1.4). The N-terminus has a pleckstrin homology (PH) domain which serves as a membrane anchor and a docking site for Gβγ binding (41). This region is followed by four elongation factors (EF) hands, each consisting of a helix-loop-helix structure, which

has been shown to bind Ca^{2+} in PLCδ (42) and is found in all family members except PLCε. The catalytic domain is formed from the X and Y regions, comprised of alternating α-helices and β-strands and is highly conserved (40-60%) among mammalian PLC family members (43). Within the catalytic domain is an insertion region that is distinct for each PLC family. The catalytic core is followed by a C2 domain and a long C-terminal tail. In PLCβ, C2 domain is responsible for strong and specific binding to activated $G\alpha_q$ subunits (44), while the C-terminal tail is required for activation by $G\alpha_q$ subunits. Additionally, the ~400 residues C-terminal tail also distinguishes the PLCβ isozymes.

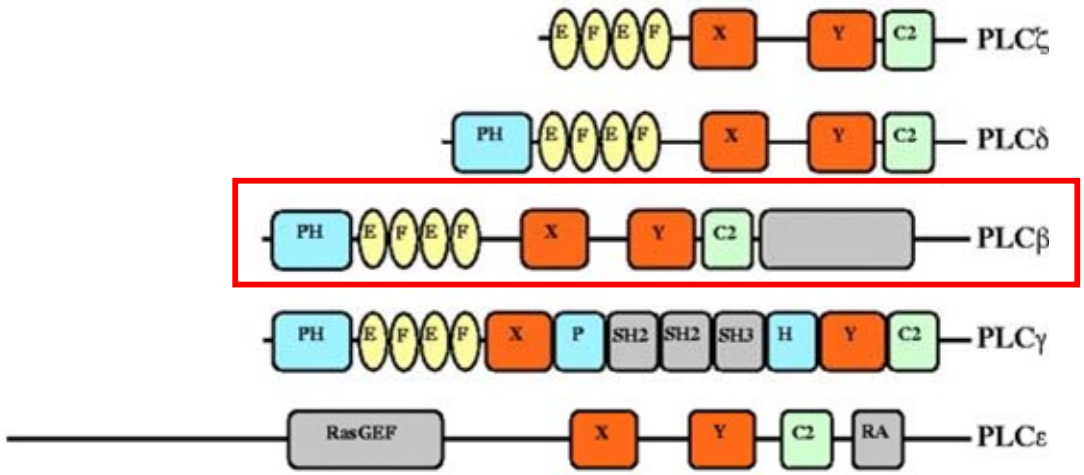

Figure1.4: Schematic diagram of the domain structure of phospholipase family members. Adapted from Saunders *et al.* (2002) (38)

Evidence of higher order signaling complexes

A number of scaffolding proteins have been identified that regulate various facets of G protein signaling, which greatly enhance signaling specificity and speed. Also, a increasing number of studies support compartmentalization of signaling components as lipid rafts (45) and caveolae (46) have been implicated in sequestering signaling components.

As mentioned above, free Gβγ subunits can active many effectors, including two members of the PLCβ family, PLCβ$_2$ and PLCβ$_3$. But how does Gβγ activate a specific effector in cytoplasmic environment where it is surrounded with many other potential partners? One of the possible reasons for signaling specificity is self-scaffolding.

Our lab has found that the addition of Gα$_{i1}$(GDP) accelerates the rate of deactivation of PLCβ$_2$- Gβγ without affecting either the *on*- or *off*- rates of PLCβ$_2$- Gβγ association (32). We propose that Gα(GDP) subunits can bind to the PLCβ$_2$- Gβγ complex and cause PLCβ$_2$ deactivation without complex dissociation. We further propose that Gα$_{i1}$(GDP) perturbs the subtle contacts between PLCβ$_2$ and Gβγ that causes activation. Since the potential activating contacts in the PLCβ$_2$- Gβγ complex is beyond the sensitivity of our fluorescence-based measurement (see below), our lab used high hydrostatic pressure to determine the volume change associated with PLCβ$_2$- Gβγ activation in the absence or presence of Gα$_s$(GDP) and found it to be very small (47).

All the above results suggest that G proteins and effectors form complexes that may remain associated during the G protein activation cycle, which can be rationalized in terms of the possible interaction sites between the proteins (32). While some peptide interference studies suggested that the Gβγ binding sites of PLCβ$_2$ and Gα are similar

(48), we suggest that the PLCβ_2 binding site on G$\beta\gamma$ is not completely occluded in the Gα_{i1}(GDP) $\beta_1\gamma_2$ heterotrimer. As mentioned, we have evidence that proteins in PLCβ-G protein signaling pathway have secondary sites to other proteins in the signaling pathway, which allow the proteins to self-scaffold on the plasma membrane thereby resulting in signal specificity and localization. Here, we will test a model in which G$\beta\gamma$ does not physically dissociate from Gα subunits upon activation, but rather rotates to activate its PLCβ effector, and exposes a second low affinity site to Gα that helps to keep the protein localized.

Literature Cited

1. Gilman, A. G. (1987) *Annu Rev Biochem* **56**, 615-649
2. Exton, J. H. (1997) *Eur J Biochem* **243**, 10-20
3. Rebecchi, M. J., and Pentyala, S. N. (2000) *Physiol Rev* **80**, 1291-1335
4. Drin, G., and Scarlata, S. (2007) *Cell Signal* **19**, 1383-1392
5. Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., Fukami, K., Kataoka, T., Yun, S., and Ryu, S. H. (2008) *BMB Rep* **41**, 415-434
6. Roberts, M. F. (1996) *FASEB J* **10**, 1159-1172
7. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058
8. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781
9. Oldham, W. M., and Hamm, H. E. (2006) *Q Rev Biophys* **39**, 117-166
10. Downes, G. B., and Gautam, N. (1999) *Genomics* **62**, 544-552
11. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J Biol Chem* **271**, 7999-8007
12. Milligan, G., and Kostenis, E. (2006) *Br J Pharmacol* **147 Suppl 1**, S46-55
13. Watson, A. J., Katz, A., and Simon, M. I. (1994) *J Biol Chem* **269**, 22150-22156
14. Watson, A. J., Aragay, A. M., Slepak, V. Z., and Simon, M. I. (1996) *J Biol Chem* **271**, 28154-28160
15. Zhang, F. L., and Casey, P. J. (1996) *Annu Rev Biochem* **65**, 241-269
16. Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F., and Vogel, Z. (1998) *FASEB J* **12**, 1019-1025
17. Fletcher, J. E., Lindorfer, M. A., DeFilippo, J. M., Yasuda, H., Guilmard, M., and Garrison, J. C. (1998) *J Biol Chem* **273**, 636-644

18. Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992) *J Biol Chem* **267**, 13807-13810
19. Clapham, D. E., and Neer, E. J. (1997) *Annu Rev Pharmacol Toxicol* **37**, 167-203
20. Graf, R., Mattera, R., Codina, J., Evans, T., Ho, Y. K., Estes, M. K., and Birnbaumer, L. (1992) *Eur J Biochem* **210**, 609-619
21. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) *Nature* **325**, 321-326
22. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. (1994) *J Biol Chem* **269**, 30898-30903
23. Mumby, S. M., and Linder, M. E. (1994) *Methods Enzymol* **237**, 254-268
24. Wise, A., Grassie, M. A., Parenti, M., Lee, M., Rees, S., and Milligan, G. (1997) *Biochemistry* **36**, 10620-10629
25. Morales, J., Fishburn, C. S., Wilson, P. T., and Bourne, H. R. (1998) *Mol Biol Cell* **9**, 1-14
26. Chen, C. A., and Manning, D. R. (2001) *Oncogene* **20**, 1643-1652
27. Smotrys, J. E., and Linder, M. E. (2004) *Annu Rev Biochem* **73**, 559-587
28. Preininger, A. M., Van Eps, N., Yu, N. J., Medkova, M., Hubbell, W. L., and Hamm, H. E. (2003) *Biochemistry* **42**, 7931-7941
29. Exton, J. H. (1993) *Adv Second Messenger Phosphoprotein Res* **28**, 65-72
30. Exton, J. H. (1994) *Annu Rev Physiol* **56**, 349-369
31. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem Sci* **17**, 502-506
32. Runnels, L. W., and Scarlata, S. F. (1999) *Biochemistry* **38**, 1488-1496
33. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature* **369**, 621-628
34. Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995) *Science* **270**, 954-960
35. Warner, D. R., Weng, G., Yu, S., Matalon, R., and Weinstein, L. S. (1998) *J Biol Chem* **273**, 23976-23983
36. Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996) *Nature* **379**, 369-374
37. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972
38. Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002) *Development* **129**, 3533-3544
39. Zhou, Y., Wing, M. R., Sondek, J., and Harden, T. K. (2005) *Biochem J* **391**, 667-676
40. Hwang, J. I., Oh, Y. S., Shin, K. J., Kim, H., Ryu, S. H., and Suh, P. G. (2005) *Biochem J* **389**, 181-186
41. Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998) *J Biol Chem* **273**, 30497-30508
42. Yamamoto, T., Takeuchi, H., Kanematsu, T., Allen, V., Yagisawa, H., Kikkawa, U., Watanabe, Y., Nakasima, A., Katan, M., and Hirata, M. (1999) *Eur J Biochem* **265**, 481-490
43. Rhee, S. G. (2001) *Annu Rev Biochem* **70**, 281-312
44. Jimenez, J. L., Smith, G. R., Contreras-Moreira, B., Sgouros, J. G., Meunier, F. A., Bates, P. A., and Schiavo, G. (2003) *J Mol Biol* **333**, 621-639

45. Brown, D. A. (2006) *Physiology (Bethesda)* **21**, 430-439
46. Cheng, Z. J., Singh, R. D., Marks, D. L., and Pagano, R. E. (2006) *Mol Membr Biol* **23**, 101-110
47. Scarlata, S. (2005) *Biophys J* **88**, 2867-2874
48. Zhang, S., Coso, O. A., Collins, R., Gutkind, J. S., and Simonds, W. F. (1996) *J Biol Chem* **271**, 20208-20212

Chapter II: Study of a self-scaffolding model of $G\alpha_{i1}$-$G\beta_1\gamma_2$-$PLC\beta_2$ complex in $G\alpha_{i1}$ activation cycle through *in vitro* and *in vivo* fluorescence binding measurements

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Dr. Urszula Golebiewska carried out the cell experiments in this study.

Introduction

Heterotrimeric G proteins are membrane-bound proteins composed of three subunits, α, β and γ (1). In the classic signaling transduction model, G protein coupled receptors (GPCRs) transduce the extracellular signals to intracellular G proteins, and catalyze the exchange of GTP for GDP on a Gα subunit. GTP-bound Gα (GTP-Gα) has a weakened affinity for Gβγ, which allows both GTP-Gα subunits and Gβγ to regulate an array of downstream effectors and initiate a set of cellular responses. This current model implies that after the activation by GPCRs, Gα subunits physically dissociate from Gβγ allowing G protein subunits diffuse freely on plasma membrane where they can encounter their downstream effectors (2-4). However, this model could not explain one key question, how GTP-Gα and Gβγ activate particular effectors after dissociation in a cytoplasmic milieu full of potential partners.

Recently, an increase mount of evidences indicate the existence of higher order complex in G protein signaling transduction that bring together specific receptors, G proteins, regulatory proteins and effectors, which might reveal key factors regulating specificity and efficiency. In a reconstituted *in vitro* system, our lab has found that GDP-Gα binds to the activated $PLC\beta_2$-Gβγ complex and rapidly turns off the activation

without physically disrupting the association (5). We also found that in cells, PLCβ_1 remains strongly associated with Gα_q through the activation cycle (6). Recent live cell studies shown that upon activation, some heterotrimers undergo structural rearrangement, rather than physical dissociation (7,8). Specifically, Bunemann and co-workers demonstrated that fluorescent-tagged Gα_i and G$\beta\gamma$ subunits remain associated during stimulation in HEK293 cells. In studies of PLCβ_1 activation, Ross and co-workers have presented kinetic data to argue that Gα_q must remain bound to receptor during turnover (9). The presence of competing proteins also seems to play a role in whether G protein dissociation occurs. For example, our lab has found that the presence of caveolin-1 promotes the dissociation of Gα_q-G$\beta\gamma$ during activation because of the higher affinity between caveolin-1 and Gα_q (10). Taken together, these studies point to a model in which receptor, the G protein heterotrimer, effector can be colocalized in signaling complexes.

However, not all studies support this model. For example, other laboratories have reported that Gα_i subunits dissociate from G$\beta\gamma$ after activation in COS cells (11), while some have observed dissociation and internalization of Gα_s upon stimulation. Thus, it is possible that protein complexes might be specific for certain types of G proteins and will form under some circumstances but not others.

In this study, we approached the question of G$\beta\gamma$ signaling specificity using the effector phospholipase Cβ (PLCβ) by fluorescence methods. PLCβ catalyzes the hydrolysis of the signaling lipid, phosphatidylinositol 4,5 bisphosphate (PI(4,5)P_2) to generate two intracellular messengers, DAG and Ins(1,4,5)P_3, which promotes activation of protein kinase C (PKC) and release of Ca^{2+} from intracellular stores, respectively. All four types of mammalian PLCβ (PLCβ_1- PLCβ_4) can be activated by Gα_q (1,12-14).

Additionally, PLCβ_2 and PLCβ_3 can be activated by G$\beta\gamma$. Gα_i inhibits adenylate cyclases, but has no direct function to PLCβ_2. In view of the known structure Gα_i(GDP)-G$\beta\gamma$ subunits (15), it is not apparent how G protein subunits activate effector without dissociation, nor is it clear how the activated Gα subunits interact with G$\beta\gamma$. Here, we first show that Gα_i-G$\beta\gamma$-PLCβ_2 exists in stable complexes in cells in the basal and activated states. We then have carried out *in vitro* studies using purified proteins to determine the changes in subunits interactions that may occur upon stimulation to allow for effector activation. We find significant changes in the orientation between the G protein subunits in the activated state that in turn exposes the Gβ site needed for PLCβ_2 activation.

Materials and Methods

Lipids

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were dried to form thin film in the bottom of a small pear shaped flask by rotary evaporation. Dried lipids were suspended by dialysis buffer (containing 150 mM NaCl and 20 mM Hepes, pH 7.2) to final concentration of 2 mM. After 5 freeze-thaw cycles in liquid nitrogen and a 37^{o}C water bath, the lipids were stored in -20^{o}C. Large Unilamellar Vesicles (LUVs), 100nm in diameter, were freshly made by extrusion.

Expression and purification of $G\alpha_{i1}$ mutants

DNAs of mutant $G\alpha_{i1}$ proteins were provided from Prof. Heidi Hamm (Dept. of Pharmacology, Vanderbilt University). In these proteins all six of the Cys were mutated and single Cys residues were introduced as indicated. These proteins behaved identically to their wild type counterparts (16,17). *Escherichia coli* cells were grown to OD600 of 0.5 units and then induced with 30 μM IPTG at room temperature for 16-20 h with gentle shaking at 200 rpm. The pellet was resuspended in buffer containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 5 mM imidazole and fresh protease inhibitors, and then disrupted by sonication. The cytosolic fractions were collected after centrifugation and incubated with 5 mL Ni-NTA agarose resin for 60 minutes at 4 ^oC and loaded to a column. The column was washed twice by wash buffer containing 5 mM imidazole and 10 mM imidazole, respectively, and then eluted with 10 mL elution buffer containing 40 mM imidazole. An additional pass through an anion exchange column was sometimes used. Eluted protein was dialyzed with exchange buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM MgCl2, 20% glycerol, 20 μM GDP, 10 mM 2-mercaptoethanol (βME), and 100 μM PMSF) then loaded to a MonoA column (Pharmacia Amersham, Piscataway, NJ). The protein was eluted by a linear gradient buffer containing 0-200 mM NaCl. The identity and purity of pooled proteins were tested by SDS-PAGE. Glycerol was added to purified protein to 10% (v/v), and the protein was aliquoted, flash-frozen with liquid nitrogen, and stored at -80 ^oC.

Expression of $G\beta_1\gamma_2$

Expression of His_6-$G\beta_1\gamma_2$ through baculovirus infection of SF9 cells has been described previously (18). This method allows for post synthetic modifications. The geranylgeranyl chain on the $G\gamma_2$ subunit was assessed by thin-layer chromatography on LK5D linear-k silica gel thin-layer chromatography (TLC) plates.

Expression of $PLC\beta_2$

His_6-$PLC\beta_2$ was expressed in Sf9 cells using a baculovirus vector provided by Alan Smrcka (Univ. of Rochester Medical School). Sf9 cells were grown to 1×10^6 cells/mL at 27 ^oC. Cells were infected with His_6-$PLC\beta_2$ recombinant baculovirus at a multiplicity of 10 for 48h at 27 ^oC and shaken at 125 rpm. Cells were harvested at 2,500 rpm for 25 minutes, washed in PBS, resuspended and spun at 4 ^oC. The pellet was resuspended ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM βME and fresh protease inhibitor cocktail) and subjected to pre-chilled nitrogen cavitation (Parr cell disruption bomb, >500 psi for 30 min with intermittent agitation). Cells were exploded into pre-chilled flask containing 40 mL lysis buffer, and the soluble fraction was recovered after ultracentrifugation at 35,000 rpm for 1h at 4 ^oC. The supernatant was loaded to an equilibrated Ni-NTA column. Bound proteins were eluted with 50 mL elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM βME) with a gradient of 10 - 150 mM imidazole.

PLC Activity Assays

Activity assays were conducted as described previously (18). Briefly, samples containing substrate (POPC:POPE:POPS (1:1:1), with 2 mol % PtdIns(4,5)P_2 and 8000 cpm/sample [^{3}H]- PtdIns(4,5)P_2), PLCβ_2 and other proteins were incubated at 30 ^{o}C for 5 minutes, and the reaction was then initiated by the addition of calcium ions. The reaction was terminated by addition of 200 μL ice cold 10% TCA and 100 μL 1% BSA. After centrifugation for 5 minutes, 300 μL of the supernatant was subjected to scintillation counting. Activity measurements of the PLCβ_2-G$\beta\gamma$–Gα complexes were carried out at 20 nM enzyme and 200 nM G protein concentrations. These high concentrations were required to insure that the ternary complexes would form. However, even at 30s measurements, we were close to the edge of the linear range. This resulted in lower values of G$\beta\gamma$ activation and higher intrinsic error in the measurements.

Gα Activation

Gα_{i1} was activated by incubation at 30 ^{o}C for 30 minutes, with the activation buffer (50 mM Hepes, 100 mM $(NH_4)_2SO_4$, 150 mM $MgSO_4$, 100 mM EDTA and 100 μM GTPγS) (16,19).

Protein Labeling

The labeling procedure has been previously described (20). Briefly, Gα_i was dialyzed against 150 mM NaCl and 20 mM Hepes buffer for 30 minutes three times to remove DTT, then labeled with 7-diethylamino-3-(4'-male-imidylphenyl)-4-methylcoumarin (CPM), which labels cysteine side chains. G$\beta\gamma$ was labeled at pH 8.0

with Dabcyl SE, which reacts with primary amines. Since the N-terminus of Gγ is blocked by prenylation (21), labeling of $G\beta_1\gamma_2$ will only allow covalent modification of the $G\beta_1$ N-terminus. Unreacted probe was removed by dialysis for three times for 30 minutes against a 100 fold excess of buffer containing DTT.

Fluorescence Measurements

Fluorescence experiments were carried out on an ISS PC1 spectrofluorometer (ISS, Urbana, IL). 10 nM CPM labeled $G\alpha_i$ was reconstituted on 200 μM Large Unilamellar Vesicles (LUV) composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (1:1:1) and the solution placed in a 3 mm microcuvette. Spectra were recorded using a 384 nm excitation wavelength and scanning the emission from 420-520 nm. The area under the curves was calculated to give the total emission intensity.

Accessibility Studies

Cys accessibility was determined by the increase in fluorescence intensity of CPM which is only fluorescent upon reacting with free thiol groups. To isolate $G\alpha_i$ Cys reactivity, we first blocked Gβγ Cys side chains by removing DTT and incubating with iodoactamide at 1:5 ratio at room temperature for 1h. Excess iodoactamide was removed by dialysis for 30 minutes three times. To test the accessibility of CPM to Cys on $G\alpha_i$ mutants, 10 nM unlabeled $G\alpha_i$, together with 200 μM LUV, blocked Gβγ and 50 nM CPM were incubated in cuvettes. Fluorescence intensity was tested at 0, 30, 60 min,

respectively. The 384 nm excitation wavelength was used and the emission from 420 - 520 nm was recorded.

N-bromosuccinimide (NBS) modification of Trp was performed by measuring the Trp intensity at λ_{ex} = 280 nm and λ_{em} = 345 nm after the addition of 20 nM freshly prepared NBS to a cuvette containing 10 nM $G\alpha_i$, and 200 μM lipid, when a constant value was reached after 8 minutes (22). In each case, the fluorescence intensity of the same amount of proteins without NBS, and the same amount of $G\alpha_i$ with NBS were used as controls.

Fluorescence-correlation-spectroscopy measurements

FCS measurements were performed on an Alba dual-channel confocal fluorescence correlation instrument (ISS, USA), equipped with an argon ion laser (Melles Griot, USA) and interfaced to an inverted microscope (TE300, Nikon). Excitation was at 488 nm and the fluorescence was recorded using an avalanche photodiode through an emission filter (HQ535/50X). Alignment and calibration were performed using freshly prepared 20 nM rhodamine 110 solutions. We used the following model to fit the experimental autocorrelation function, $G(\tau) = \Sigma a_i (1+\tau/\tau_D)^{-1}$, in which τ_D is the residence time of a molecule in FCS observation volume and a_i is the relative contribution of the ith species in the autocorrelation. Information about the size of a particle can be obtained by measuring the diffusion constant D and using the Stokes-Einstein relationship, $\tau_D = r^2/4D$, where r is the lateral radius of the excitation and detection volume (see (23)).

Cross-linking

Proteins were dialyzed in HMSE (50 mM Hepes, pH 7.5, 6 mM $MgCl_2$, 75 mM sucrose and 1 mM EDTA) buffer 3 x 30 min to remove DTT. Concentrated proteins (1 μM) were placed in 50 μL HMSE buffer. Cross-linking was initiated by the addition of 2.0 μL of freshly prepared 50 mM bismaleimide hexane (BMH) (Pierce) in DMSO. In control uncross-linked samples, 20 mM DTT was added to the solution prior to the reagent BMH. After incubation at room temperature for 1h, the reaction was quenched by adding DTT and incubated for 15 minutes at room temperature (24). The samples were boiled and resolved by SDS-PAGE on 8% gels.

Cell Culture and Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/mL of penicillin, and 50 g/mL streptomycin sulfate at 37 ^oC in a 5% CO_2 incubator. The cells were transfected using calcium phosphate coprecipitation in which cells were grown on 60 mm dishes for 24 – 48h to achieve 80 - 90% confluence, the media was then replaced, and 5 μg of eYFP-$G\alpha_i$ and eCFP-$G\beta_1$ and 10 μg of HA$G\gamma_7$ plasmids were mixed with 120 mM $CaCl_2$ and HBS buffer (21 mM Hepes, 123 mM NaCl, 5 mM KCl, and 0.9 mM Na_2HPO_4, pH 7.1), incubated on ice for 10 min, and added to cells drop wise. The cells were then incubated at 37 ^oC and the media replaced after 8 – 14h. The cells were allowed to recover for 8 - 14h and split into 35 mm glass bottom Mattek dishes and imaged 48 – 72 h later. For

PLCβ_2-G protein FRET measurements, cells were transfected with labeled G proteins 48h prior to microinjections with Alexa546 labeled PLCβ_2.

Microinjections

Transfected cells were grown in Mattek dishes for 48 h to achieve 70-80% confluence. Prior to microinjecting, we changed the media to phenol-free Leibovitz's-15 (L-15). We used an InjectMan NI2 with FemtoJet pump from Eppendorf to microinject the solutions into cytoplasm. We typically set the injection pressure Pi = 27 - 25 hPa, and kept the compensation pressure Pc = 14 hPa. The injection time was t = 0.4 s. Typically, we injected about 10-25 cells within a 10 - 20 minute period. We examined the microinjected cells under the phase microscope (Axiovert 200M from Zeiss with 40 × phase 2 objective) to select viable cells. We then transferred the cells to the Zeiss LSM 510 Meta/confocor 2 apparatus (Jena, Germany) and collected images.

In vivo single cell FRET measurements

In vivo FRET experiments were performed on Zeiss LSM 510 Meta/confocor2 apparatus (Jena, Germany) by monitoring the sensitized emission. The optical settings were: λ_{ex} = 458 nm and λ_{em}= 475 - 525 nm for eCFP, λ_{ex} = 514 nm and λ_{em} = 560 - 615 nm for eYFP, λ_{ex} =458 nm and λ_{em} = 560 - 615 nm for FRET. Details of the experimental set-up procedure and analysis have been described (25). For GFP - Alexa546 FRET we used: λ_{ex} = 458 nm and λ_{em} = 585 - 615 nm.

Results

$G\alpha_{i1}$ and $G\beta\gamma$ are stably complexed through the activation cycle in HEK 293 cells

We have previously found that $PLC\beta_1$ is stably bound to $G\alpha_q$ through the activation cycle (6). Here, we tested whether $PLC\beta_2$, which can be activated by $G\beta\gamma$ subunits released from $G\alpha_i$, remains complexed to G proteins during activation. In initial studies, we transfected HEK293 cells with eYFP-$G\alpha_i$, eCFP-$G\beta_1$ and HA-$G\gamma_7$ and tested whether the extent of FRET between the eCFP and eYFP tags changed upon activation. We find that eYFP-$G\alpha_i$ and eCFP-$G\beta_1$ exhibits a FRET of 0.36 ± 0.03 ($n = 11$) (Figure 2.1). This value is significantly higher than negative controls using either free CFP and YFP or non-interacting membrane associated proteins (15%) and lower than positive controls using eCFP and eYFP attached by a peptide linker (70%) (25). The intermediate FRET value observed for the eYFP- $G\alpha_i$ / eCFP-$G\beta\gamma$ is slightly lower but close to values obtained for eCFP-$G\alpha_q$ / eYFP-$G\beta\gamma$ (0.50 ± 0.04). Stimulation of the cells with isoproterenol to generate activated $G\alpha_i$ subunits did not affect the magnitude of FRET suggesting that $G\alpha_i$ and $G\beta\gamma$ remain associated in the basal and stimulated states.

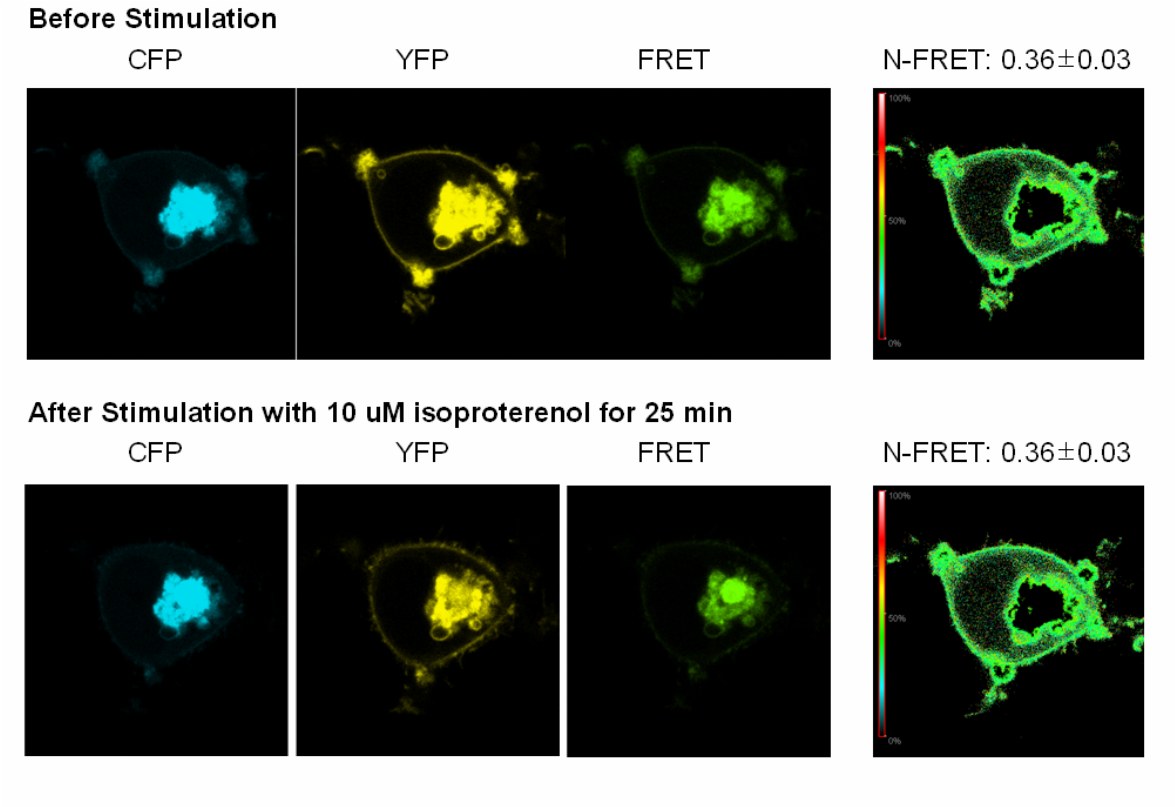

Figure 2.1: Representative images of HEK 293 cell expressing eCFP-Gβ_1 and eYFP-Gα_i before and after stimulation by isoproterenol.

We then transfected HEK 293 cells with GFP-Gβ_1 and HA-Gγ_7, and microinjected purified Alexa546-PLCβ_2 into the cells (Figure 2.2). The injected PLCβ_2 distributed in the cytosol and plasma membrane in accord with previous studies (26). The amount of FRET from the plasma membrane population of PLCβ to Gβγ was then measured in basal and stimulated cells. We obtain FRET values of 0.35 ± 0.04 (n = 30) in basal state and this value was unaltered upon stimulation. These studies suggest that the plasma membrane population of PLCβ_2 remains localized with G protein subunits in the basal and stimulated states.

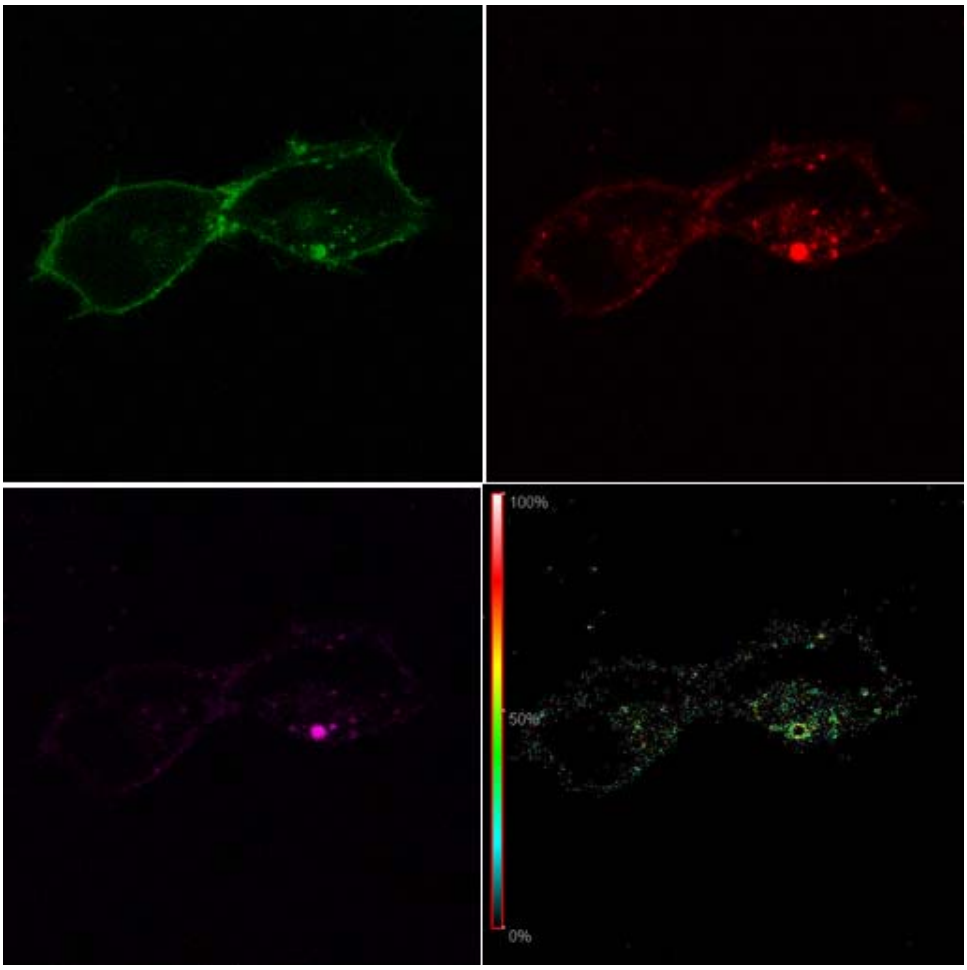

Figure 2.2: Representative images of HEK 293 cell expressing (A) eCFP-Gβ_1 / HA-Gγ_7, (B) Alexa 546 – PLCβ_2, (C) calculated FRET and the (D) normalized FRET (N-FRET)

Characterization of the binding of purified Gα_{i1} to G$\beta\gamma$

The above studies along with previous work support the idea that heterotrimeric G proteins and effectors can exist in complexes that remain bound through the activation cycle (6). To understand the molecular changes that might allow for interactions between activated G protein subunits and effectors, we carried out a series of *in vitro* studies.

We initially measured the decrease in affinity between Gα_i and G$\beta\gamma$ subunits upon activation. These studies were carried out using wild type Gα_{i1} and a series of single Cys mutants (Figure 2.3) (16). The proteins were labeled with the probe CPM

since it becomes fluorescent upon covalent attachment to Cys side chains and its emission intensity is sensitive to the local dielectric environment. We reconstituted the CPM-$G\alpha_i$ proteins onto large, unilamellar vesicles (LUVs) consisting of POPC:POPE:POPS (1:1:1) and note that even though the bacterially expressed $G\alpha_i$ subunits are not myristoylated or palmitotylated, they still bind to membranes with high affinity (20).

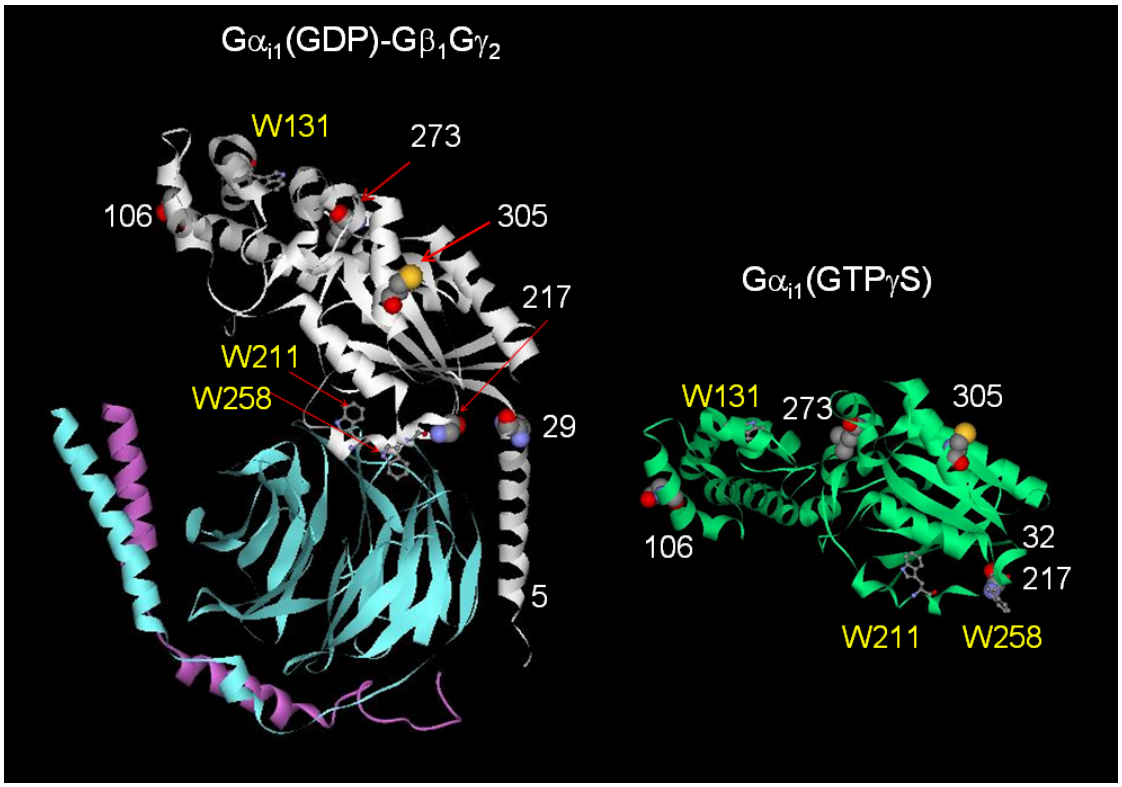

Figure 2.3: Left panel--Locations of single Cys residues in $G\alpha_{i1}$ shown in CPK from the crystal structure of $G\alpha_{i1}(GDP)G\beta_1\gamma_2$ ($G\alpha$, $G\beta$ and $G\gamma$ are shown in white, blue and pink, respectively). The 3 Trp residues in $G\alpha_{i1}$ are shown in ball and stick form.
Right panel—Crystal structure of $G\alpha_{i1}(GTP\gamma S)$ that starts from Arg32. Cys residues are shown in CPK, and Trps are shown in ball and stick form.
Images were generated using DSViewer Pro 6.0.

We measured the changes in fluorescence intensity as freshly thawed and dialyzed Gβγ subunits were incrementally added relative to control sample where buffer was substituted for Gβγ. In all samples, we observed a significant increase in fluorescence intensity from 0 - 10 nM Gβγ that leveled off at higher Gβγ concentrations. This change in intensity, which has been previously observed in studies using the same mutants labeled with a different probe (16), is interpreted as being due to protection of the CPM probe from solvent quenching due to association of Gβγ subunits. In Figure 2.4 we show the change in normalized fluorescence for wild type $G\alpha_i$ as a function of Gβγ concentration. Fitting the initial changes to a bimolecular dissociation constant gives a similar value for all of the mutants (1.8+/-0.3 nM). This value is comparable to similar fluorescence studies of $G\alpha_q$-Gβγ association and comparable to the apparent Kd of 1.18-1.34nM obtained by flow cytometry (27).

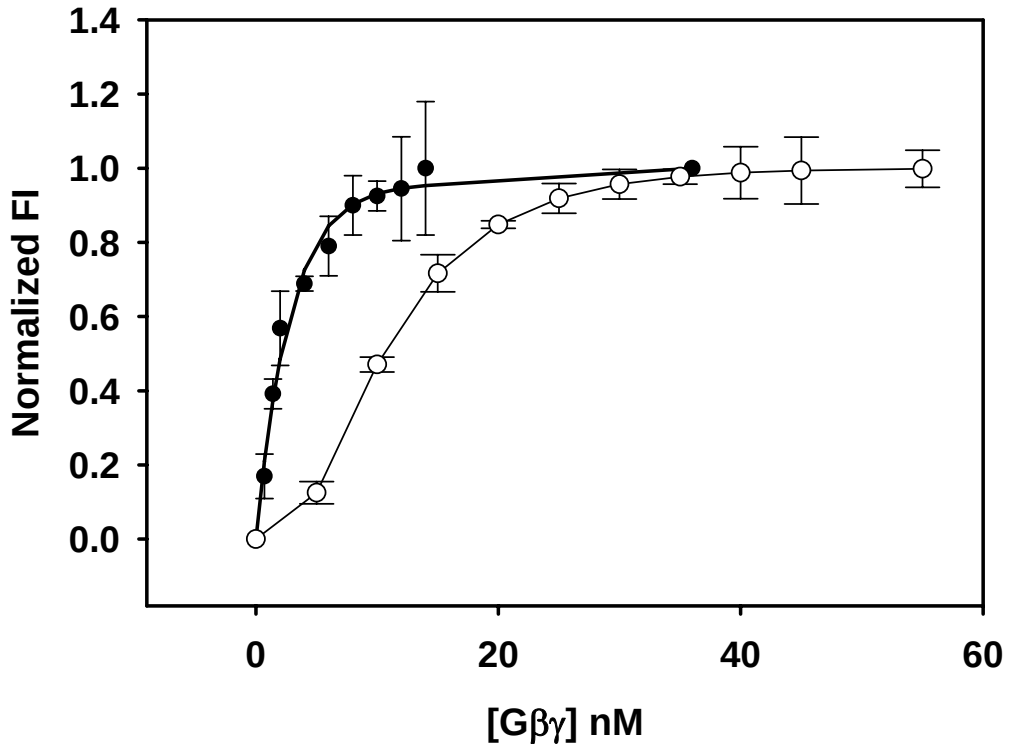

Figure 2.4: Comparison of the binding of Gβγ subunits to $G\alpha_i$(GDP) and $G\alpha_i$(GTPγS) on lipid membranes. Plot showing the change in Gβγ affinity upon $G\alpha_i$ activation as determined by the fluorescence change of 10 nM CPM-$G\alpha_i$ (wild type) reconstituted onto 200 μM PC:PE:PS (1:1:1) large, unilamellar vesicles as unlabeled Gβγ subunits are added where (●) corresponds to $G\alpha_i$(GDP) and (○) corresponds to $G\alpha_i$ (GTPγS). Data were normalized from the 18% change in CPM-$G\alpha_i$ fluorescence. Similar results were obtained using the 29C mutant. Each measurement was performed in triplicate and standard deviation is shown.

It is interesting to compare the extent of intensity changes for the single Cys $G\alpha_i$ mutants. All of the mutants showed an increase in CPM intensity with the addition of small amounts of Gβγ subunits to give similar apparent values of Kd ranging from 0.8-1.2 nM. The extent of this increase varied with each mutant, most likely due to differences in solvent exposure as Gβγ subunits bind. The data in Table 1 show that 3C and 29C, which are on the N-terminal arm of $G\alpha_i$ (see Figure 2.3) display a modest

change in intensity upon Gβγ binding most likely due to their proximity to the membrane surface thus reducing the change in dielectric upon binding. 106C and 273C, which are not close to the interaction region, also show a small intensity increase. In contrast, a large intensity increase is seen for 217C $G\alpha_i$ most likely because it is located close to the Gβγ association site. Interestingly, 305C $G\alpha_i$, which is far from the Gβγ binding site showed a large increase in binding. The reason for this increase is unclear and may be due to changes in membrane proximity/orientation of the subunit or other conformational changes.

We activated the CPM-$G\alpha_i$ mutants and measured their affinity to Gβγ subunits. As expected, the titration curves were shifted to an apparent Kd ranging from 34 to 45 nM confirming a weaker association in the activated heterotrimer (e.g. Figure 2.4**)**. These results suggest that a measurable population of activated $G\alpha_i$ associates to Gβγ subunits with a reduced but strong affinity. We found the mutants also showed an increase in fluorescence intensity with Gβγ binding. However, in the activated state a different pattern of the mutants was observed (Table 1) with 273C now showing a very large increase whereas 217C and 305C now showing a more modest increase. These results suggest large changes in contacts that $G\alpha_i$ makes with Gβγ subunits in the activated state.

$G\alpha_i$(GDP)	3C	29C	106C	217C	273C	305C
% increase	9.0±0.8	5.7±0.3	3.7±0.2	20.2±0.1	10.1±0.2	23.7±0.5
$G\alpha_i$(GTPγS)	3C	29C	106C	217C	273C	305C
% increase	10.0±1.2	43.8±10.2	11.0±2.4	5.6±0.3	73.8±5.6	12.0±0.3

Table 2.1: Summary of the fluorescence increase percentage of CPM-$G\alpha_i$(GDP) and CPM-$G\alpha_i$(GTPγS) mutants after the addition of Gβγ subunits. n = 3-6.

FRET between single Cys Ga mutants and Gβγ is not observed in the deactivated state

We conducted studies to determine the extent of orientational changes between the two G protein subunits in the activated state. We first measured the ability of CPM placed on the different Cys sites of the $G\alpha_i$ mutants to transfer fluorescence energy to a non-fluorescent energy transfer acceptor placed on the N –terminus of Gβ (Dabcyl SE, see Methods). The probability of energy transfer depends on the 6^{th} power of the distance between the two probes. For the CPM - Dabcyl pair, the distance at which 50% of the donor fluorescence is lost to transfer is 22Å (28). According to the crystal structure (29), the distances between the N-terminus of Gβ and the Cys sites of the $G\alpha_i$ (GDP) mutants should be out of range for FRET with the exception of 106C which would show a FRET of 6% assuming randomly oriented probes. Thus, if activation causes reorientation of the subunits, then it may be visible by the onset of FRET.

We carried out these studies by measuring the decrease in CPM intensity from the single $G\alpha_i$ mutants when Dabcyl-Gβγ was added. In general, the extent of FRET varied but was close to or less than 10% for all of the $G\alpha_i$ mutants which is within the error our measurements. The absence of FRET is in agreement with the crystal structure.

These studies were then repeated using activated $G\alpha_i$ proteins. Unlike $G\alpha_i$ (GDP) mutants, which did not participate in FRET with Dabcyl-$G\beta\gamma$, all of the mutants except for 3C were capable of FRET in the activated state (Figure 2.5). These results support the idea that the G protein subunits are oriented differently when $G\alpha_i$ is activated.

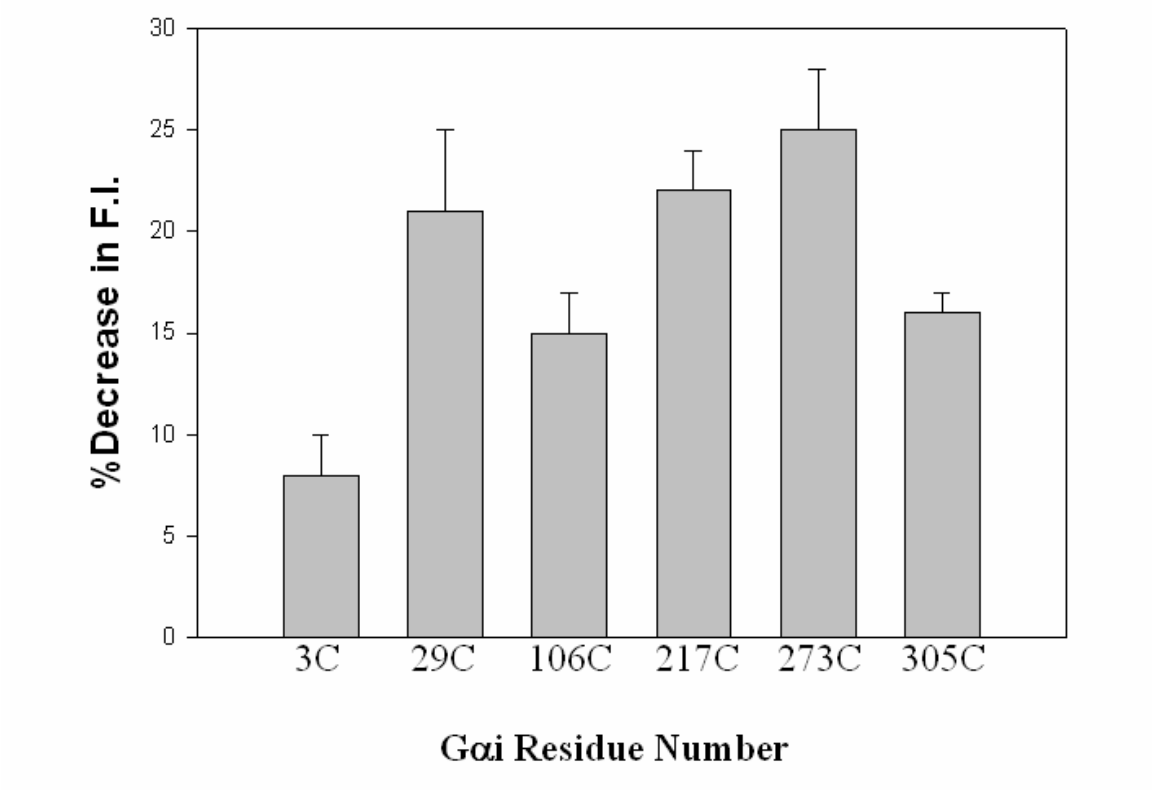

Figure 2.5: FRET meausurements in terms of relative loss in donor (i.e. CPM) fluorescence between $G\alpha_i$(GTPγS) single Cys mutants (10nM) reconstituted onto 200 μM PC:PE:PS (1:1:1) large, unilamellar vesicles, as 80nM $G\beta\gamma$ labeled on the N-terminus with a non-fluorescent FRET acceptor, Dabcyl, is added. All measurements were corrected for control samples which substituted unlabeled $G\beta\gamma$ for Dabcyl-$G\beta\gamma$ where n = 6 and standard deviation is shown.

Changes in Trp and Cys accessibilities upon $G\alpha_i$ activation

To gain more insight into the changes in G protein subunit orientation associated with activation, we monitored changes in the accessibility of the 3 Trp residues of $G\alpha_i$ to N-bromo-succidimide (NBS). NBS reacts with indole residues rendering them unable to

absorb and emit radiation. The rationale for these studies is that the exposure of one and possibly two of the Trp residues (W211 and W258, respectively, see Figure 2.3) changes upon activation whereas the third residue (W131) appears buried in both states. We first monitored changes in Trp exposure of $G\alpha_i$ (GDP) in the absence of Gβγ subunits. Treatment with NBS results in an 11 +/- 1% reduction in fluorescence. In contrast, addition of NBS to the activated protein causes a 5.6 +/- 1.5% reduction. This protection of the Trp side chains with activation may be due to protection of these residues by the N-terminal (29,30).

To better assess the orientation between the G protein subunits in activated form, we determined the accessibility of the Cys in the single Cys mutants to CPM which becomes fluorescent upon covalent linkage. For these studies, we focused on 217C and 273C since our fluorescence intensity studies (Table 2.1) suggest pronounced changes in the local environment of these residues upon activation. These studies were performed by first blocking solvent accessible Cys residues on Gβγ subunits with iodoacetamide (see methods) and measuring the increase in intensity as CPM reacts with the Cys217 or 273 on the mutants in the absence and presence of blocked Gβγ subunits over a 30 minute period. As a control, we tested 106C since this residue is outside the Gβγ binding region.

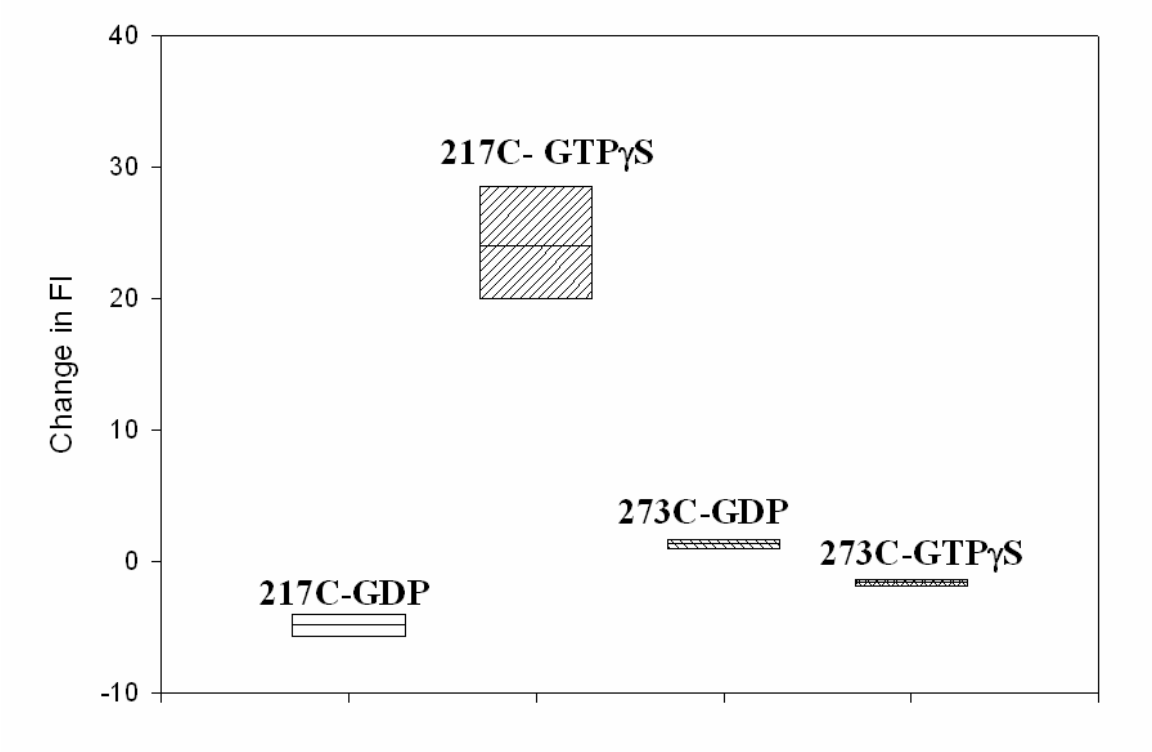

Figure 2.6: Accessibility of 217C and 273C to covalent labeling with CPM in the deactivated and activated states, where the accessibility was monitored by the increase in fluorescence intensity due to CPM covalent attachment after 30 minutes where the change in fluorescence intensity was calculated using ((Fi-Fo)/Fo). Intensities were subtracted from those without bound Gβγ. the amount of Gβγ added at 10 nM for the $G\alpha_i$ (GDP) mutants and 80nM for the $G\alpha_i$ (GTPγS) mutants. In these plots, the box displays the highest and lowest values as taken from the standard deviation while the line inside the box is the mean.

As expected, the accessibility of 106C was identical in the absence and presence of 10 nM blocked Gβγ. Residue 273C, which is outside the $G\alpha_i$-Gβγ interface but more buried in the protein interior, also shows modest decrease in accessibility in the presence of 10nM blocked Gβγ (Figure 2.6) presumably due to the effective increase in the protein matrix the CPM must diffuse through for labeling. In contrast, activation of 217C resulted in a large increase in its accessibility. This result suggests a significant change in the $G\alpha_i$-Gβγ interface upon activation.

PLCβ_2 binds to the heterotrimer

The above studies show that Gα_i-G$\beta\gamma$ can remain associated in the activated state with a different orientation between the two subunits. We next verified that this change in orientation will allow for effector binding and activation. To assess binding, we measured the change in intensity of one of the Gα_i mutants, CPM-29C, as G$\beta\gamma$ was added up to a stoichiometric amount to allow the formation of Gα_i (GDP)-G$\beta\gamma$ (Figure 2.7). We then added PLCβ_2. Not only did PLCβ_2 not disrupt the Gα_i–G$\beta\gamma$ complex, but it binds directly to it as observed by a second titration curve (Figure 2.7). These results imply that PLCβ_2 may also bind weakly to Gα_i (GDP) as well as G$\beta\gamma$. This idea was directly tested by measuring the binding of CPM-Gα_i (GDP) to PLCβ_2 (see Figure 2.7 *insert*). We find that even though PLCβ_2 is not activated by Gα_i, the two proteins still associate. This weaker association between related, but not functionally reactive proteins supports the notion of self-scaffolding.

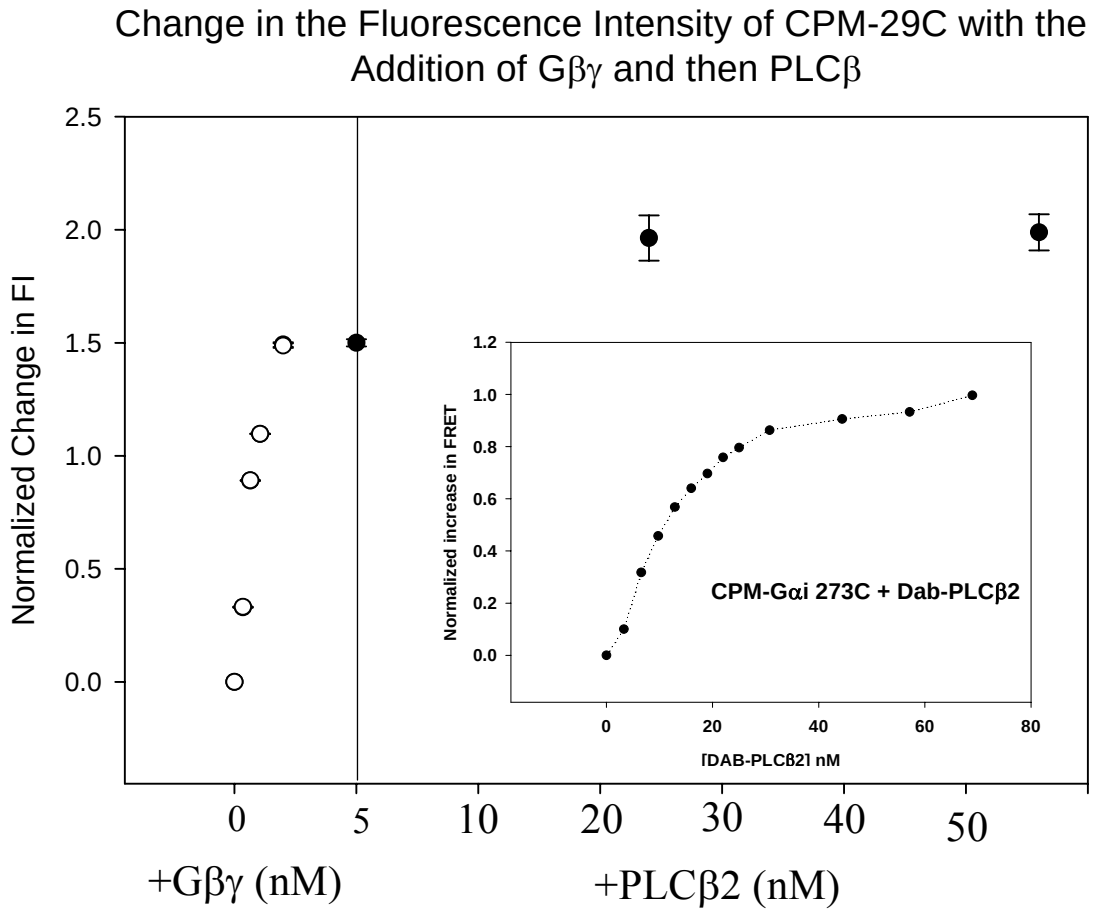

Figure 2.7: Plot showing the association of PLCβ_2 to the Gα_i-G$\beta\gamma$ complex. The open circles show the increase in fluorescence intensity as unlabled G$\beta\gamma$ subunits are added to 10 nM CPM-Gα_i(GDP) (29C) reconstituted onto 200 μM PC:PE:PS (1:1:1) large, unilamellar vesicles. Addition of unlabeled PLCβ2 (closed circles) to the Gα_i(GDP)-G$\beta\gamma$ complex does not reverse the increased intensity. The insert figure shows the association between CPM- Gα_i 29C with the PLCβ_2 where n = 3.

We verified formation of the Gα_i(GDP)-G$\beta\gamma$-PLCβ_2 by two methods. In the first, we labeled one of the mutants 106C with Alexa488 and measured the increase in the diffusion constant (tauD) as unlabeled G$\beta\gamma$ subunits, and then PLCβ_2 were added by fluorescence correlation spectroscopy (FCS). These data were taken in the absence of lipid vesicles. We find that tauD for Alexa488-Gα_i increases from 732 $\pm$ 9 to 756 $\pm$ 14 /s

(S.E.) when Gβγ was added. This small increase is consistent with the increase in effective mass in forming the heterotrimer. Addition of PLCβ$_2$ increased this value to 835 ±16 /s. Keeping in mind that the diffusion constant is inversely related to the molecular size and shape of the species, and that PLCβ$_2$ is an ellipsoid (31), these data are consistent with the formation of the higher order, Gα$_i$(GDP)-Gβγ-PLCβ$_2$ complex.

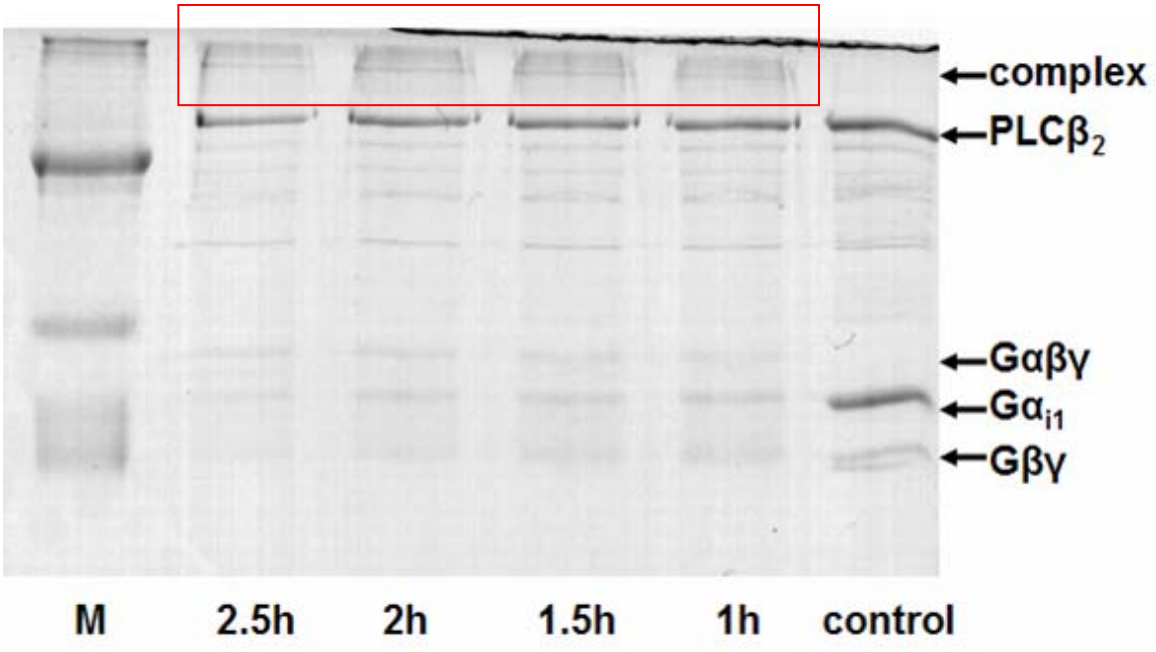

Figure 2.8: SDS gel showing the increase in molecular weight when a cross-linking agent is added to Gα$_i$, Gβγ and PLCβ$_2$.

We confirmed the presence of Gα$_i$(GDP)-Gβγ-PLCβ$_2$ complexes by chemical cross-linking. We find that addition of cross-linker to a mixture of Gα$_i$(GDP), Gβγ and PLCβ$_2$ generates bands with masses that correspond to Gα$_i$-Gβγ and Gα$_i$-Gβγ-PLCβ$_2$ complexes (Figure 2.8). These studies suggest that PLCβ$_2$ cross-links with the Gα$_i$-Gβγ heterotrimer.

PLCβ_2 can only be activated by the Gα_q family of G proteins and not by other families of Gα (32). Previous studies have shown that if Gα_i(GDP) will bind to activated PLCβ_2-G$\beta\gamma$ causing deactivation without disrupting the complex (18). We determined whether PLCβ_2 can be activated by G$\beta\gamma$ subunits while bound to Gα_i(GTPγS) by activity measurements. We note that in these studies, we needed to carry out the measurements at relatively high concentrations of proteins in order to form higher order complexes which sometimes caused the measurements to be above the linear range in the shortest measurement times thus causing G$\beta\gamma$ activated to be reduced. The measurements were also hampered by loss of protein due to dialysis to remove the metals in the buffers required to activate Gα_i and the potential loss of nucleotide. In Figure 2.9 we show the results of a compilation of 4 independent measurements of the samples in triplicate where the activities were normalized to PLCβ_2 alone. We find that the activity of the enzyme consistently increases with G$\beta\gamma$ and this increase is eliminated in the presence of Gα_i(GDP) but not Gα_i(GTPγS). Even with the concerns listed above, we conclude that Gα_i(GTPγS) is capable of supporting activation of the G$\beta\gamma$-PLCβ_2 complex.

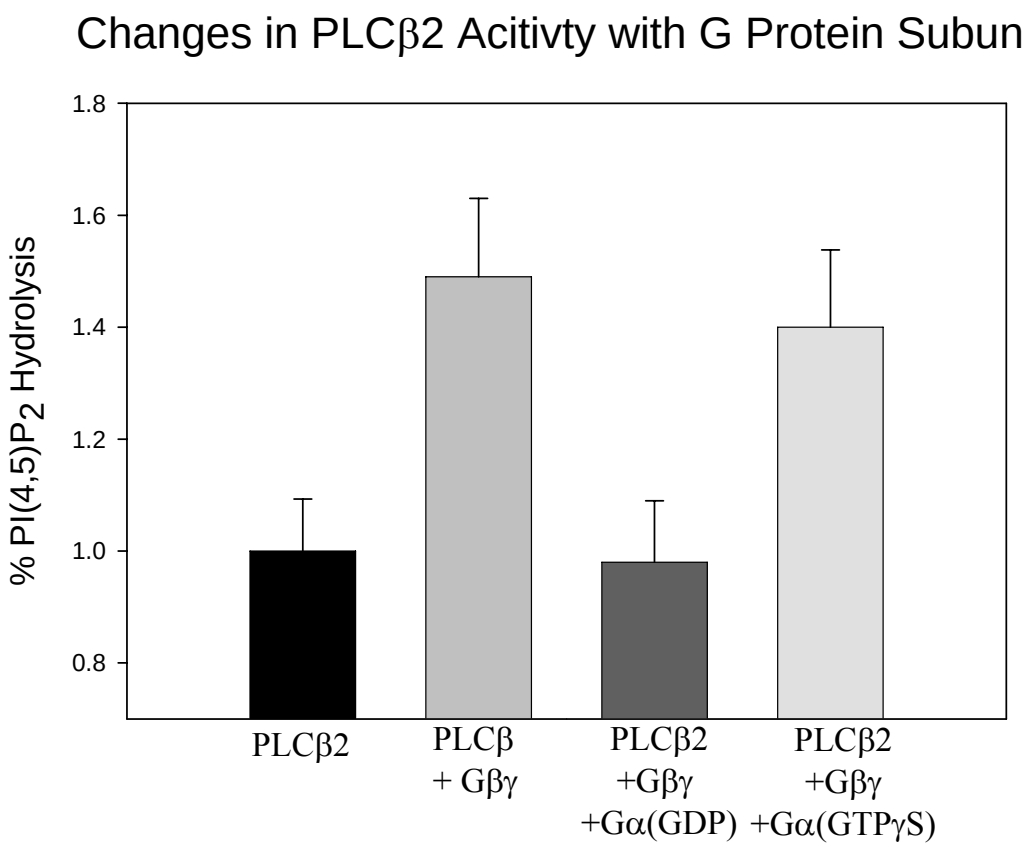

Figure 2.9: Changes in $PLC\beta_2$ activity where substrate, 2 mol% PtdIns(4,5)P_2, was immersed in 133 mM PC:PE:PS (1:1:1) small, unilamellar vesicles (see methods). Measurements were taken of the isolated enzyme and in the presence of 200 nM Gβγ, 200 nM $G\alpha_i$ (GDP) and/or 200 nM $G\alpha_i$(GTPγS) where each point is taken from the average of 4 data sets where each sample was measured in triplicate.

Discussion

The traditional view of signal transduction is that binding of GTP to the Gα subunit of a heterotrimeric G protein results in dissociation of Gα from Gβγ subunits (32-35). The dissociated G protein subunits are then free to individually bind to effector enzymes and change their catalytic activities. This scenario is well supported by a variety of in vitro studies, such as the large drop in measured affinity between the G protein subunits with a concomitant increase in G protein - effector affinity (e.g. (36)). In this

study, we have shown that G protein subunits and effectors can remain associated through the activation cycle. Specifically, we show that in HEK293 cells, $G\alpha_i$ and $G\beta\gamma$ subunits remain associated through the activation cycle, and this observation correlates well with other studies (7,37). We also show that PLCβ effectors are pre-bound to G protein subunits and that the extent of this binding does not change during the activation cycle similar to the result obtained for $PLC\beta_1$ and $G\alpha_q$ (6). Consistent with this idea of pre-bound G proteins-effectors, we find that cytosolic $PLC\beta_2$ is not recruited to the membrane by $G\beta\gamma$ subunits during activation. This result correlates well with our previous observation that $PLC\beta_1$ is not recruited to the membrane upon activation of $G\alpha_q$ in PC12 and HEK293 cells (6).

The resulting effect of signaling complexes is to direct signals along a particular pathway in a rapid manner. We find here that $PLC\beta_2$ resides in complexes that contain $G\alpha_i$-$G\beta\gamma$. This finding is surprising because $PLC\beta_2$ is the main effector of $G\alpha_q$ and its activation by $G\beta\gamma$ has been assumed to be secondary only through $G\beta\gamma$ released from $G\alpha_i$ as it interacts with its main effector, adenylyl cyclase. It is possible that these proteins are localized in large complexes that contain the effectors of both G protein subunits. However, it is also possible that complexes with only a single effector type exist leaving open the possibility that $G\beta\gamma$ may activate its effectors without concomitant activation of $G\alpha_i$ effectors. It is intriguing to suggest that separate pools of $G\alpha_i$-$G\beta\gamma$ exist specifically for $G\beta\gamma$ effectors to allow for signaling specificity in the same way that separate pools of $G\alpha_q$ exists for its two effectors, PLCβ and phosphoinositide 3-kinase (25).

There are many potential interactions that can stabilize signaling complexes. We have previously reported that proteins involved in the G protein-PLCβ signaling pathway

have high affinity interaction sites for their functional partner, and may also contain lower affinity sites for other pathway proteins (38). Here, we find that even though PLCβ_2 is not a functional partner of Gα_i, it is capable of binding to this protein, and this secondary interaction may serve to stabilize the Gα_i-G$\beta\gamma$-PLCβ complex. This observation supports the notion that these proteins have the ability to self-scaffold through secondary, non-functional sites thereby keeping the proteins localized in the complex through the activation cycle.

The finding from these and other studies that G proteins and effectors can remain bound in the basal and activated states in cells raises the question of how the interactions between these proteins change upon GTP binding to Gα that allow for effector activation. This idea is especially intriguing for G$\beta\gamma$ which does not undergo conformational changes during the activation cycle. We approached this question by first measuring the energy involved in activation that must be overcome for GTPγS-Gα_i to remain bound to G$\beta\gamma$ subunits. We find that activation reduces their affinity approximately an order of magnitude, which correlates well with previous studies (27). However, this interaction is still fairly strong and it is likely that under many cellular conditions, the local concentrations of the G proteins may be high enough to sustain interaction between the subunits. We postulate that the discrepancies in G protein subunit dissociation behavior seen under different cellular conditions may be attributed to differences in affinities, such as the weaker affinity between Gα_s and G$\beta\gamma$ as compared to Gα_q and Gα_i, (27,37) or to the presence of high local amounts competing proteins, such as caveolin-1 which binds to activated Gα_q (39,40).

The molecular interactions that allow activated G protein subunits to remain associated are unclear. Our studies here suggest large scale conformational changes in Gα that are consistent with the crystal structures (29,30). The major change upon activation involves movement of the N-terminal arm from Gβγ to make contacts within $G\alpha_i$. This change results in solvent protection of Trp side chains (W211 and W258) from reacting with N-bromosuccidimide which is observed here.

The nature of the $G\alpha_i$ (GTPγS)-Gβγ structure is unclear. By placing fluorescence tags at different locations on $G\alpha_i$ using single Cys mutants, we find that the orientation between $G\alpha_i$ and Gβγ subunits differ extensively in the deactivated and activated states. Experiments comparing the intensity changes when Gβγ binds to $G\alpha_i$(GDP) or to $G\alpha_i$(GTPγS) show large differences on 29C, which resides on the edge of the N-terminal arm, 217C which lies close to Gβγ in the GDP-bound state and 273C, which is far from the Gβγ interface in the deactivated state (Figure 2.3). We also find that activation of $G\alpha_i$ results in a decrease in the distances between the N-terminus of Gβγ and residues 29, 217 and 273 as determined by onset of FRET suggesting that these residues move within 30 Angstroms of each other. Interestingly, we find that 217C becomes highly accessible to CPM upon activation suggesting that the side chain of this residue no longer protected by bound Gβγ but is exposed in the activated state.

Our results suggest that $G\alpha_i$(GTPγS)-Gβγ can support $PLC\beta_2$ activation. If this is the case, then Gβ86-105, which is the region responsible $PLC\beta_2$ activation (41), must become exposed. Exposure of this region is consistent with movement of the N-terminal arm of $G\alpha_i$. Since the N-terminus of $G\alpha_i$ is palmitoylated and myristolyated (see (21)),

then the N-terminus is expected to maintain its contacts with the membrane surface while allowing the helical domain to rotate towards Gβγ.

Using GRAMM (42), we have constructed potential models of the $G\alpha_i$(GTPγS)-Gβγ complex by docking the structure of $G\alpha_i$(GTPγS) and $G\alpha_i$(GDP) derived from their complexes (29,30). Several low energy structures were obtained. However, of the top 16 structures only 3 showed exposure of Gβγ86-105 and only one of these correlated to the FRET and the CPM results. This structure shows interaction between the C-terminal helical regions of Gγ and helix 330-346 of $G\alpha_i$. This interaction would require Gβγ to rotate on the membrane surface to the opposite end of $G\alpha_i$, which would likely be mediated by the N-terminal arm. While this model is possible, it is important to note that it was constructed with an activated $G\alpha_i$ structure that is missing the first 31 residues.

The results presented here show that $G\alpha_i$-Gβγ-PLCβ_2 complexes exist in cells and are capable of signaling while remaining associated. While the molecular details of the orientation between the G protein subunits in the activated form remains to be solved, it is clear that the traditional view of G protein signaling that involve subunit dissociation and subsequent effector binding may only occur under certain specific cellular circumstances. Efforts are underway to better identify the molecular interface between the G protein subunits in the activated form.

Literature Cited

1. Exton, J. H. (1993) *Adv Second Messenger Phosphoprotein Res* **28**, 65-72

2. Birnbaumer, L. (2007) *Biochim Biophys Acta* **1768**, 772-793
3. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781
4. Neer, E. J. (1995) *Cell* **80**, 249-257
5. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) *J Biol Chem* **271**, 27209-27212
6. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014
7. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082
8. Hynes, T. R., Mervine, S. M., Yost, E. A., Sabo, J. L., and Berlot, C. H. (2004) *J Biol Chem* **279**, 44101-44112
9. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J Biol Chem* **271**, 7999-8007
10. Sengupta, P., Philip, F., and Scarlata, S. (2008) *J Cell Sci* **121**, 1363-1372
11. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) *Science* **291**, 2408-2411
12. Exton, J. H. (1994) *Annu Rev Physiol* **56**, 349-369
13. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem Sci* **17**, 502-506
14. Runnels, L. W., and Scarlata, S. F. (1999) *Biochemistry* **38**, 1488-1496
15. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058
16. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972
17. Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem.* **271**, 413-424
18. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry* **37**, 15563-15574
19. Chiadac, P., Mavkin, V. S., and Ross, E. M. (1999) *Biochem.Pharm.* **58**, 39-48
20. Philip, F., and Scarlata, S. (2004) *Biochem.* **43**, 11691-11700
21. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J.Biol.Chem.* **270**, 503-506
22. Spade, T., and Witkop, B. (1967) *Meth.Enzymol.* **11**, 498-506
23. Schwille, P. (2000) *Cross-correlation analysis in FCS in Fluorescence correlation spectroscopy: Theory and applications*, Springer, Berlin
24. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) *J. Biol. Chem.* **273**, 16265-16272
25. Golebiewska, U., and Scarlata, S. (2008) *Biophys. J.* **95**, 2575-2582
26. Illenberger, D., Walliser, C., Strobel, J., Gutman, O., Niv, H., Gaidzik, V., Kloog, Y., Gierschik, P., and Henis, Y. (2003) *J.Biol.Chem.* **278**, 8645-8652
27. Sarvazyan, N. A., Lim, W. K., and Neubig, R. R. (2002) *Biochemistry* **41**, 12858-12867
28. van der Meer, W., Coker G. and Chen, S. S.-Y. (1994) *Resonance Energy Transfer, Theory and Data*, VCH Publishers, Inc., New York
29. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058
30. Tesmer, J., Berman, D., Gilman, A., and Sprang, S. (1997) *Cell* **89**, 251-261
31. Jezyk, M. R., Snyder, J. T., Gershberg, S., Worthylake, D. K., Harden, T. K., and Sondek, J. (2006) *Nat.Struc.& Mol.Biol.* **13**, 1135-1139
32. Birnbaumer, L. (2007) *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**, 772-793

33. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1994) *Molecular Biology of the Cell*, Garland, New York
34. Exton, J. H. (1997) *European Journal of Biochemistry* **243**, 10-20
35. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781
36. Runnels, L. W., and Scarlata, S. (1999) *Biochemistry* **38**, 1488-1496
37. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proceedings of the National Academy of Sciences* **103**, 17789-17794
38. Dowal, L., Elliott, J., Popov, S., Wilkie, T. M., and Scarlata, S. (2001) *Biochemistry* **40**, 414-421
39. Sengupta, P., Philip, F., and Scarlata, S. (2008) *J Cell Sci* **121**, 1363-1372
40. Murthy, K. S., and Makhlouf, G. M. (2000) *J.Biol.Chem.* **39**, 30211-30219
41. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) *Science* **283**, 1332-1335
42. Vakser, I., Matar, O., and Lam, C. (1999) *PNAS* **96**, 8477-8482

Chapter III: $G\alpha_{i1}$(GDP) Subunits Have a Second, High Affinity Gβγ Binding Site

This work has been submitted to Journal of Biological Chemistry: Jingting Wang, Parijat Sengupta, Yuanjian Guo, Urszula Golebiewska and Suzanne Scarlata. *$G\alpha_{i1}$(GDP) Subunits Have a Second, High Affinity Gβγ Binding Site.*
Dr. Parijat Sengupta contributed to FCS work, Dr. Yuanjian Guo and Dr. Urszula Golebiewska contributed significantly to microinjection.

Introduction

The plasma membranes of cells are organized as a series of protein-rich and lipid-rich domains (1-3). Many of protein-rich domains, in particular those organized by caveolae, are thought to be complexes of functionally related proteins that transduce extracellular signals (2). There is increasing evidence that heterotrimeric G proteins exist in pre-formed membrane complexes with their receptors and their intracellular effectors (4-8). The G protein signaling system is initiated when an extracellular agonist binds to its specific G protein coupled receptor or GPCR (for reviews see (9-12)). The ligand-bound receptor will then catalyze the exchange of GTP for GDP on the Gα subunit in the G protein heterotrimer. In the basal state, Gα(GDP) binds strongly to Gβγ, but in the GTP-bound state this affinity is reduced, allowing Gα(GTP) and Gβγ subunits to individually bind to a host of specific intracellular enzymes and change their catalytic activity.

While the interactions between G protein subunits have been studied extensively *in vitro*, their behavior in cells may differ. For example, in pure or semi-pure systems, activation of Gα(GDP) sufficiently weakens its affinity for Gβγ resulting in dissociation (13). However, in cells separation of the heterotrimer is observed under some circumstances, but not others (7,14-17). The reason for these differences in behavior is

not clear. There are 4 families of Gα subunits that each contain several members and additionally, there are many subtypes of Gβγ subunits (18). It is possible that differences in dissociation behavior reflects differences in affinity between G protein subunit subytpes(19), the presence of various protein partners and/or differences in post-synthetic modifications of the subunits(20).

The mechanism that allows activated G proteins to remain bound is not apparent from the crystal structure (21,22). If G protein subunits do not dissociate in cells, then their interaction must change in such a manner as to expose the effector interaction site(s). We have found that phospholipase $C\beta_1$ ($PLC\beta_1$), an important effector of $G\alpha_q$(23) is bound to $G\alpha_q$ prior to activation and throughout the activation cycle (6) implying that $G\alpha_q$(GDP) interacts with $PLC\beta_1$ in a non-functional manner.

We have evidence that signaling complexes are stabilized by a series of secondary interactions. Using purified proteins and model membranes, we have found that members of the $G\alpha_q$-Gβγ/ $PLC\beta_1$/RGS4 signaling system have secondary, weaker binding sites to members of this signaling system in addition to their high affinity site(s) to their functional partner(s). We speculate that secondary contacts allow for self-scaffolding of signaling proteins. To understand the nature of these secondary contacts, we have studied the ability of the $G\alpha_{i1}$(GDP)-Gβγ heterotrimer to remain complexed through the activation cycle. Here, we present evidence that $G\alpha_{i1}$(GDP) has two distinct Gβγ binding sites that only differ in affinity by an order of magnitude and may allow for continued association between the subunits upon activation. We also find that this site plays an important role in stabilizing G protein associations in cells and provides a mechanism of self-scaffolding.

Materials and Methods

Protein expression and purification

His_6-$G\alpha_{i1}$ proteins were expressed *Escherichia coli* and purified as previously described. The DNAs of these proteins were kindly provided by Dr. Heidi Hamm (Dept. of Pharmacology, Vanderbilt University). Dr. Hamm has shown that the single Cys mutants behave identically to wild type (see 23; 24 for full description of their properties as well as their expression and purification). $G\alpha_{i1}$ (GDP) was activated by incubation at 30 ^{o}C for 30 minutes, with the activation buffer (50 mM Hepes, 100 mM $(NH_4)_2SO_4$, 150 mM $MgSO_4$, 100 mM EDTA and 100 μM GTPγS) 23; 25. His_6-$G\beta_1\gamma_2$ was expressed in SF9 cells cells through baculovirus infection 26. This method allows for post synthetic modifications. The geranylgeranyl chain on the $G\gamma_2$ subunit was assessed by thin-layer chromatography on LK5D linear-k silica gel thin-layer chromatography (TLC) plates. The preparation of His_6-$PLC\beta_2$ from a baculovirus – SF9 expression system has been described.

Protein Labeling

$G\alpha_{i1}$ proteins were labeled with the thiol-reactive probe, 7-diethylamino-3-(4'-male-imidylphenyl)-4-methylcoumarin (CPM) at a 1:1 probe to protein ratio as determined by absorption spectroscopy. Unreacted probe was removed by dialysis for three times for 30 min. against a 100 fold excess of buffer containing DTT. Gβγ was labeled at pH 8.0 with Dabcyl SE or Alexa488 TFP, which reacts with primary amines to give a labeling ratio of 1:5 probe: protein as determined by absorption. This low level of labeling is due to acetylation of a large portion of the protein as determined by trypsin

digestion followed by mass spectrometry. Unreacted probe was removed by gel chromatography. Labeling was also verified by measuring the diffusion coefficient of Alexa-Gβγ by fluorescence correlation spectroscopy.

Fluorescence Measurements

Fluorescence experiments were carried out on an ISS PC1 spectrofluorometer (ISS, Urbana, IL). 10nM CPM labeled $G\alpha_i$ was reconstituted on 200 μM large unilamellar vesicles (LUV) composed of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L- serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphoethanolamine (POPE) (1:1:1) and the solution placed in a 3 mm microcuvette. Spectra were recorded using a 384 nm excitation wavelength and scanning the emission from 420 - 520nm. The area under the curves was calculated to give the total emission intensity.

Raw data files generated during data acquisition using ISS software were exported to raw data in LFD format one by one. These files were then opened in SIMFCS program (Enrico Gratton, LFD, UCI). Histograms were generated using "Large Vector Correlation" routine of SIMFCS and saved as .his files. Globals software (Enrico Gratton, LFD, UCI) was used to analyze these histograms. The data were fit to an equation describing Gaussiun beam waist PCH distribution involving 2 components with brightness ratio. The brightness ratio of the 1st and 2nd component was fixed at 2 (24). The fractions of components were calculated from the relative population ratio of the two species determined by the fitting routine.

Cell Culture and Transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/mL of penicillin, and 50 μg/mL streptomycin sulfate at 37 °C in a 5% CO_2 incubator. The cells were transfected using calcium phosphate coprecipitation in which cells were grown on 60 mm dishes for 24 – 48 h to achieve 80 – 90% confluence, the media was then replaced, and 5 μg of eYFP-$G\alpha_{i1}$ and eCFP-$G\beta_1$ and 10 μg of HA-$G\gamma_7$ plasmids were mixed with 120 mM $CaCl_2$ and HBS buffer (21 mM Hepes, 123 mM NaCl, 5 mM KCl, and 0.9 mM Na_2HPO_4, pH 7.1), incubated on ice for 10 min, and added to cells drop wise. The cells were then incubated at 37 °C and the media replaced after 8 – 14h. The cells were allowed to recover for 8 – 14h and split into 35 mm glass bottom Mattek dishes and imaged 48 – 72 h later. Membrane preparations from cells transfected with eYFP- $G\alpha_{i1}$ and eCFP-$G\beta_1$ activate PLCβ effectors at a ~50% higher level that membrane prepared from cells transfected with empty vector.

Microinjection

Transfected cells were grown in Mattek dishes for 48h at 70-80% confluence. Prior to microinjecting, the media was changed to phenol-free Leibovitz's-15 (L-15). The sample microinjection solutions consisted of 130 nM peptide with 0.2% deoxycholic acid in 20nM Hepes, 160 mM NaCl, pH 7.2 with trace amounts of Cy5, and the control solution was identical except that peptide was omitted. We used an InjectMan NI2 with FemtoJet pump from Eppendorf to microinject the solutions into cytoplasm. We typically set the injection pressure Pi = 30 hPa, and kept the compensation pressure Pc = 15 hPa. The injection time was t = 0.4 s. Typically, we injected about 10-25 cells within a 10–20 minute period. We examined the microinjected cells under the phase microscope

(Axiovert 200M from Zeiss with 40 × phase 2 objective) to select viable cells. We then transferred the cells to the Zeiss LSM 510 Meta/confocor 2 apparatus (Jena, Germany) and collected images.

Results

FRET studies suggest that $G\alpha_{i1}$ binds multiple $G\beta\gamma$ subunits

In a previous study, Hamm and coworkers developed a series of single Cys mutants of $G\alpha_{i1}$ to monitor conformational changes that occur upon activation (25). We have previously used these mutants to measure the affinity between $G\beta\gamma$ and $G\alpha_{i1}$(GDP) on 200 μM lipid bilayers to be Kd~1nM (Wang et al., *submitted*). To determine the changes in orientation between $G\alpha_{i1}$ and $G\beta\gamma$ that occur upon activation, we labeled each of these single Cys $G\alpha_{i1}$ mutants with an environmentally-sensitive fluorescent probe (CPM) and monitored the ability of these mutants to transfer excited stated energy (i.e. FRET) to $G\beta\gamma$ subunits labeled on the N-terminus of $G\beta\gamma$ with the non-fluorescent FRET acceptor, Dabcyl SE (D-). We measured the labeling ratio to be 0.3:1 probe to protein and note that this low ratio is due to significant fraction of aceylated protein as determined by mass spectrometry. We note that there is uncertainty of the labeling species due to the difficulty in assessing the small $G\gamma$. Therefore; we are using FRET as a general indicator of binding.

Because Dabcsyl SE is a non-fluorescent energy transfer acceptor, transfer from CPM (C-) will be seen as a decrease in donor fluorescence. The R_o for these probes, which corresponds to the distance at which 50% of donor fluorescence is lost to transfer, is 20Å (26). Keeping in mind that the amount of FRET follows the 6^{th} power of the

distance, then from the crystal structure (27), only the 106C mutant will be close enough to the N-terminus of Gβ to participate in FRET in the deactivated state barring a large amount of free rotation of the N-terminus of Gγ. Unexpectedly, the titration curves for all of the $G\alpha_{i1}$ mutants showed a biphasic behavior (Figure 3.1). At low Gβγ concentrations, an increase in C-$G\alpha_{i1}$ donor intensity was observed, while at higher concentrations, a decrease in intensity, indicative of FRET, was seen. This decrease was observed for all mutants and saturated at approximately 80 nM Gβγ. However, if the data obtained for the unlabeled Gβγ is subtracted from D-Gβγ, this increase is not observed. We have previously reported this increase in CPM- $G\alpha_{i1}$(GDP) intensity with the addition of unlabeled Gβγ and interpret it to be due to protection of the CPM from solvent quenching as Gβγ subunits bind to $G\alpha_{i1}$ forming the heterotrimer. Thus, the initial parts of the titration curves are interpreted to represent the primary Gβγ binding site of $G\alpha_{i1}$(GDP).

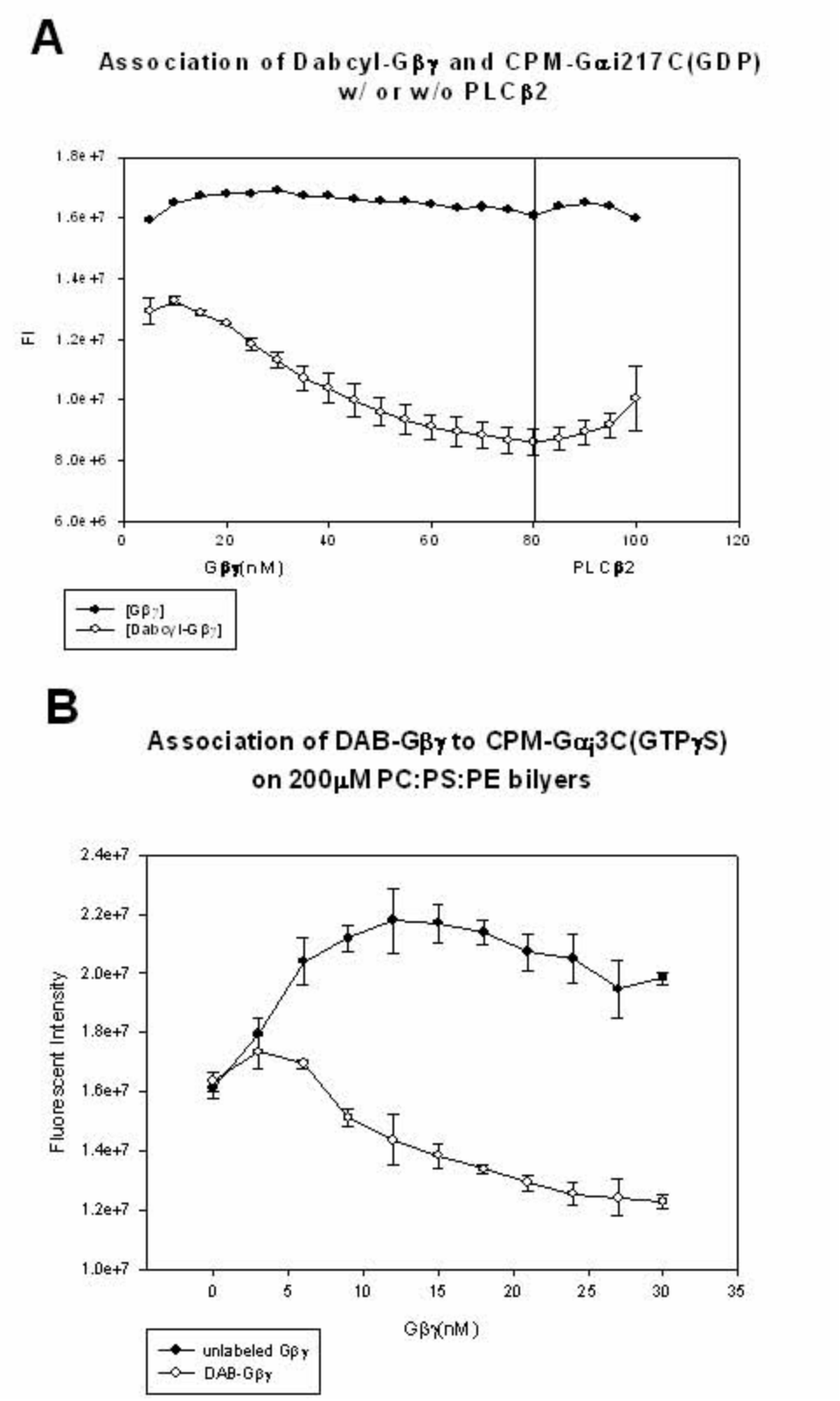

Figure 3.1: **A**. Raw data of a single set of titrations comparing the change in fluorescence intensity of C-Gα217C with the addition of unlabeled Gβγ or D-Gβγ, and then $PLC\beta_2$.

Note that the intensity values of the unlabeled were offset to show that both samples show a similar rise at low Gβγ concentrations. **B**. The results using activated, C-Gα 3C.

Above 5 nM D-Gβγ, the decreases in C-$G\alpha_{i1}$ intensities are not seen when unlabeled Gβγ subunits are used. We interpret the loss in C-$G\alpha_{i1}$ intensity to be due to FRET to D-Gβγ. We find that the extent of this decrease varied for each mutant (Figure 3.2), although the concentration dependence of the titration curves was similar. Fitting the titration curves to a bimolecular association constant gives a similar apparent dissociation constant for all of the single $G\alpha_{i1}$ mutants ranging from 21 to 34 nM giving an average of Kd=23+/-5 nM. These apparent Kd values are ~20 fold weaker than those measured for $G\alpha_{i1}$(GDP)-Gβγ by other methods.

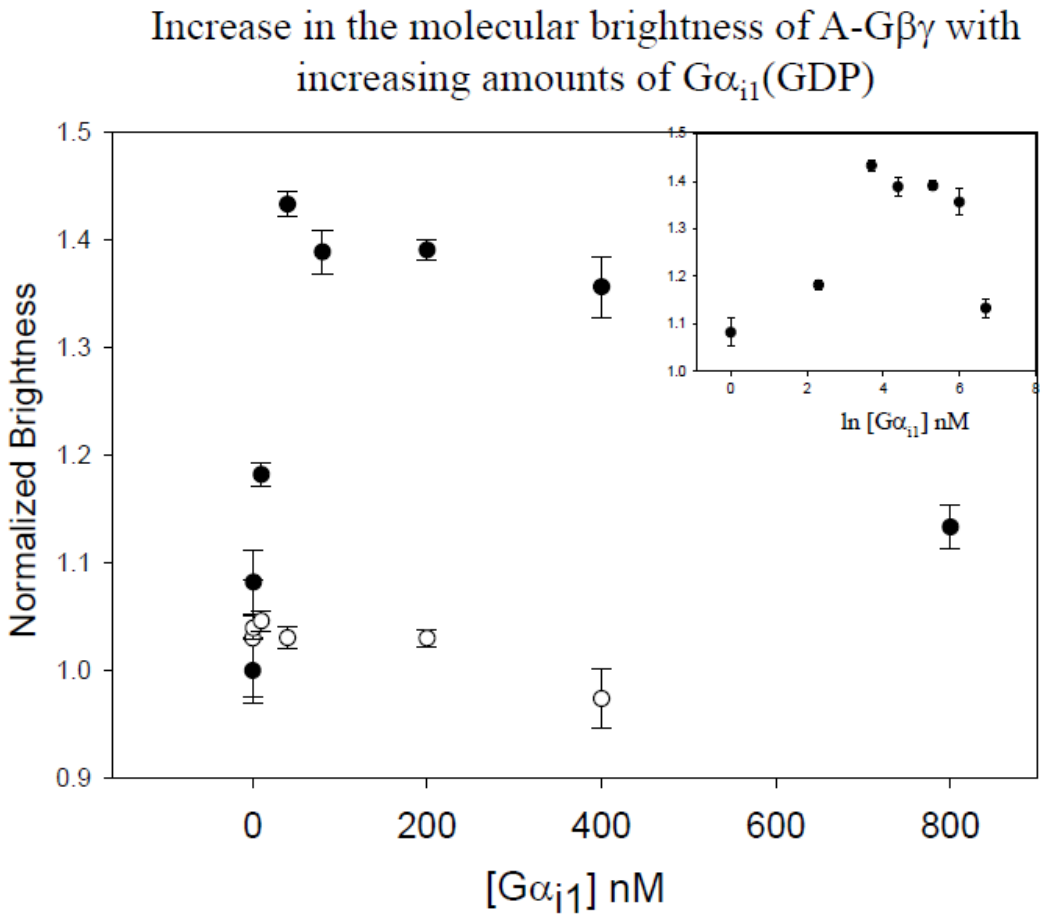

Figure 3.2: Dependence of molecular brightness of 80 nM A Gβγ at different $G\alpha_{i1}$(GDP) concentrations (closed circles) and $G\alpha_{i1}$(GTPγS) (open circles) where each point is an average of 18-23 measurements and SD is shown. *Inset* – plot of the ln of the $G\alpha_{i1}$(GDP) versus concentration.

The results of the FRET titrations are surprising because all mutants display a significant amount of FRET that begins above concentration at which the $G\alpha_{i1}$–Gβγ heterotrimer should have formed (i.e. apparent Kd~1nM), and also because, with one exception, all of the probe positions should be out of FRET range according to the crystal structure (27) or only display a small amount of FRET if the N-terminus of Gγ is very flexible. We do not believe that this weaker apparent affinity is due to the presence of

fluorescent tags since the location of the probes are not interfacial and since the apparent Kd values obtained for the mutants are close. The simplest interpretation of these data is that a second Gβγ molecule is binding to the heterotrimer which is in close enough proximity to participate in FRET with the single Cys sites (i.e. within 20Å).

We repeated the FRET titrations using the activated forms of the 29C and 106 C mutants. We could not detect binding of at low Gβγ concentrations in accord with the reduced affinity that accompanies activation (e.g. Figure 3.1B). We do, however, clearly observe a weaker association that is on the same order as the apparent second site observed using FRET (K_d(app)= 45 +/-5 nM for 29C and 62+/- 11 nM for 106C). These results suggest that in the activated state, the high affinity site is replaced by a second, weaker site.

Brightness analysis shows that $G\alpha_{i1}$ can bind to multiple $G\beta\gamma$ subunits.

To verify the existence of a second site, we labeled Gβγ with Alexa488 TFP (A-) and measured the molecular brightness of the molecules in solutions containing different stoichiometric amounts of $G\alpha_{i1}$ (Figure 3.2) (28) on an FCS instrument without lipid vesicles which would interfere with diffusion measurements. In the absence of $G\alpha_{i1}$, the brightness of A-Gβγ, expressed as counts per second per molecule, was ~40% lower than free Alexa 488 most likely due to the presence of local quenching groups.

At low amounts of $G\alpha_{i1,}$ the brightness of A-Gβγ does not significantly change. However, as the amount of $G\alpha_{i1}$ added to A-Gβγ increases, the brightness also increases. The only mechanism that can increase this value is the presence of oligomers that contain more than one A-Gβγ molecules. As the amount of $G\alpha_{i1}$ rises 5 to 10 fold above A-Gβγ,

the brightness decreases as the equilibrium shifts to a single Gβγ molecule per $G\alpha_{i1}$ subunit. The corresponding diffusion coefficients measured by FCS averaged 5.9 $\pm$0.8 (n=9) fold slower than free Alexa488 suggesting that the complexes are small and not larger aggregates.

We fit the data from the 80 nM A-Gβγ : 40 nM $G\alpha_{i1}$ in Figure 3.2 to a model in which one A-Gβγ binds to the A-Gβγ-$G\alpha_{i1}$ complex. The results show that at this stoichiometry, 30% of the Gβγ is in a Gβγ-$G\alpha_{i1}$-Gβγ complex. The labeling ratio of A-Gβγ is 1:5 probe:protein and so a significant population of unlabeled material is present which is optically silent. Thus, the amount of Gβγ-$G\alpha_{i1}$-Gβγ is expected to be far greater than 30%. We note also that presence of lipids would be expected to increase the amount of higher order complexes due to the effective reduction of dimensionality (see (29)).

Our FRET titration curves suggest that in the activated state, the primary high affinity binding site of Gβγ to $G\alpha_{i1}$ is replaced by a second, weaker site. To determine whether activated $G\alpha_{i1}$ is capable of binding 1 or 2 A-Gβγ subunits, we repeated the above studies using activated $G\alpha_{i1}$. We find that the brightness is constant at through a wide range of $G\alpha_{i1}$ concentrations (Figure 3.2) suggesting that in the activated state, only one Gβγ binding site is available.

FRET studies between Gβγ subunits support a Gβγ-$G\alpha_{i1}$-Gβγ complex

To support the idea that $G\alpha_{i1}$ is capable of binding 2 Gβγ subunits, we carried out a FRET study in which we added unlabeled $G\alpha_{i1}$ to CPM-labled Gβγ(C-Gβγ). Then added

Dabcyl-labeled Gβγ(D-Gβγ) to the stoichiometric amount of C-Gβγ–$G\alpha_{i1}$(GDP) complex and measured the ability of the two proteins to FRET when unlabeled $G\alpha_{i1}$ is added. We find that $G\alpha_{i1}$ produces FRET between the Gβγ subunits (Figure 3.3) supporting the idea that $G\alpha_{i1}$ binds multiple Gβγ subunits.

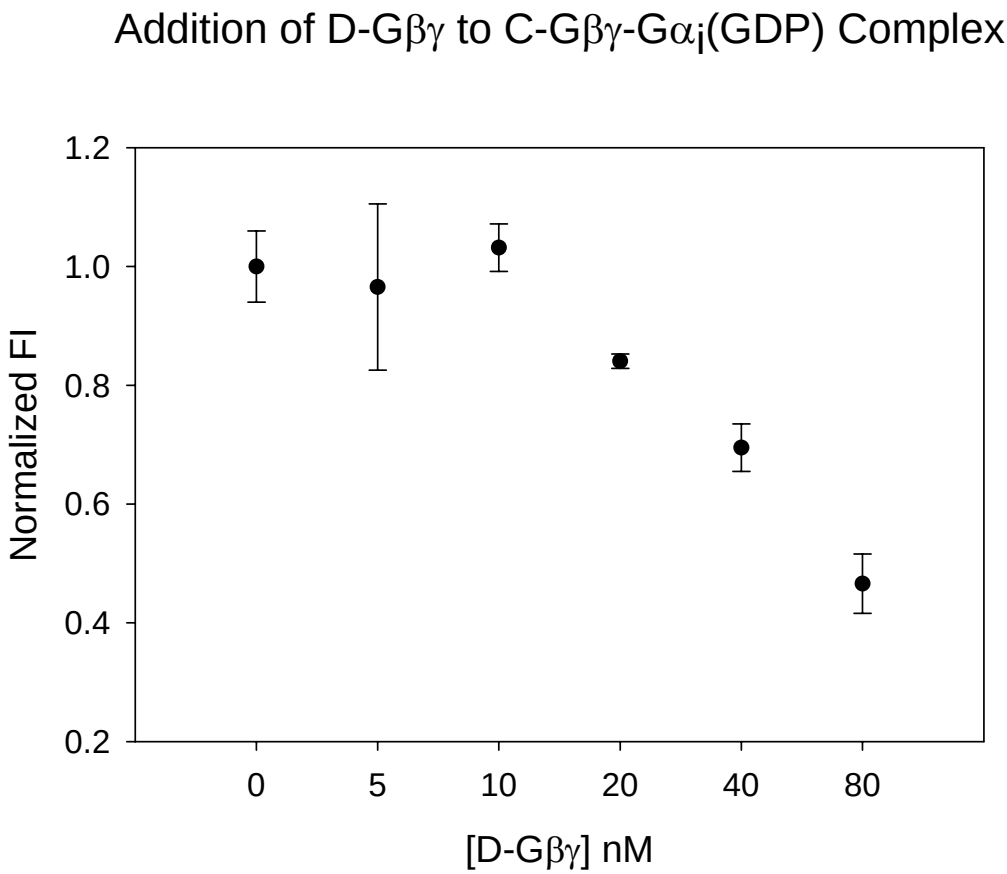

Figure 3.3: Titration showing the onset of FRET, as seen by a decrease in fluorescence, as D-Gβγ is added to (C-Gβγ)-$G\alpha_{i1}$(GDP) showing that the two Gβγ subunits are in FRET distance where n = 3 and S.D. is shown.

The second Gβγ site does not occlude PLCβ_2 effector binding

PLCβ_2 is strongly activated by $G\alpha_q$ but not $G\alpha_{i1}$ subunits, and it is also an effector of Gβγ subunits (for review see (30,31)). We determined whether PLCβ_2 could displace the second Gβγ from the $G\alpha_{i1}$–Gβγ heterotrimer by adding PLCβ_2 to the (C-$G\alpha_{i1}$)-(D-Gβγ) complex and measuring the change in the amount of FRET. Addition of

80 nM PLCβ_2 to solutions containing 10 nM Gα_{i1} and 80 nM G$\beta\gamma$ had no significant affect the amount of FRET most likely due to binding of the enzyme to unbound G$\beta\gamma$ subunits (Figure 3.1 and 3.4**)**. However, addition of 236 nM PLCβ_2 resulted in a partial loss in FRET for 106C, 217C and 305C, which had a high degree of FRET, and a complete loss in FRET for 3C, 29C and 273C which had a lower degree of FRET. Thus, either the PLCβ_2 directly displaces the second G$\beta\gamma$ site or addition of excess PLCβ_2 causes dissociation of G$\beta\gamma$ by a shift in equilibrium. To distinguish between these mechanisms, we added D-G$\beta\gamma$ to a solution C-Gα_{i1} in the presence of PLCβ_2 where the PLCβ_2 was at a concentration high enough to be completely bound to G$\beta\gamma$ (i.e. 20 nM). We find that PLCβ_2 does not affect binding implying that the site of interaction between the second G$\beta\gamma$ and Gα_{i1}(GDP) does not overlap with the effector binding site (*data not shown*).

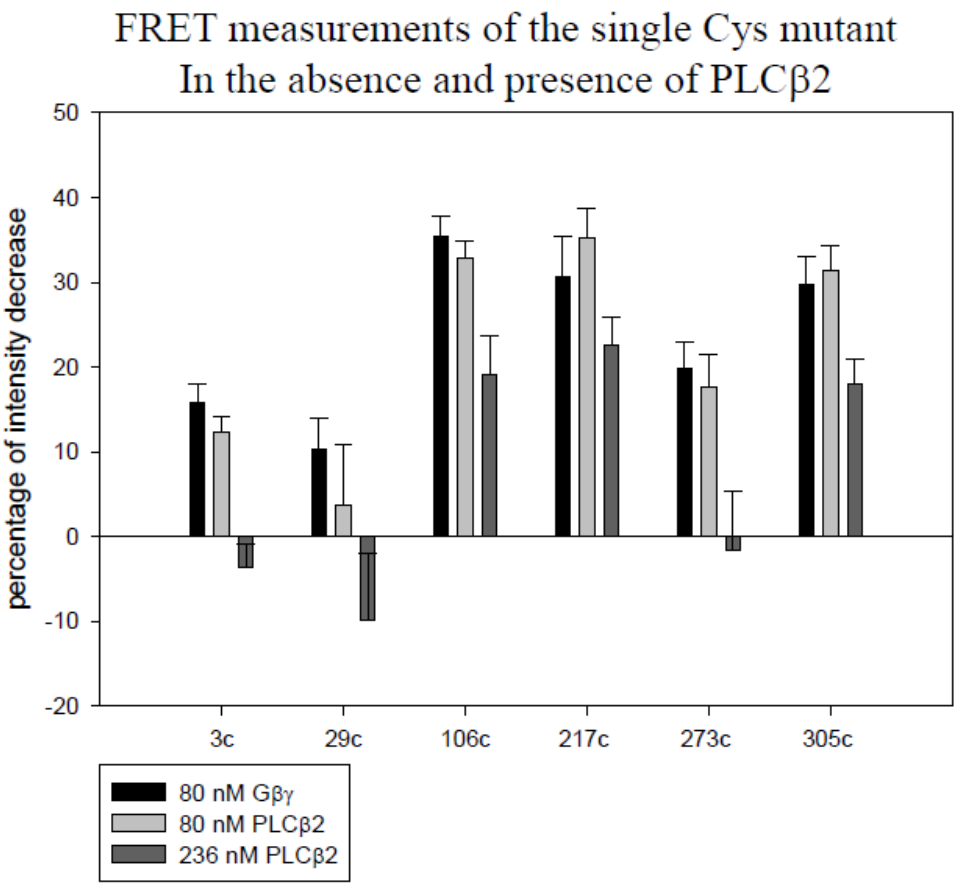

Figure 3.4: Compiled results of the fluorescence decreases in single Cys CPM-$G\alpha_{i1}$(GDP) mutants with the addition of 80 nM Gβγ and 80 and 236 nM of $PLC\beta_2$ is shown where n = 3-6 and S.D. is shown.

Identification of the second interaction site

To identify the nature of the second binding site, we used GRAMM (http://vakser.bioinformatics.ku.edu/resources/gramm, Dr. Vasker, Univ. of Kansas) to dock Gβγ to the $G\alpha_{i1}G\beta\gamma$ using the crystal structure of Sprang and coworkers (27). Of the 20 lowest energy conformations, approximately half involve direct Gβγ-Gβγ interactions, which is inconsistent with the FRET and FCS data. Of the remaining models, we eliminated several on the basis of the FRET results, the partial occlusion of Gβ 86-105 which comprises the $PLC\beta_2$ binding site (32-34), and the possible membrane orientation

of the proteins (35,36). We then carried out another study to distinguish between the remaining models based on differences in the solvent accessibility of the single Cys in the $G\alpha_{i1}$ mutants when the first and second Gβγ subunits is bound using the thiol-reactive probe, CPM. In its unreacted state, CPM is not fluorescent but becomes highly fluorescent upon covalent linkage to a thiol group (see http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) and thus, Cys accessibility can be judged by the onset of fluorescence. We then added CPM to a solution containing iodoacetamide-treated Gβγ subunits to $G\alpha_{i1}$(GDP) bound to lipid membranes. We compared the amount of fluorescence at 0 and 30 minutes for the 106C, 217C and 273C of $G\alpha_{i1}$ at Gβγ concentrations where the first should be primarily occupied (10 nM Gβγ) and at concentrations the second site would be occupied (80 nM Gβγ). 3C and 29C were not tested due to their close proximity to the first site and the membrane interface. We find that 217C and 273C were more (~38 $\pm$ 5%) shielded (i.e. exhibited less) CPM fluorescence at 80 nM Gβγ as compared to 10 nM. However, 106C shows the same amount of CPM fluorescence at both concentrations suggesting that it is exposed when the second Gβγ is bound. This finding narrows down the potential models to a set of three similar models (the lowest energy one is shown in (Figure 3.5**)**. This model also correlates well with the FRET measurements.

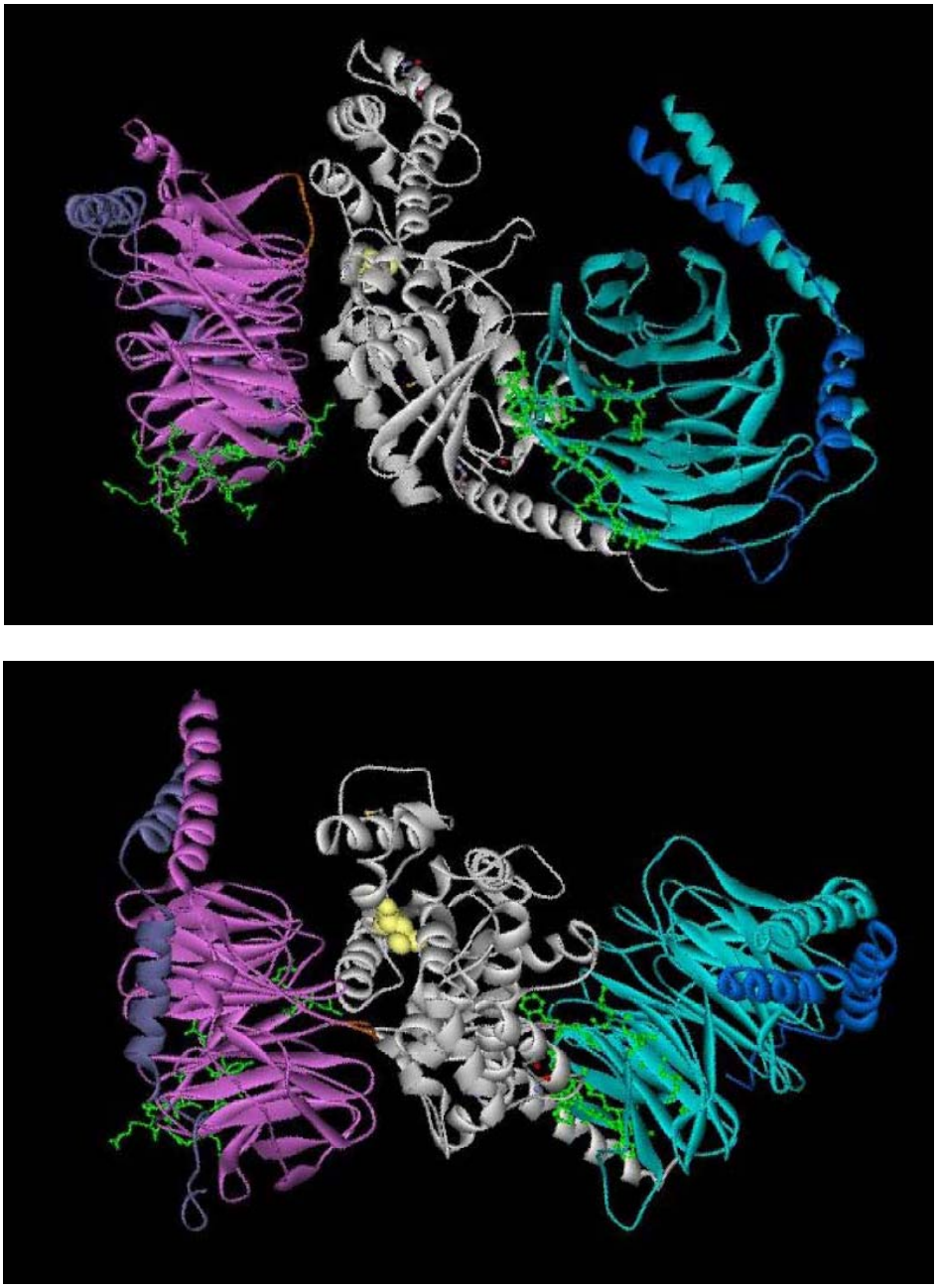

Figure 3.5: Two views of the lowest energy molecular model of the Gβγ-$G\alpha_{i1}$(GDP)-Gβγ complex as predicted by GRAMM that best fits the fluorescence measurements.

According to this model, the most prominent contact made between $G\alpha_{i1}$(GDP) and the second Gβγ subunit involves residues 267-271 of Gβγ. We prepared a peptide that has this sequence and should compete with binding of the second Gβγ. We note that

since this contact region is small, the test peptide may not be as effective for competition with the second binding site as compared to larger peptides (e.g.(37)). Two types of assays were carried out. In the first, we the added D-Gβγ to C-$G\alpha_{i1}$ until FRET was clearly observed indicating the formation of (D-Gβγ)-(C-$G\alpha_{i1}$)-Gβγ complex. We then added peptide to the complex, and found a complete reversal of FRET suggesting dissociation of the D-Gβγ subunit (Figure 3.6 *top*). In a second series of studies, we titrated D-Gβγ into a solution containing C-$G\alpha_{i1}$ in the absence and presence of 10 μM peptide. We find the peptide shifts the binding of the second D-Gβγ to higher concentrations from K_d(app) = 15.4 $\pm$ 2.7 nM to 32 $\pm$ 9.6 nM and reduced the extent of binding as judged by the amount of FRET (Figure 3.6 *bottom*). These results suggest that the peptide competitively binds to the second site.

Our FRET and FCS studies show that in the activated state, $G\alpha_{i1}$ only binds one Gβγ subunit, and our titration curves show this binding has an affinity *on par* with the second Gβγ site seen for $G\alpha_{i1}$ in the deactivated state. To determine whether the second Gβγ binding site in the deactivated state overlaps with the single binding site in the activated state, we carried out a titration study of D-Gβγ to C-$G\alpha_{i1}$(GTPγS) in the presence of 10 μM peptide. We find that the presence of peptide has little affect on the binding of D-Gβγ to C-$G\alpha_{i1}$ suggesting that the loop encompassing the second binding sites has little or no overlap with the $G\alpha_{i1}$(GTP) site. However, it is still possible that even though there is no direct overlap, binding of the full length proteins could be competitive.

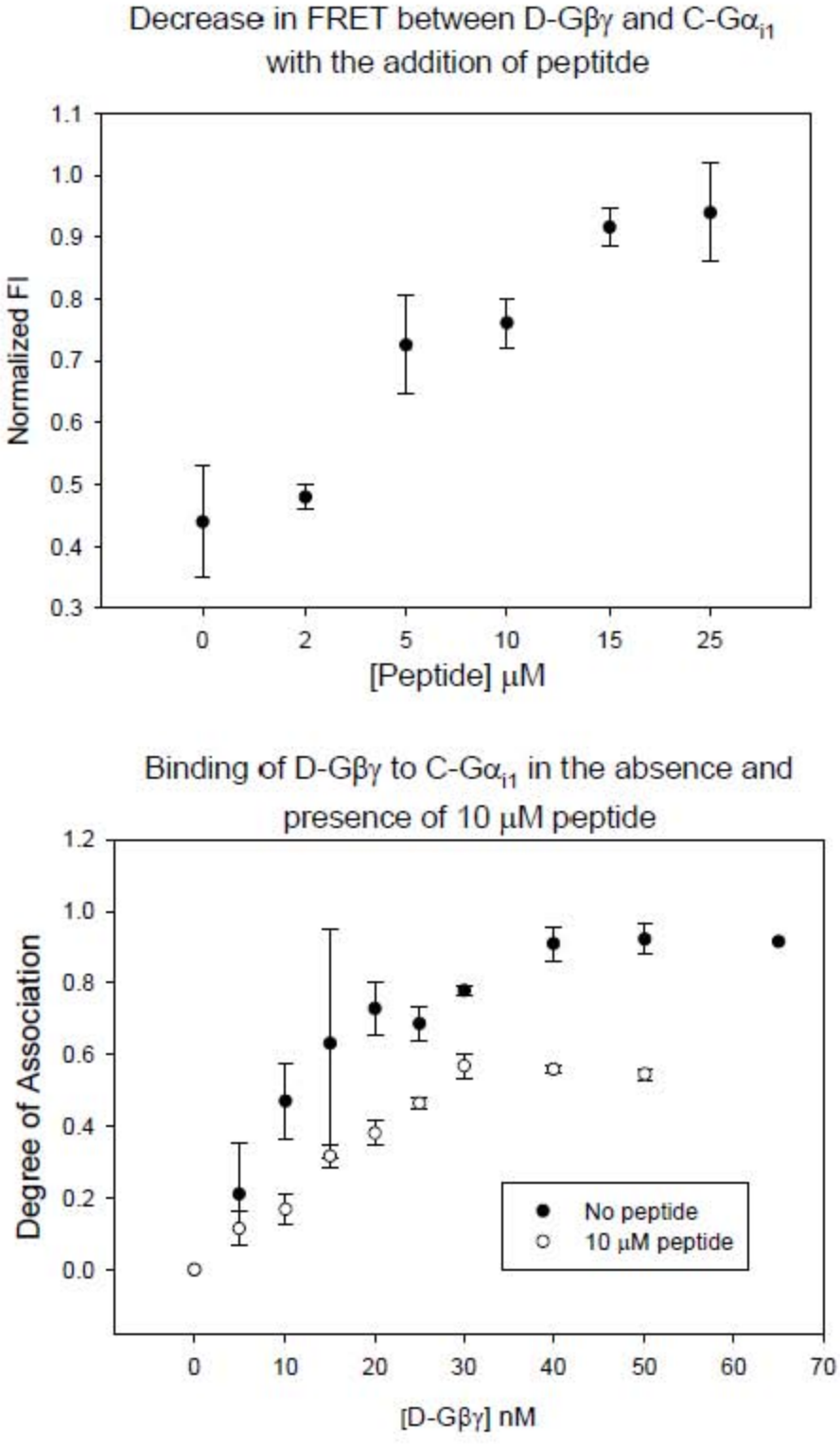

Figure 3.6: (*Top*) Recovery of CPM fluorescence due to the reversal of FRET when Gβ267−271 is added to the (D-Gβγ)–(C-Gα_{i1}(GDP)) complex. (*Bottom*) Reduction in the

association between D-Gβγ and C-$G\alpha_{i1}$(GDP) due to a large excess of Gβ267-271 peptide.

Addition of peptide reduces $G\alpha_i$-$G\beta\gamma$ in cells

To determine if the second site has functional significance, we determined whether the peptide could disrupt Gβγ-$G\alpha_{i1}$ association in HEK293 cells. These studies were carried out by transfecting HEK293 cells with the FRET pair, eCFP-$G\beta_1\gamma_7$ and eYFP-$G\alpha_{i1}$ and determining the change in FRET after microinjecting the peptide. We note that we added a tracer, the red dye (Cy5), whose emission is out of range of the CFP/YFP channels, to allow us to identify the microinjected cells. The microinjected cells were viewed within one hour after injection and showed no visible changes in morphology (Figure 3.7). We compared the amount of FRET between eYFP-$G\alpha_{i1}$(GDP) and eCFP-Gβγ in microinjected cells that contained peptide versus cells microinjected with the carrier solution. The results from 3 independent studies show that peptide causes a reduction in eYFP-$G\alpha_{i1}$(GDP) and eCFP-Gβγ (Figure 3.8). We note that the large variability in the FRET values of the peptide samples are most likely due to the variation in the amount of peptide delivered into the cells. On the whole, these studies show that peptide disrupts G protein subunits in cells.

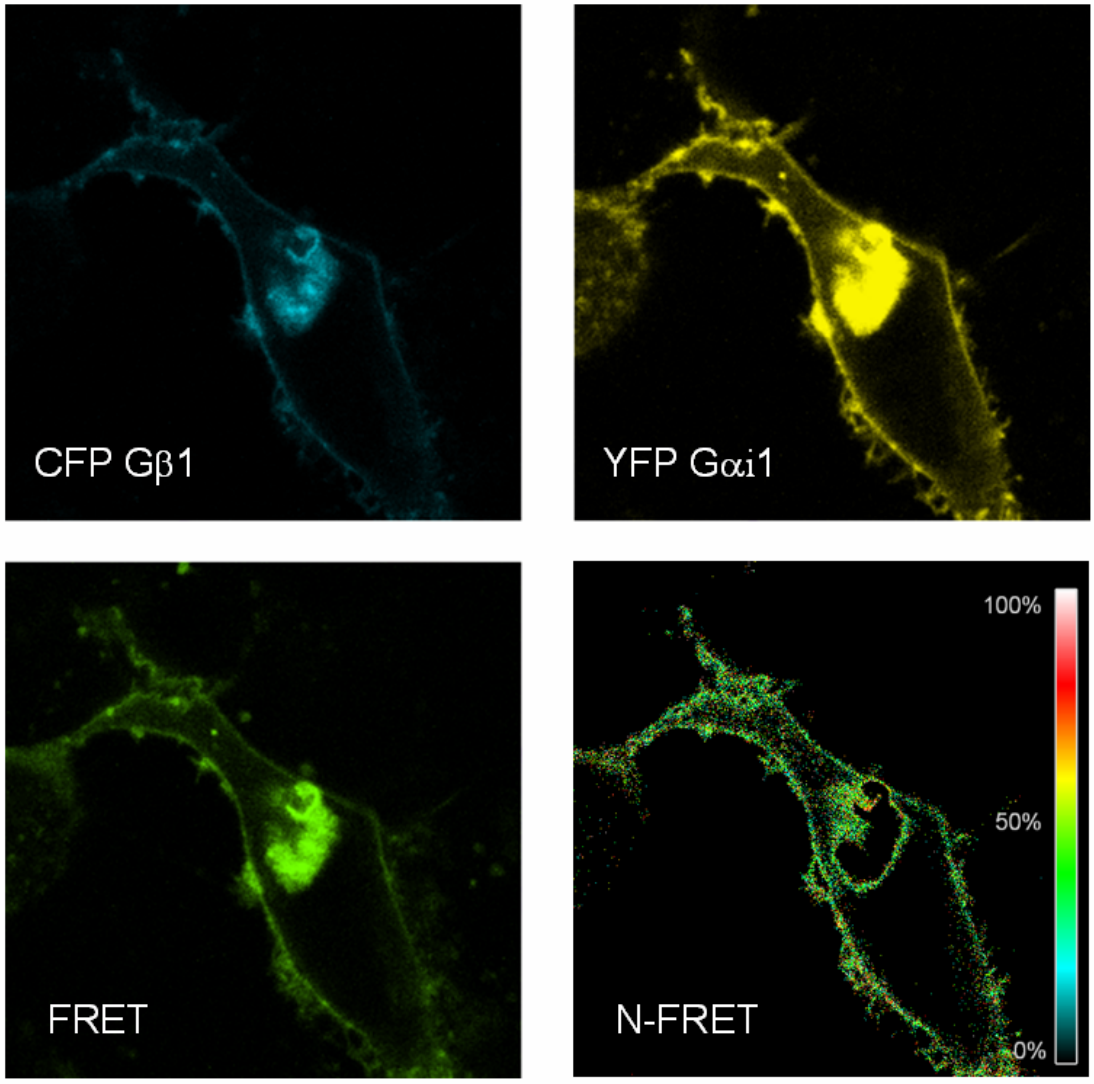

Figure 3.7: Images of HEK293 cells expressing eYFP-$G\alpha_{i1}$ and eCFP-$G\beta\gamma$ with microinjection of Cy5 alone and with Gβ267-271 peptide.

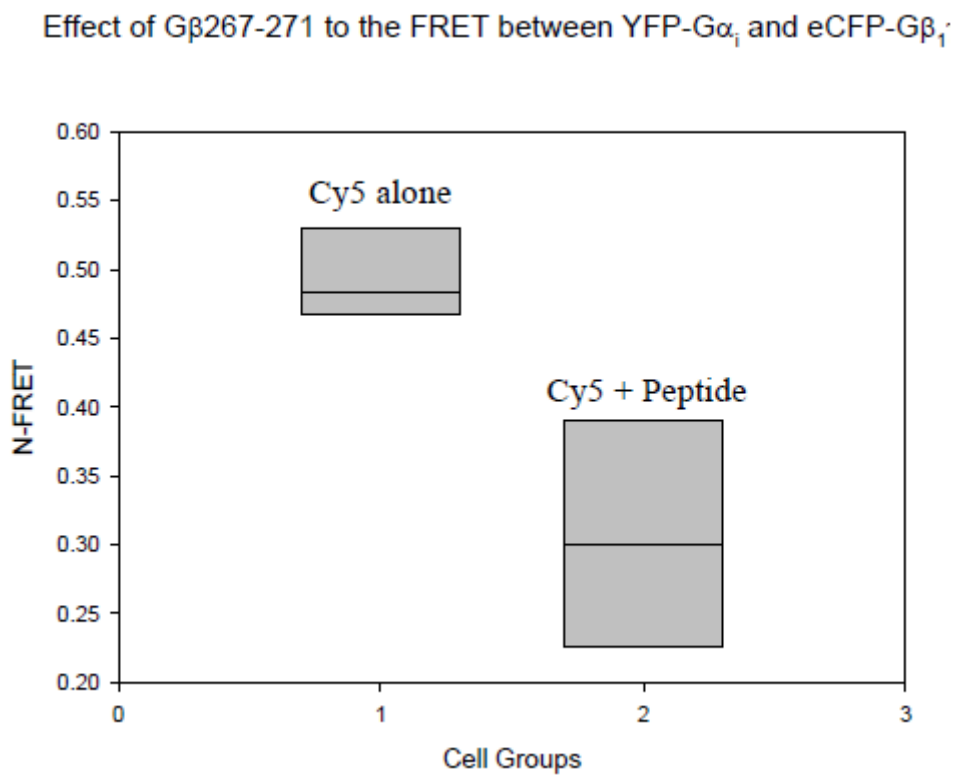

Figure 3.8: Effect of Gβ267-271 on the amount of FRET between eYFP-Gα_{i1} and eCFP-Gβγ.

Discussion

In this study, we have shown that Gα_{i1} subunits contain a second binding site for Gβγ subunits through a combination of fluorescence methods. Molecular modeling coupled with fluorescence and accessibility studies suggests that this site encompasses residues 267-271 of Gβ. Activation of Gα_{i1} eliminates the high affinity site but promotes the formation of a low affinity site that does not share the identical interface with the secondary site. The Gβγ affinity of this binding site is only 10 fold lower than its functional one and injection of a competitive peptide into cells reduces this association.

The structural changes that occur upon G protein activation have been intensely studied (see 38). In the deactivated heterotrimer, $G\alpha_{i1}$ (GDP) makes three critical contacts with $G\beta\gamma$ subunits including one that encompasses the first ~30 residues of $G\alpha_{i1}$ (GDP) resulting in a high affinity interaction (28). Exchange of GDP for GTP produces conformational changes in three key regions that result in a ~10 fold loss in affinity (13; 21). This drop in affinity may be too small to cause subunit dissociation in cells especially considering G proteins are associated with receptors and most likely other partners which may stabilize the heterotrimer. There is evidence that this is not necessarily the case in cells (e.g. 4; 7; 15). Additionally, we have found that activation of $G\alpha_{i1}$ changes its orientation with respect to $G\beta\gamma$ subunits allowing exposure of $PLC\beta_2$ interaction sites without dissociation of the heterotrimer (Wang et al., *submitted*).

Previous studies of subunit interactions in the G protein heterotrimer have typically utilized stoichiometric amounts of subunits or used methods that masked this interaction. Thus, the presence of a second $G\beta\gamma$ site association has not yet been observed despite the strong affinity. In the studies carried out here, association between the subunits was promoted by the effective reduction in dimensionality by carrying out the measurements on membrane-bound proteins. It is thus important to consider the affinities reported here as apparent affinities and the true affinities are expected to be lower. A lower affinity was qualitatively observed in the brightness measurements which were carried out in the absence of membranes (Figure 3.4).

Elementary docking studies of the second site suggest that there are several contact points along the surface of $G\beta\gamma$ that are responsible for binding to the second site and in particular, a unique contact encompassing the loop residues 267-271. This

interaction is not found in the crystal structures of Gβγ bound to GRK or the peptide binding region of adenylyl cyclase. This region is not involved in PLCβ_2 binding. We note, however, that the second site involves multiple interactions over four Gβγ blades, two of which are close to the PLCβ_2 binding site.

The observation that the affinity of the second Gβγ for deactivated Gα_{i1} is in range of the affinity seen for the only Gβγ site in activated Gαi1 (although lower affinity sites may exist) leads to the speculation that the sites overlap. If this were the case, then Gα_{i1} activation may cause Gβγ to move to this site stabilizing the heterotrimeric state. However, our data indicate that the interface of the second binding site encompassing residues 267-271 is not operative in the activated state as indicated by the inability of the peptide to displace Gβγ from deactivated Gα_{i1} but not from activated Gα_{i1}. Since Gα_{i1} subunits undergo large conformational changes

upon activation, it is likely that the Gβγ267-271-Gα_{i1} interaction is weakened or replaced by other contacts.

While it is unclear whether some, if any, of the contacts between the second site Gβγ site and Gα_{i1}(GDP) are utilized by activated Gα_{i1}, our studies using cultured cells show that the 267-271 interface stabilizes Gα_{i1}(GDP)-Gβγ association in the basal state. This observation suggests that this second site serves to organize higher order G protein complexes on the plasma membrane serving a scaffolding function. It is also possible that the second binding site serves to increase the density of Gβγ effector binding. The comparative affinities of the primary and secondary binding sites sets a window of the concentration range that Gβγ will be released in cells to dissociation can contact effectors that are distance from the original heterotrimeric complex.

Literature Cited

1. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1994) *Molecular Biology of the Cell*, Garland, New York
2. Edinin, M. (2003) *Annu.Rev.Biophy.Biomolec.Struc.* **32**, in press
3. Anderson, R. G. (1998) *Annu.Rev.Biochem.* **67**, 199-225
4. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082
5. Nobles, M., Benians, A., and Tinker, A. (2005) *Proc Natl Acad Sci U S A* **102**, 18706-18711
6. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014
7. Philip, F., Sengupta, P., and Scarlata, S. (2007) *J.Biol.Chem.* **282**, 19203-19216
8. Golebiewska, U., and Scarlata, S. (2008) *Biophys. J.* **95**, 2575-2582
9. Neer, E. (1995) *Cell* **80**, 249-257
10. Neer, E. J. (1994) *Protein Science* **3**, 3-14
11. Birnbaumer, L. (2007) *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**, 772-793
12. Exton, J. H. (1997) *European Journal of Biochemistry* **243**, 10-20
13. Runnels, L. W., and Scarlata, S. (1999) *Biochemistry* **38**, 1488-1496
14. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) *Science* **291**, 2408-2411
15. Hughes, T. E., Zhang, H., Logothetis, D., and Berlot, C. H. (2001) *J.Biol.Chem.* **276**, 4227-4235
16. Hynes, T. R., Mervine, S. M., Yost, E. A., Sabo, J. L., and Berlot, C. H. (2004) *J.Biol.Chem.* **279**, 44101-44112
17. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proceedings of the National Academy of Sciences* **103**, 17789-17794
18. Hildebrandt, J. D. (1997) *Biochem. Pharmacol.* **54**, 325-329
19. Sarvazyan, N. A., Lim, W. K., and Neubig, R. R. (2002) *Biochemistry* **41**, 12858-12867
20. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J.Biol.Chem.* **270**, 503-506
21. Tesmer, J., Berman, D., Gilman, A., and Sprang, S. (1997) *Cell* **89**, 251-261
22. Tesmer, J. J. G., Sunahara, R., Gilman, A. G., and Sprang, S. R. (1997) *Science* **278**, 1907-1916
23. Rebecchi, M. J., and Pentyala, S. N. (2000) *Physiol.Rev.* **80**, 1291-1335
24. Chen, Y., Muller, J. D., So, P. T., and Gratton, E. (1999) *Biophys J* **77**, 553-567
25. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972
26. van der Meer, W., Coker G. and Chen, S. S.-Y. (1994) *Resonance Energy Transfer, Theory and Data*, VCH Publishers, Inc., New York
27. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058
28. Schwille, P. (2001) *Cell.Biochem.Biophys.* **34**, 382-408
29. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry* **37**, 15563-15574

30. Exton, J. H. (1994) *Annu. Rev. physiol.* **56**, 349-369
31. Suh, P., Park, J., Manzoli, L., Cocco, L., Peak, J., Katan, M., Fukami, K., Kataoka, T., Yun, S., and Ryu, S. (2008) *BMB reports* **41**, 415-434
32. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) *J. Biol. Chem.* **273**, 16265-16272
33. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998) *J Biol Chem* **273**, 28298-28304
34. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) *Science* **283**, 1332-1335
35. Murray, D., McLaughlin, S., and Honig, B. (2001) *J. Biol. Chem.* **276**, 45153-45159
36. Drin, G., and Scarlata, S. (2007) *Cellular Signalling* **19**, 1383-1392
37. Drin, G., Douguet, D., and Scarlata, S. (2006) *Biochemistry* **45**, 5712-5724

Chapter IV: General Conclusion

The concept of self-scaffolding proteins is widely accepted in different areas of cellular signaling pathways (1) and is a hot area of G protein research. The formation of signaling networks that brings together specific receptors, G proteins, regulatory proteins, enzymes, and substrates, provides specificity of signaling and enhances the interactions between components of a particular signaling module (2,3).

In this study, we report two more self-scaffolding cases in G protein signaling pathway. In the first series of studies, we carried out Forster Resonance Energy Transfer (FRET) studies and found that eYFP-$G\alpha_i$ and eCFP-$G\beta\gamma$ remain associated after stimulation in HEK293 cells. This result correlates well with works from Bunemann and co-workers (4) and other studies (5), and contrasts the traditional understanding of signal transduction that activation of $G\alpha$ subunit of a heterotrimeric G protein results in dissociation of $G\alpha$ from $G\beta\gamma$ subunits (6-9). We also found that the level of FRET between Alex546-$PLC\beta_2$ and eGFP-$G\beta\gamma$ is significant and unchanged throughout the activation cycle in HEK293 cells thus showing that these proteins can localize into stable signaling complexes, which is similar to the result obtained for $PLC\beta_1$ and $G\alpha_q$ (10). The resulting effect of signaling complexes is to direct signals along a particular pathway in a rapid manner. We found here that $PLC\beta_2$ resides in complexes that contain $G\alpha_i$-$G\beta\gamma$. This finding is surprising because $PLC\beta_2$ is the main effector of $G\alpha_q$ and its activation by

Gβγ has been assumed to be secondary only through Gβγ released from $G\alpha_i$ as it interacts with its main effector, adenylyl cyclase.

To understand the basis for this stabilization, we carried out *in vitro* studies using a series of single Cys mutants (11) labeled with fluorescence tags and monitored their interaction with Gβγ subunits and changes in their fluorescence properties and accessibility upon activation and Gβγ binding. These studies suggest a model of the orientation changes between G protein subunits that occur upon activation that allow the G proteins to remain complexed but still activate effectors.

In the initial FRET experiments of the first project, we observed a biphasic behavior in the titration curves for all of the $G\alpha_{i1}$ mutants, which was unexpected. Hence, we studied the complexes using brightness analysis of FCS measurements (12). We found that $G\alpha_{i1}$(GDP) can bind a second Gβγ subunit with an affinity only 10 fold weaker than the primary site and close to the affinity between activated $G\alpha_{i1}$(GTP) and Gβγ subunits. Also, we found that phospholipase C β_2, an effector of Gβγ, does not compete with the second binding site implying that effectors can be bound to the $G\alpha_{i1}$(GDP)-$(G\beta\gamma)_2$ complex. Biophysical measurements and molecular docking studies suggest that this second site is distant from the primary one. A synthetic peptide having this sequence competes with binding of the second Gβγ subunit. Injection of this peptide into HEK293 cells expressing eYFP-$G\alpha_{i1}$(GDP) and eCFP-Gβγ reduces the overall association of the subunits suggesting this site is operative in cells. We propose that this second binding site serves to promote and stabilize G protein subunit interactions in the presence of competing cellular proteins.

Although the above studies indicates the existence of higher order complexes in G protein signaling pathway, there are still many unanswered questions and future directions for the study of the interaction of the self-scaffolding proteins and the second binding site in $G\alpha_i$ for $G\beta\gamma$. First, even though we found the affinity of the second binding site is close to the affinity between activated $G\alpha_i$ and $G\beta\gamma$ subunits, we do not have enough solid evidence to show there are identical binding sites in $G\alpha_i$ subunits. Second, the function of the second binding site is still unclear. We performed cell culture experiments to show that the 267-271 interface stabilizes $G\alpha_{i1}$(GDP)-$G\beta\gamma$ association in the basal state, which suggests that the second $G\beta\gamma$ might be critical in helping the formation of higher order complexes on the plasma membrane. It is also possible that a region of $G\beta\gamma$ might allow contact between $G\alpha_{i1}$(GTP) subunits and their downstream effectors that are distant from the primary heterotrimeric complex, since $G\beta\gamma$ have two different binding affinities to $G\alpha_{i1}$(GDP) while only one to $G\alpha_{i1}$(GTP). Third, our data do not indicate reveal orientation information of $PLC\beta_2$ in the $G\alpha_i$-$G\beta\gamma$-$PLC\beta_2$ complex. Stepping fluorescence experiment using $PLC\beta_2$ mutants might help to solve the question. For example, we could make a series of Cys $PLC\beta_2$ mutants and perform FRET experiment to understand how $PLC\beta_2$ bind to $G\alpha_i$-$G\beta\gamma$ complex. Also we are trying to better identify how G protein subunits are orientated in activated form.

It is very interesting that a number of studies that support scaffolding phenomena have been identified in the recent years, and expectably more and more will be found in the near future. However, it is clear that the traditional view of G protein signaling that involves subunit dissociation and subsequent effector binding may only occur under certain specific cellular circumstances. The emerging picture of G protein signaling

pathway is becoming increasingly complex. Clearly, detailed knowledge of the composition of possible complexes is essential to fully understand the precise signaling pathways involved.

Literature Cited

1. Faux, M. C., and Scott, J. D. (1996) *Cell* **85**, 9-12
2. Burack, W. R., and Shaw, A. S. (2000) *Curr Opin Cell Biol* **12**, 211-216
3. Hall, R. A., and Lefkowitz, R. J. (2002) *Circ Res* **91**, 672-680
4. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082
5. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proc Natl Acad Sci U S A* **103**, 17789-17794
6. Birnbaumer, L. (2007) *Biochim Biophys Acta* **1768**, 772-793
7. Alberts, A. S., Montminy, M., Shenolikar, S., and Feramisco, J. R. (1994) *Mol Cell Biol* **14**, 4398-4407
8. Exton, J. H. (1997) *Eur J Biochem* **243**, 10-20
9. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781
10. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014
11. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972
12. Chen, Y., Muller, J. D., So, P. T., and Gratton, E. (1999) *Biophys J* **77**, 553-567

Bibliography

1. Gilman, A. G. (1987) *Annu Rev Biochem* **56**, 615-649

2. Exton, J. H. (1997) *Eur J Biochem* **243**, 10-20

3. Rebecchi, M. J., and Pentyala, S. N. (2000) *Physiol Rev* **80**, 1291-1335

4. Drin, G., and Scarlata, S. (2007) *Cell Signal* **19**, 1383-1392

5. Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., Fukami, K., Kataoka, T., Yun, S., and Ryu, S. H. (2008) *BMB Rep* **41**, 415-434

6. Roberts, M. F. (1996) *FASEB J* **10**, 1159-1172

7. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058

8. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781

9. Oldham, W. M., and Hamm, H. E. (2006) *Q Rev Biophys* **39**, 117-166

10. Downes, G. B., and Gautam, N. (1999) *Genomics* **62**, 544-552

11. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J Biol Chem* **271**, 7999-8007

12. Milligan, G., and Kostenis, E. (2006) *Br J Pharmacol* **147 Suppl 1**, S46-55

13. Watson, A. J., Katz, A., and Simon, M. I. (1994) *J Biol Chem* **269**, 22150-22156

14. Watson, A. J., Aragay, A. M., Slepak, V. Z., and Simon, M. I. (1996) *J Biol Chem* **271**, 28154-28160

15. Zhang, F. L., and Casey, P. J. (1996) *Annu Rev Biochem* **65**, 241-269

16. Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F., and Vogel, Z. (1998) *FASEB J* **12**, 1019-1025

17. Fletcher, J. E., Lindorfer, M. A., DeFilippo, J. M., Yasuda, H., Guilmard, M., and Garrison, J. C. (1998) *J Biol Chem* **273**, 636-644

18. Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992) *J Biol Chem* **267**, 13807-13810

19. Clapham, D. E., and Neer, E. J. (1997) *Annu Rev Pharmacol Toxicol* **37**, 167-203

20. Graf, R., Mattera, R., Codina, J., Evans, T., Ho, Y. K., Estes, M. K., and Birnbaumer, L. (1992) *Eur J Biochem* **210**, 609-619

21. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) *Nature* **325**, 321-326

22. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. (1994) *J Biol Chem* **269**, 30898-30903

23. Mumby, S. M., and Linder, M. E. (1994) *Methods Enzymol* **237**, 254-268

24. Wise, A., Grassie, M. A., Parenti, M., Lee, M., Rees, S., and Milligan, G. (1997) *Biochemistry* **36**, 10620-10629

25. Morales, J., Fishburn, C. S., Wilson, P. T., and Bourne, H. R. (1998) *Mol Biol Cell* **9**, 1-14

26. Chen, C. A., and Manning, D. R. (2001) *Oncogene* **20**, 1643-1652

27. Smotrys, J. E., and Linder, M. E. (2004) *Annu Rev Biochem* **73**, 559-587

28. Preininger, A. M., Van Eps, N., Yu, N. J., Medkova, M., Hubbell, W. L., and Hamm, H. E. (2003) *Biochemistry* **42**, 7931-7941

29. Exton, J. H. (1993) *Adv Second Messenger Phosphoprotein Res* **28**, 65-72

30. Exton, J. H. (1994) *Annu Rev Physiol* **56**, 349-369

31. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem Sci* **17**, 502-506

32. Runnels, L. W., and Scarlata, S. F. (1999) *Biochemistry* **38**, 1488-1496

33. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature* **369**, 621-628

34. Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995) *Science* **270**, 954-960

35. Warner, D. R., Weng, G., Yu, S., Matalon, R., and Weinstein, L. S. (1998) *J Biol Chem* **273**, 23976-23983

36. Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996) *Nature* **379**, 369-374

37. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972

38. Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002) *Development* **129**, 3533-3544

39. Zhou, Y., Wing, M. R., Sondek, J., and Harden, T. K. (2005) *Biochem J* **391**, 667-676

40. Hwang, J. I., Oh, Y. S., Shin, K. J., Kim, H., Ryu, S. H., and Suh, P. G. (2005) *Biochem J* **389**, 181-186

41. Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y., and Lemmon, M. A. (1998) *J Biol Chem* **273**, 30497-30508

42. Yamamoto, T., Takeuchi, H., Kanematsu, T., Allen, V., Yagisawa, H., Kikkawa, U., Watanabe, Y., Nakasima, A., Katan, M., and Hirata, M. (1999) *Eur J Biochem* **265**, 481-490

43. Rhee, S. G. (2001) *Annu Rev Biochem* **70**, 281-312

44. Jimenez, J. L., Smith, G. R., Contreras-Moreira, B., Sgouros, J. G., Meunier, F. A., Bates, P. A., and Schiavo, G. (2003) *J Mol Biol* **333**, 621-639

45. Brown, D. A. (2006) *Physiology (Bethesda)* **21**, 430-439

46. Cheng, Z. J., Singh, R. D., Marks, D. L., and Pagano, R. E. (2006) *Mol Membr Biol* **23**, 101-110

47. Scarlata, S. (2005) *Biophys J* **88**, 2867-2874

48. Zhang, S., Coso, O. A., Collins, R., Gutkind, J. S., and Simonds, W. F. (1996) *J Biol Chem* **271**, 20208-20212

49. Exton, J. H. (1993) *Adv Second Messenger Phosphoprotein Res* **28**, 65-72

50. Birnbaumer, L. (2007) *Biochim Biophys Acta* **1768**, 772-793

51. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781

52. Neer, E. J. (1995) *Cell* **80**, 249-257

53. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) *J Biol Chem* **271**, 27209-27212

54. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014

55. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082

56. Hynes, T. R., Mervine, S. M., Yost, E. A., Sabo, J. L., and Berlot, C. H. (2004) *J Biol Chem* **279**, 44101-44112

57. Biddlecome, G. H., Berstein, G., and Ross, E. M. (1996) *J Biol Chem* **271**, 7999-8007

58. Sengupta, P., Philip, F., and Scarlata, S. (2008) *J Cell Sci* **121**, 1363-1372

59. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) *Science* **291**, 2408-2411

60. Exton, J. H. (1994) *Annu Rev Physiol* **56**, 349-369

61. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem Sci* **17**, 502-506

62. Runnels, L. W., and Scarlata, S. F. (1999) *Biochemistry* **38**, 1488-1496

63. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058

64. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972

65. Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem.* **271**, 413-424

66. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry* **37**, 15563-15574

67. Chiadac, P., Mavkin, V. S., and Ross, E. M. (1999) *Biochem.Pharm.* **58**, 39-48

68. Philip, F., and Scarlata, S. (2004) *Biochem.* **43**, 11691-11700

69. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J.Biol.Chem.* **270**, 503-506

70. Spade, T., and Witkop, B. (1967) *Meth.Enzymol.* **11**, 498-506

71. Schwille, P. (2000) *Cross-correlation analysis in FCS in Fluorescence correlation spectroscopy: Theory and applications*, Springer, Berlin

72. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) *J. Biol. Chem.* **273**, 16265-16272

73. Golebiewska, U., and Scarlata, S. (2008) *Biophys. J.* **95**, 2575-2582

74. Illenberger, D., Walliser, C., Strobel, J., Gutman, O., Niv, H., Gaidzik, V., Kloog, Y., Gierschik, P., and Henis, Y. (2003) *J.Biol.Chem.* **278**, 8645-8652

75. Sarvazyan, N. A., Lim, W. K., and Neubig, R. R. (2002) *Biochemistry* **41**, 12858-12867

76. van der Meer, W., Coker G. and Chen, S. S.-Y. (1994) *Resonance Energy Transfer, Theory and Data*, VCH Publishers, Inc., New York

77. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058

78. Tesmer, J., Berman, D., Gilman, A., and Sprang, S. (1997) *Cell* **89**, 251-261

79. Jezyk, M. R., Snyder, J. T., Gershberg, S., Worthylake, D. K., Harden, T. K., and Sondek, J. (2006) *Nat.Struc.& Mol.Biol.* **13**, 1135-1139

80. Birnbaumer, L. (2007) *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**, 772-793

81. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1994) *Molecular Biology of the Cell*, Garland, New York

82. Exton, J. H. (1997) *European Journal of Biochemistry* **243**, 10-20

83. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781

84. Runnels, L. W., and Scarlata, S. (1999) *Biochemistry* **38**, 1488-1496

85. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proceedings of the National Academy of Sciences* **103**, 17789-17794

86. Dowal, L., Elliott, J., Popov, S., Wilkie, T. M., and Scarlata, S. (2001) *Biochemistry* **40**, 414-421

87. Sengupta, P., Philip, F., and Scarlata, S. (2008) *J Cell Sci* **121**, 1363-1372

88. Murthy, K. S., and Makhlouf, G. M. (2000) *J.Biol.Chem.* **39**, 30211-30219

89. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) *Science* **283**, 1332-1335

90. Vakser, I., Matar, O., and Lam, C. (1999) *PNAS* **96**, 8477-8482

91. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1994) *Molecular Biology of the Cell*, Garland, New York

92. Edinin, M. (2003) *Annu.Rev.Biophy.Biomolec.Struc.* **32**, in press

93. Anderson, R. G. (1998) *Annu.Rev.Biochem.* **67**, 199-225

94. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082

95. Nobles, M., Benians, A., and Tinker, A. (2005) *Proc Natl Acad Sci U S A* **102**, 18706-18711

96. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014

97. Philip, F., Sengupta, P., and Scarlata, S. (2007) *J.Biol.Chem.* **282**, 19203-19216

98. Golebiewska, U., and Scarlata, S. (2008) *Biophys. J.* **95**, 2575-2582

99. Neer, E. (1995) *Cell* **80**, 249-257

100. Neer, E. J. (1994) *Protein Science* **3**, 3-14

101. Birnbaumer, L. (2007) *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**, 772-793

102. Exton, J. H. (1997) *European Journal of Biochemistry* **243**, 10-20

103. Runnels, L. W., and Scarlata, S. (1999) *Biochemistry* **38**, 1488-1496

104. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) *Science* **291**, 2408-2411

105. Hughes, T. E., Zhang, H., Logothetis, D., and Berlot, C. H. (2001) *J.Biol.Chem.* **276**, 4227-4235

106. Hynes, T. R., Mervine, S. M., Yost, E. A., Sabo, J. L., and Berlot, C. H. (2004) *J.Biol.Chem.* **279**, 44101-44112

107. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proceedings of the National Academy of Sciences* **103**, 17789-17794

108. Hildebrandt, J. D. (1997) *Biochem. Pharmacol.* **54**, 325-329

109. Sarvazyan, N. A., Lim, W. K., and Neubig, R. R. (2002) *Biochemistry* **41**, 12858-12867

110. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J.Biol.Chem.* **270**, 503-506

111. Tesmer, J., Berman, D., Gilman, A., and Sprang, S. (1997) *Cell* **89**, 251-261

112. Tesmer, J. J. G., Sunahara, R., Gilman, A. G., and Sprang, S. R. (1997) *Science* **278**, 1907-1916

113. Rebecchi, M. J., and Pentyala, S. N. (2000) *Physiol.Rev.* **80**, 1291-1335

114. Chen, Y., Muller, J. D., So, P. T., and Gratton, E. (1999) *Biophys J* **77**, 553-567

115. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972

116. van der Meer, W., Coker G. and Chen, S. S.-Y. (1994) *Resonance Energy Transfer, Theory and Data*, VCH Publishers, Inc., New York

117. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell* **83**, 1047-1058

118. Schwille, P. (2001) *Cell.Biochem.Biophys.* **34**, 382-408

119. Runnels, L. W., and Scarlata, S. F. (1998) *Biochemistry* **37**, 15563-15574

120. Exton, J. H. (1994) *Annu. Rev. physiol.* **56**, 349-369

121. Suh, P., Park, J., Manzoli, L., Cocco, L., Peak, J., Katan, M., Fukami, K., Kataoka, T., Yun, S., and Ryu, S. (2008) *BMB reports* **41**, 415-434

122. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) *J. Biol. Chem.* **273**, 16265-16272

123. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998) *J Biol Chem* **273**, 28298-28304

124. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) *Science* **283**, 1332-1335

125. Murray, D., McLaughlin, S., and Honig, B. (2001) *J. Biol. Chem.* **276**, 45153-45159

126. Drin, G., and Scarlata, S. (2007) *Cellular Signalling* **19**, 1383-1392

127. Drin, G., Douguet, D., and Scarlata, S. (2006) *Biochemistry* **45**, 5712-5724

128. Faux, M. C., and Scott, J. D. (1996) *Cell* **85**, 9-12

129. Burack, W. R., and Shaw, A. S. (2000) *Curr Opin Cell Biol* **12**, 211-216

130. Hall, R. A., and Lefkowitz, R. J. (2002) *Circ Res* **91**, 672-680

131. Bunemann, M., Frank, M., and Lohse, M. J. (2003) *Proc Natl Acad Sci U S A* **100**, 16077-16082

132. Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006) *Proc Natl Acad Sci U S A* **103**, 17789-17794

133. Birnbaumer, L. (2007) *Biochim Biophys Acta* **1768**, 772-793

134. Alberts, A. S., Montminy, M., Shenolikar, S., and Feramisco, J. R. (1994) *Mol Cell Biol* **14**, 4398-4407

135. Exton, J. H. (1997) *Eur J Biochem* **243**, 10-20

136. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr Rev* **24**, 765-781

137. Dowal, L., Provitera, P., and Scarlata, S. (2006) *J Biol Chem* **281**, 23999-24014

138. Medkova, M., Preininger, A. M., Yu, N. J., Hubbell, W. L., and Hamm, H. E. (2002) *Biochemistry* **41**, 9962-9972

139. Chen, Y., Muller, J. D., So, P. T., and Gratton, E. (1999) *Biophys J* **77**, 553-567