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A SELF-SCAFFOLDING MODEL FOR G PROTEIN SIGNALING

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SUMMARY

Activation of heterotrimeric G proteins is generally believed to induce dissociation of $G\alpha$ and $G\beta\gamma$ subunits, which are then free to bind to and change the catalytic activity of a variety of intracellular enzymes. We have previously found that in cells $G\alpha_q$ subunits remain complexed with its major effector, phospholipase C β 1 (PLC β 1) through the activation cycle. To determine whether this behavior may be operative in other systems, 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. We also found that the level of FRET between Alex546-PLC β 2 and eGFP- $G\beta\gamma$ is significant and unchanged upon activation in HEK293 cells thus showing that these proteins can localize into stable signaling complexes. To understand the basis for this stabilization, we carried out *in vitro* studies using a series of single Cys mutants labeled with fluorescence tags and monitored their interaction with $G\beta\gamma$ subunits and changes in their fluorescence properties and accessibility upon activation and $G\beta\gamma$ binding. Our studies suggest a significant change in the orientation between G protein subunits upon activation that allow the G proteins to remain complexed while activating effectors.

Keywords

G proteins; cell signaling; fluorescence spectroscopy; protein complexes; G protein effectors

INTRODUCTION

Heterotrimeric G proteins are involved in signaling pathways that respond to a large and diverse set of extracellular agents. Signaling begins when an extracellular agonist binds to its specific G protein coupled receptor (GPCR) spanning the plasma membrane^{1; 2}. The ligand-bound receptor then relays this association to a group of membrane-bound intracellular heterotrimeric G proteins. Heterotrimeric G proteins consist of an α and a $\beta\gamma$ subunit. In the unstimulated state, GDP is bound to the α subunit allowing it to make high affinity contacts with $G\beta\gamma$ subunits. Ligand binding to its specific GPCR causes the receptor to catalyze the exchange of GTP for GDP on $G\alpha$ subunits. This exchange disrupts high affinity contacts between $G\alpha$ and $G\beta\gamma$ subunits and allows the $G\alpha$ (GTP) subunits and the $G\beta\gamma$ subunits to individually bind to a host of specific intracellular enzymes and affect their activity (see 3; 4; 5). $G\alpha$ subunits have a GTPase activity and as GTP is converted to GDP, the high affinity contacts between $G\alpha$ and $G\beta\gamma$ subunits return and the signal is turned-off.

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There are 4 families of $G\alpha$ subunits that are coupled to different GPCRs which in turn regulate a specific set of effector enzymes⁶. In contrast, there are many subtypes of $G\beta\gamma$ subunits and *in vitro* studies suggest there is little specificity in regards to the family of $G\alpha$ subunits they bind or the specific effectors they regulate⁷. However, this promiscuity does not generally occur in cells and it is unclear how $G\beta\gamma$ subunits activate specific effectors in a cytoplasmic environment surrounded by many other potential partners.

In this study, we have approached the question of $G\beta\gamma$ signaling specificity using the effector phospholipase $C\beta$ ($PLC\beta$). $PLC\beta$ enzymes catalyze the hydrolysis of the signaling lipid, phosphatidylinositol 4,5 biphosphate ($PI(4,5)P_2$), which gives rise to an increase in intracellular Ca^{2+} and activation of protein kinase C^{8; 9; 10; 11}. There are 4 known subtypes of $PLC\beta$ that are all activated by $G\alpha_q$ ^{12; 13; 14}. $PLC\beta_2$ and β_3 are additionally activated by $G\beta\gamma$ subunits, although not concomitantly with $G\alpha_q$ ¹⁵. $PLC\beta$ activation by $G\beta\gamma$ subunits can occur in response to agonists that specifically activate $G\alpha_q$, such as carbachol, angiotension II, bradykinin, etc., and also by a wide range of agonists through release of $G\beta\gamma$ subunits by other $G\alpha$ families. Thus, it is not clear how the specificities of $PLC\beta_2$ and β_3 in cells are maintained. One mechanism that may explain signal specificity in cells is self-scaffolding of the proteins to form stable signaling complexes.

This current model of G protein signaling implies that activation results in physical separation between $G\alpha(GTP)$ and $G\beta\gamma$ resulting in diffusion of the G protein subunits on the plasma membrane where they can encounter one or more effectors before deactivation (e.g. 1; 7; 16). However, there is increasing evidence that stable signaling complexes exist in cells. In the basal state, G proteins appear to be complexed with their specific receptors (e.g. 17; 18). Our lab has found that $G\alpha_q$ - $G\beta\gamma$ subunits are associated with G protein coupled receptors (GPCRs) in the basal state, and remain in heterotrimeric form after dissociation and internalization of the receptor during cell stimulation¹⁸. Also, $G\alpha_q$ remains strongly associated to its effector, $PLC\beta_1$ through the activation cycle¹⁹. The behavior of $G\alpha_q$ correlates well with observations showing that the $G\alpha_i$ - $G\beta\gamma$ heterotrimer remain associated during stimulation in HEK293 cells²⁰ but contrasts the dissociation of $G\alpha_i G\beta\gamma$ seen in COS cells and the dissociation and internalization of $G\alpha_s$ in HEK293 cells^{21; 22; 23; 24}. Thus, the tendency of G proteins to dissociate may be specific for certain types of G proteins and under certain circumstances as detailed by Digby and coworkers²⁵. The presence of competing proteins may also play a role in G protein dissociation. For example, we have found that the presence of caveolin-1 promotes the dissociation of $G\alpha_q$ - $G\beta\gamma$ during activation due to strong interactions between activated $G\alpha_q$ and caveolin-1²⁶. Taken together, these studies suggest that G proteins exist in signaling complexes with receptors and effectors and that under many cellular circumstances G proteins can remain associated but still can activate effectors. We have previously proposed that G proteins signaling complexes are stabilized by secondary interactions that allow the proteins to self-scaffold into stable complexes²⁷. These self-scaffolded complexes would limit the direction of the signal due to a fixed composition of proteins and would explain $G\beta\gamma$ signaling specificity.

In viewing the known structure $G\alpha_i(GDP)$ - $G\beta\gamma$ subunits²⁸, it is not apparent how effector activation could occur without subunit dissociation, nor is it clear how $G\alpha_i(GTP\gamma S)$ might interact with $G\beta\gamma$. In this study, we first show that $G\alpha_i$ - $G\beta\gamma$ - $PLC\beta_2$ form stable complexes in cells in the basal and activated states. Then, by a series of *in vitro* studies using purified proteins, we show changes in subunits interactions that occur upon stimulation which 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 $G\beta$ site needed for $PLC\beta_2$ activation.

RESULTS

Gai1 and Gβγ are stably complexed through the activation cycle in HEK293 cells

We have previously found that PLCβ1 is stably bound to Gαq through the activation cycle¹⁹. Here, we tested whether PLCβ2, which can be activated by Gβγ subunits released from Gai, remains complexed to G proteins during activation. In initial studies, we transfected HEK293 cells with eYFP-Gai, eCFP-Gβ1 and HA-Gγ7 and tested whether the extent of FRET between the eCFP and eYFP tags changed upon activation. Although Gγ7 behaves identically to Gγ1 *in vitro*, we used the HA-Gγ7 construct in these studies since its expression and cellular characteristics are well established, e.g.^{21; 22; 23}. We find that eYFP-Gai and eCFP-Gβ1 exhibits a FRET value of 0.36 ± 0.03 ($n = 11$). This value is significantly higher than negative controls using either free CFP and YFP or non-interacting membrane associated proteins (15%) and lower than eCFP and eYFP attached by a peptide linker (70%)²⁹. The intermediate FRET value observed for the eYFP-Gai/eCFP-Gβγ is slightly lower but close to values obtained for eCFP-Gαq/eYFP-Gβγ (0.50 ± 0.04). Stimulation of the cells by the addition of 1 μM isoproterenol to generate activated Gai subunits did not affect the magnitude of FRET (FRET = 0.035 ± 0.03 , $n=14$) suggesting that Gai and Gβγ remain associated in the basal and stimulated states. To insure that all Gα subunits were activated (i.e. that activation was not limited by the amount of available receptor), we repeated the measurements using aluminum fluoride³⁰. Aluminum fluoride non-specifically activates all Gα (GDP) subunits by mimicking the γ-phosphate of GTP. Treatment of the cells with aluminum fluoride caused rapid movement and morphological changes in the cells, but did not result in a change in FRET. This result supports the idea that the G protein heterotrimer is capable of remaining associated upon activation.

We then transfected HEK293 cells with GFP-Gβ1 and HA-Gγ7, and microinjected purified Alexa546-PLCβ2 into the cells (Fig. 1). The injected PLCβ2 distributed in the cytosol and plasma membrane in accord with previous studies³¹. The amount of FRET from the plasma membrane population of PLCβ to Gβγ was then measured in basal and stimulated cells. We obtain an average FRET value of 0.35 ± 0.04 ($n = 30$) in basal state and which was unaltered upon stimulation. These studies suggest that the plasma membrane population of PLCβ2 remains localized with Gβγ subunits in the basal and stimulated states.

Characterization of the binding of purified Gai1 to Gβγ

The above studies support the idea that heterotrimeric G proteins and effectors can exist in complexes that remain bound through the activation cycle¹⁹. 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 Gβγ subunits upon activation. These studies were carried out using wild type Gα_{i1} and a series of single Cys mutants (Fig. 2) as previously described³². These proteins have been shown to have the same functional properties as the wild type protein. The proteins were labeled with CPM. This probe becomes fluorescent upon covalent attachment to Cys side chains and its emission intensity is sensitive to the local dielectric environment. We reconstituted the CPM-Gai proteins onto large, unilamellar vesicles (LUVs) consisting of POPC:POPE:POPS (1:1:1). We note that even though the bacterially expressed Gai subunits are not myristoylated or palmitoylated, they bind to membranes with a high affinity (Scarlati et al., *unpublished results*).

We measured the changes in fluorescence intensity as freshly thawed and dialyzed Gβγ subunits were incrementally added relative to controls 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 probe³², is interpreted as being due to protection of the CPM probe from solvent quenching due to association of G $\beta\gamma$ subunits. In Fig. 3 we show the change in normalized fluorescence for wild type G α_i as a function of G $\beta\gamma$ concentration. Fitting the initial changes to a bimolecular dissociation constant gives a similar value for the wild type and all of the mutants as expected (1.8 $\pm$ 0.3 nM). This value is comparable to similar fluorescence studies of G α_q -G $\beta\gamma$ association and comparable to the apparent K_d of 1.18–1.34 nM obtained by flow cytometry³³. Additionally, the observation that all of the single Cys G α_i constructs have similar G $\beta\gamma$ affinity serves as a good check of the integrity of the folding of the mutants.

It is interesting to compare the extent of intensity changes for the single Cys G α_i mutants. All of the mutants showed an increase in CPM intensity with the addition of small amounts of G $\beta\gamma$ subunits. The extent of this increase varied with each mutant, most likely due to differences in solvent exposure as G $\beta\gamma$ subunits bind. The data in Table 1 show that 3C and 29C, which are on the N-terminal arm of G α_i (see Fig. 2) display a modest change in intensity upon G $\beta\gamma$ 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 α_i most likely because it is located close to the G $\beta\gamma$ association site. Interestingly, 305C G α_i , which is far from the G $\beta\gamma$ 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 α_{i1} mutants and measured their affinity to G $\beta\gamma$ subunits. Again, all of the mutants all showed gave similar K_d values suggesting their activated states are properly folded. As expected, the titration curves were shifted to an apparent K_d ranging from 34 to 45 nM and confirming a weaker association in the activated state (e.g. Fig 3). While the mutants showed differences in fluorescence intensity with G $\beta\gamma$ binding, the extent of the increase differed in the activated versus unactivated states (Table 1) with 217C now showing a very large increase whereas 273 showing a decrease in intensity. These results suggest large changes in contacts between G α_i and G $\beta\gamma$ subunits upon activation.

FRET between single Cys G α mutants and G $\beta\gamma$ 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 α_i mutants to transfer fluorescence energy to a non-fluorescent energy transfer acceptor placed on the N-termini of G $\beta\gamma$ (Dabcyl, see Methods) which are in close proximity. The probability of energy transfer depends on the 6th 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 22Å³⁴. According to the crystal structure²⁸, the distances between the N-terminus of G β and the Cys sites of the G α_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, it may be visible by the onset of FRET. We note that at the lipid and protein concentrations used in this study, FRET will reflect specific associations (*see methods*).

We carried out these studies by measuring the decrease in CPM intensity from the single G α_i mutants when Dabcyl- G $\beta\gamma$ was added. In general, the extent of FRET varied but was close to or less than 10% for all of the G α_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 α_i proteins. Unlike G α_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 (Fig. 4). These results support the idea that the G protein subunits are oriented differently when G α_i is activated.

Changes in Trp and Cys accessibilities upon G α_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 α_i to N-bromosuccinimide (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 Fig. 2) changes upon activation whereas the third residue (W131) appears buried in both states. We first monitored changes in Trp exposure of G α_i GDP in the absence of G $\beta\gamma$ subunits. Treatment with NBS results in an 11 $\pm$ 1% reduction in fluorescence. In contrast, addition of NBS to the activated protein causes a 5.6 $\pm$ 1.5% reduction. This protection of the Trp side chains with activation may be due to protection of these residues by the N-terminus^{28; 35}. Since W211 is in the switch II region, it is probable that this region contributes greatly to the accessibility changes seen here.

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 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 G $\beta\gamma$ subunits with iodoacetamide (*see methods*) and measuring the increase in intensity as CPM reacts with the Cys 217 or 273 on the mutants in the absence and presence of blocked G $\beta\gamma$ subunits over a 30 minute period. As a control, we tested 106 since this residue is outside the G $\beta\gamma$ binding region.

As expected, the accessibility of 106C was identical in the absence and presence of 10 nM blocked G $\beta\gamma$. Residue 273C, which is outside the G α_i -G $\beta\gamma$ interface but more buried in the protein interior, also shows modest decrease in accessibility in the presence of 10nM blocked G $\beta\gamma$ (Fig. 5) 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 is consistent with the CPM intensity changes in Table 1 and suggests a significant change in the G α_i -G $\beta\gamma$ interface upon activation.

PLC β 2 binds to the heterotrimer

The above studies show that G α_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 G α_i mutants, CPM-29C, as G $\beta\gamma$ was added up to a stoichiometric amount to allow the formation of G α_i (GDP)-G $\beta\gamma$ (Fig. 6). We then added PLC β 2. If PLC β 2 disrupts the G α_i -G $\beta\gamma$ complex, we would observe a reversal of the titration curve, but this did not occur. Instead, PLC β 2 appears to bind directly to G protein complex (Fig 6). These results imply that PLC β 2 may also bind weakly to G α_i (GDP) as well as G $\beta\gamma$ subunits. This idea was directly tested by measuring the binding of CPM- G α_i (GDP) to PLC β 2 (see Fig. 6 *insert*). We find that even though PLC β 2 is not activated by G α_i , the two proteins still associate. This weaker association between related, but not functionally reactive proteins, supports the notion of self-scaffolding.

PLC β 2 can only be activated by the G α_q family of G proteins and not by other families of G α ³. Previous studies have shown that G α_i (GDP) will bind to activated PLC β 2-G $\beta\gamma$ causing deactivation without disrupting the complex³⁶. We determined whether PLC β 2 can be activated by G $\beta\gamma$ subunits while bound to G α_i (GTP γ S) by activity measurements. We carried

out these studies at protein concentrations high enough that the ternary complex would be formed. Since this required relatively high concentrations of proteins to form higher complexes, our measurements were slightly above the linear range causing the extent of G $\beta\gamma$ activation of PLC β 2 to be reduced. We also note that we needed to dialyze out the metals used to activate G α i since they interfere with PLC β 2 activity. In Figure 7 we show the results of a compilation of 4 independent measurements of the samples in triplicate where the activities were normalized to PLC β 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 G α i(GDP) but not G α i(GTP γ S). Even with the concerns listed above, we conclude that G α i(GTP γ S) does not interfere with activation of the G $\beta\gamma$ -PLC β 2 complex.

DISCUSSION

The traditional view of signal transduction is that binding of GTP to the G α subunit of a heterotrimeric G protein results in dissociation of G α from G $\beta\gamma$ subunits^{1; 2; 3; 4}. 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. 37). In this study, we have shown that G protein subunits and G protein subunits and effectors can remain associated in the basal and activated states. Specifically, we show that in HEK293 cells, G α i and G $\beta\gamma$ subunits remain associated through the activation cycle. These results are in accord with other studies^{20; 25}. We also show that PLC β effectors are pre-bound to G $\beta\gamma$ subunits and that the extent of this binding does not change during the activation cycle similar to the result obtained for PLC β 1 and G α q¹⁹. Consistent with this idea of pre-bound G proteins – effectors, we find that cytosolic PLC β 2 is not recruited to the membrane by G $\beta\gamma$ subunits during activation similar to the behavior seen for PLC β 1 upon activation of G α q in PC12 and HEK293 cells¹⁹.

The resulting effect of signaling complexes is to direct signals along a particular pathway in a rapid manner. We find here that PLC β 2 localizes with G $\beta\gamma$ subunits and that PLC β 2 has the ability to bind to G α i *in vitro*. This leaves open the possibility that PLC β 2 may localize in complexes with G α i-G $\beta\gamma$. PLC β 2 is the main effector of G α q and its activation by G $\beta\gamma$ has been assumed to be secondary only through G $\beta\gamma$ released from G α 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 α i effectors. It is intriguing to suggest that separate pools of G α 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 α q exist for its two effectors, PLC β and phosphoinositide 3-kinase²⁹.

There are many potential interactions that can stabilize signaling complexes. We have previously reported that proteins involved in the G protein-PLC β signaling pathway have high affinity interaction sites for their functional partner, and may also contain lower affinity sites for other pathway proteins²⁷. This idea appears to be the case for PLC β 2 and G α i, and this secondary interaction may serve to stabilize the G α i-G $\beta\gamma$ -PLC β complex. This observation supports the notion that these proteins have the ability to self-scaffold through secondary, nonfunctional sites thereby keeping the proteins localized in the complex through the activation cycle. It is important to note that the cellular FRET measurements and the *in vitro* measurements were taken in either the deactivated or activated states without resolution of the time dependence of the interactions. Implicit in the self-scaffolding model is that effector interactions with G protein subunits are stable throughout the G protein activation cycle. While there is precedent for this idea in model systems^{36; 38}, the effective off-rates of these

interactions have yet to be accurately determined in cells although our previous cell studies showing stable G protein-effector interactions^{18; 19} support this notion.

The finding 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 $G\alpha$ 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 $G\alpha i$ -GTP γ S 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 studies³³. 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 $G\alpha s$ and $G\beta\gamma$ as compared to $G\alpha q$ and $G\alpha i$,^{25; 33} or to the presence of high local amounts competing proteins, such as caveolin-1 which binds to activated $G\alpha q$ ^{26; 39}.

The molecular interactions that allow activated G protein subunits to remain associated are unclear. Our studies here suggest large scale conformational changes in $G\alpha$ that are consistent with the crystal structures^{28; 35}. The major change upon activation involves movement of the N-terminal arm from $G\beta\gamma$ to make contacts within $G\alpha i$. This switch results in solvent protection of Trp side chains (W211) from reacting with N-bromosuccinimide which is observed here.

The nature of the $G\alpha i$ (GTP γ S)- $G\beta\gamma$ 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 $G\beta\gamma$ subunits differ extensively in the deactivated and activated states. Experiments comparing the intensity changes when $G\beta\gamma$ binds to $G\alpha i$ GDP or to $G\alpha i$ GTP γ S show large differences on 29C, which resides on the edge of the N-terminal arm, 217C which lies close to $G\beta\gamma$ in the GDP-bound state and also residue 273C, which is far from the $G\beta\gamma$ interface in the deactivated state (Fig. 2). We also find that activation of $G\alpha i$ results in a decrease in the distances between the N-termini of $G\beta\gamma$ and residues 29, 217 and 273 as determined by onset of FRET. 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 $G\beta\gamma$ but is exposed in the activated state.

Our results suggest that $G\alpha$ GTP γ S- $G\beta\gamma$ can support PLC β 2 activation. If this is the case, then $G\beta$ 86–105, which is the region responsible PLC β 2 activation⁴⁰, 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 myristoylated (see 41), then the N-terminus is expected to maintain its contacts with the membrane surface while allowing the helical domain to rotate towards $G\beta\gamma$.

Using GRAMM⁴², we have constructed potential models of the $G\alpha i$ (GTP γ S) – $G\beta\gamma$ complex by docking the structures of $G\alpha i$ (GTP γ S) and of $G\beta\gamma$ derived from its complex with $G\alpha i$ (GDP)^{28; 35}. It is important to consider that the crystal structure is missing the first 31 residues which may play a role in $G\beta\gamma$ association. Several low energy structures were obtained. We eliminated all but 3 of the 16 lowest energy structures by requiring exposure of $G\beta$ 86–105 is exposed based on our observation that PLC β 2 is activated by $G\beta\gamma$ under conditions where it is bound to $G\alpha i$ (GTP γ S). In Fig. 8 we show the model that best fits the FRET and accessibility results. In this model, the C-terminal region $G\gamma$ interacts with helix 330–346 of $G\alpha i$. If this model is correct, this interaction would require $G\beta\gamma$ to rotate on the membrane surface to the opposite end of $G\alpha i$, which would likely be mediated by the $G\alpha i$ (GTP γ S) N-terminal arm. While this

change in orientation between the G protein subunits is possible, it is not clear whether this large scale movement could occur in protein complexes in cells.

Of special note is the role of the membrane surface in stabilizing signaling complexes. It has been shown that $G\beta\gamma_i$ attach to membranes through a large basic patch⁴³ which appears to be common to most $G\beta\gamma$ dimers. It is likely that this orientation is preserved upon activation of $G\alpha$ subunits. The accessibility studies carried out here were carried out using membrane-bound species and the observed changes may reflect changes in membrane interaction as well as changes in $G\beta\gamma$ interaction. Future studies on the membrane orientation of $G\alpha$ in the deactivation and activated states alone and complexed with $G\beta\gamma$ subunits would help the interpretation these studies and are planned. These results are expected to lead to more intelligent models of the activated complex.

The results presented here show that $G\alpha_i$ - $G\beta\gamma$ -PLC β 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.

The results of this study have only focused on the thermodynamics of protein interactions and have not addressed the kinetics of the dissociation/association events. Theoretically, if the lifetime of the G protein- effector complex exceeds the rate of G protein deactivation, then it is possible that the G protein subunits can exchange between other effectors. The model presented here suggests that multiple, secondary interactions as well as confinement on the membrane surface sufficiently localizes the proteins allowing them to remain associated in a complex. Interestingly, the rate of $G\alpha$ deactivation is regulated by its intrinsic GTPase activity as well as RGS proteins and proteins with RGS activity such as PLC β enzymes⁴⁴. If the RGS activity of PLC β 2 affects the GTPase activity of $G\alpha_i$, then it may directly as well as indirectly control the rate of its activation by $G\beta\gamma$ subunits. The contribution of secondary interactions in controlling these rates is complex and is presently being addressed using functional studies.

MATERIAL AND METHODS

Expression and purification of $G\alpha_i$ mutants

DNAs of mutant $G\alpha_i$ 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 (see 32; ⁴⁵ for full description of their properties as well as their expression and purification). *Escherichia coli* cells were grown to OD₆₀₀ of 0.5 units and then induced with 30 μ M IPTG at room temperature for 16–20h with gentle shaking at 200rpm. The pellet was resuspended in buffer containing 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 5 mM imidazole and fresh protease inhibitors, then disrupted by sonication. The cytosolic fractions were collected after centrifugation and incubated with 5mL Ni-NTA agarose resin for 60 min at 4 °C and loaded to a column. The column was washed twice by wash buffer containing 5 mM imidazole and 10 mM imidazole, respectively, then eluted with 10 ml elution buffer containing 40mM 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 μ M GDP, 10 mM 2-mercaptoethanol, and 100 μ M PMSF) then loaded to a MonoA column (Pharmacia Amersham, Piscataway, NJ). The protein was eluted by a linear gradient buffer containing 0–200mM 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 °C.

Expression of G β γ ₂

Expression of His₆-G β γ ₂ through baculovirus infection of Sf9 cells has been described previously³⁶. This method allows for post synthetic modifications. The geranylgeranyl chain on the G γ ₂ subunit was assessed by thin-layer chromatography on LK5D linear-k silica gel thin-layer chromatography (TLC) plates.

Expression of PLC β ₂

His₆-PLC β 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 °C. Cells were infected with His₆-PLC β ₂ recombinant baculovirus at a multiplicity of 10 for 48h at 27 °C and shaken at 125 rpm. Cells were harvested at 2,500 rpm for 25 minutes, washed in PBS, resuspended and spun at 4 °C. The pellet was suspended in ice-cold lysis buffer (20mM Tris-HCl, pH 8.0, 300mM NaCl, 10mM β ME 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 40ml lysis buffer, and the soluble fraction was recovered after ultracentrifugation at 35,000 rpm for 1h at 4 °C. The supernatant was loaded to an equilibrated Ni-NTA column. Bound proteins were eluted with 50 ml elution buffer (20mM Tris-HCl, pH 8.0, 500mM NaCl, 10mM β ME) with a gradient of 10–150 mM imidazole.

PLC Activity Assays

Activity assays were conducted as described previously³⁶. Briefly, samples containing substrate (POPC:POPE:POPS (1:1:1), with 2 mol % PtdIns(4,5)P₂ and 8000cpm/sample [3H]-PtdIns(4,5)P₂), PLC β ₂ and other proteins were incubated at 30 °C for 5 minutes, and the reaction was then initiated by the addition of calcium ions. The reaction was terminated by addition of 200 μ l ice cold 10% TCA and 100 μ l 1% BSA. After centrifugation for 5 minutes, 300 μ l of the supernatant was subjected to scintillation counting. Activity measurements of the PLC β ₂-G β γ -G α complexes were carried out at 20nM 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 β γ activation and higher intrinsic error in the measurements.

G α Activation

G α i was activated by incubation at 30 °C for 30 minutes, with the activation buffer (50mM Hepes, 100mM (NH₄)₂SO₄, 150mM MgSO₄, 100mM EDTA and 100 μ M GTP γ S)^{32; 46}.
ALUM FL ACTIVATION

Protein Labeling

The labeling procedure for these proteins has been previously described⁴⁷ and yield fully functional proteins labeled between 0.3 and 1.2 probe:protein as determined by absorption measurements. Briefly, PLC β ₂ or G α i were dialyzed against 150mM NaCl and 20mM Hepes buffer for 30min three times to remove DTT, then labeled with Alexa546 (PLC β ₂) or with 7-diethylamino-3-(4'-male-imidylphenyl)-4-methylcoumarin, CPM, G α i, which labels cysteine side chains. G β γ was labeled at pH 8.0 with Dabcyl SE, which reacts with primary amines. Labeling was also verified by the diffusion coefficient using fluorescence correlation spectroscopy. Unreacted probe was removed by dialysis for three times for 30 min. against a 100 fold excess of buffer containing DTT or by gel chromatography.

Fluorescence Measurements

Fluorescence experiments were carried out on an ISS PC1 spectrofluorometer (ISS, Urbana, IL). 10nM CPM labeled G α i was reconstituted on 200 μ M 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 3mm microcuvette. Spectra were recorded using a 384nm excitation wavelength and scanning the emission from 420–520nm. The area under the curves was calculated to give the total emission intensity.

In vitro FRET Studies

FRET measurements were carried out by reconstituting 10 nM CPM-G α i onto 200 μ M POPC:POPS:POPE large, unilamellar vesicles and measuring the decrease in integrated intensity as 10 nM Dabcyl-G β γ subunits, or 80 nM for activated CPM- G α i, are added as compared to the changes seen using identical amounts of unlabeled G β γ subunits. Since we are viewing associations on proteins confined to membrane surfaces, there is the possibility that non-specific association may occur. To determine the importance of non-specific encounters, we note that at 200 μ M lipid, the proteins to diffuse on a surface area of $\sim 10^{17} \text{ \AA}^2$. We assume that the proteins encounter each other diffusing in the length 50 $\text{\AA}$ from the membrane surface into the solvent. Considering the G protein subunits are at a concentration of 20nM each and are $\sim 2.5 \times 10^5 \text{ \AA}^3$. If FRET occurs when the proteins are within a radius of $2500 \text{ \AA}^2$ of each other, then amount of membrane far exceeds the probability of diffusional encounters (almost 60 fold). This point has been previously discussed along with control studies using non-interacting proteins^{36; 48; 49}.

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 α i Cys reactivity, we first blocked G β Cys side chains by removing DTT and incubating with iodoacetamide at 1:5 ratio at room temperature for 1h. Excess iodoacetamide was removed by dialysis for 30 min three times. To test the accessibility of CPM to Cys on G α i mutants, 10nM unlabeled G α i, together with 200 μ M LUV, blocked G β and 50nM CPM were incubated in cuvettes. Fluorescence intensity was tested at 0, 30, 60 min, respectively. The 384nm excitation wavelength was used and the emission from 420–520nm was recorded.

N-bromosuccinimide (NBS) modification of Trp was performed by measuring the Trp intensity at $\lambda_{\text{ex}}=280\text{nm}$ and $\lambda_{\text{em}}=345\text{nm}$ after the addition of 20 μ M freshly prepared NBS to a cuvette containing 10nM G α i, and 200 μ M lipid, when a constant value was reached after 8 minutes⁵⁰. In each case, the fluorescence intensity of the same amount of proteins without NBS, and the same amount of G α i with NBS were used as controls.

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 μ g/ml streptomycin sulfate at 37 °C in a 5% CO₂ 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 μ g of eYFP-G α i and eCFP-G β 1 and 10 μ g of HAG γ 7 plasmids were mixed with 120 mM CaCl₂ and HBS buffer (21 mM Hepes, 123 mM NaCl, 5 mM KCl, and 0.9 mM Na₂HPO₄, 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 – 72h later. For PLC β 2 - G protein FRET measurements, cells were transfected with labeled G proteins 48h prior to microinjections with Alexa546 labeled PLC β 2.

The integrity of the expressed G protein subunits was assessed by preparing membrane fractions from the transfected cells and measuring their ability to support activation of PLC β 2. We find that cells transfected with eYFP-G α i and eCFP-G β 1 activate PLC β 2 at a ~50% higher level than membrane prepared from cells transfected with empty vector.

Microinjections

Transfected cells were grown in Mattek dishes for 48h 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 $P_i = 27 - 25$ hPa, and kept the compensation pressure $P_c = 14$ 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 $\times$ 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. This system uses laser excitation allowing little bleed-through from the CFP channel. The optical settings were: $\lambda_{exc} = 458$ nm and $\lambda_{em} = 475 - 525$ nm for eCFP, $\lambda_{exc} = 514$ nm and $\lambda_{em} = 560 - 615$ nm for eYFP, $\lambda_{exc} = 458$ nm and $\lambda_{em} = 560 - 615$ nm for FRET. Details of the experimental set-up procedure and analysis have been described²⁹. For GFP – Alexa546 FRET we used: $\lambda_{exc} = 458$ nm, and $\lambda_{exc} = 585 - 615$ nm. FRET measurements entailed taking an image through a donor, acceptor and FRET filter at identical instrument settings (detector gain and laser intensity). We expressed fluorescent constructs CFP and YFP (for the CFP/YFP FRET) and expressed GFP only and injected Alexa546 to nontransfected cells (for the GFP-Alexa546 FRET) to determine the amount of bleed-through between channels. Data were analyzed using Zeiss software that takes into account bleed-through and detector sensitivity.

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ABBREVIATIONS

FRET

Forster Resonance Energy Transfer

CPM

7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin

Dabcyl-SE

4-((4-(dimethylamino)phenyl)azo)benzoic acid succinimidyl ester

LUVs

large unilamellar vesicles

PLC

mammalian phosphoinositide-specific phospholipase C

PI(4,5)P₂

phosphatidylinositol 4,5-bisphosphate

POPE

1-palmitoyl-2-oleoylphosphatidylethanolamine

POPS

1-palmitoyl-2-oleoylphosphatidylserine

POPC

1-palmitoyl-2-oleoylphosphatidylcholine

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Figure 1. Representative image of two HEK293 cells in the basal state expressing (A) eGFP-G β 1/HaG γ 7, (B) Alexa546-PLC β 2, (C) calculated FRET, (D) normalized FRET or N-FRET. Note the injection spot is clearly visible at the bottom of the cell and was removed from the N-FRET calculation.

Figure 2.

Left -Location of the single Cys residues in CPK from the crystal structure ²⁸ of $G_{\alpha i1}$ (green) mutants in wild type $G_{\alpha i1}(GDP)G\beta_1\gamma_2$ where $G\beta$ is in light blue and $G\gamma$ in dark blue. Also shown are the three $G_{\alpha i1}$ Trp residues in ball and stick form. *Right* – Structure of $G_{\alpha i1}(GTP\gamma S)$ showing the Cys locations of the mutants in CPK and the Trp residues in ball and stick form, taken from the crystal structure which begins at residue Arg32 ⁵¹. Images were generated using DSViewer Pro 6.0.

Comparison the Binding of $G\beta\gamma$ Subunits to $G\alpha i(\text{GDP})$ and $G\alpha i(\text{GTP}\gamma\text{S})$ on Lipid Membranes

Figure 3.

Plot showing the loss in $G\beta\gamma$ 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 $G\beta\gamma$ subunits are added where (●) corresponds to $G\alpha i(\text{GDP})$ and (○) corresponds to $G\alpha i(\text{GTP}\gamma\text{S})$. Data were normalized from the 18% change in CPM- $G\alpha i$ fluorescence. Each measurement was performed in triplicate and standard deviation is shown.

Figure 4.

FRET measurements in terms of relative loss in donor (i.e. CPM) fluorescence between 10 nM G α i(GTP γ S) single Cys mutants (10nM) reconstituted onto 200 μ M PC:PE:PS (1:1:1) large, unilamellar vesicles, as 80nM G $\beta\gamma$ with the non-fluorescent FRET acceptor, Dabcyl is added. All measurements were corrected for control samples which substituted unlabeled G $\beta\gamma$ for DAB-G $\beta\gamma$ where n=6 and standard deviation is shown.

CPM accessibility of 217C and 273C bound to G $\beta\gamma$ in the basal and activated forms

Figure 5.

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 calculated by $((F_i - F_o)/F_o)$ due to CPM covalent attachment after 30 minutes. Intensities were subtracted from those without bound G $\beta\gamma$ where the amount of G $\beta\gamma$ added was 10 nM for the G α_i (GDP) mutants and 80nM for the G α_i (GTP γ S) 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.

Figure 6.

Plot showing the association of PLCβ2 to the Gαi-Gβγ complex. The open circles show the increase in fluorescence intensity as unlabeled Gβγ subunits were added to 10 nM CPM-Gαi (GDP) (29C) reconstituted onto 200 μM PC:PE:PS (1:1:1) large, unilamellar vesicles. Addition of unlabeled PLCβ2 (●) to the heterotrimer does not reverse the increased intensity of CPM-Gβi. The insert figure shows the weak association between CPM-Gαi29C with the PLCβ2, n=3 in the absence of Gβγ subunits.

Changes in PLC β 2 Activity with G Protein Subunits

Figure 7.

Changes in PLC β 2 activity as determined by reconstituting the enzyme in 1.3 mM PC:PE:PS (1:1:1) and 2 mol% PtdIns(4,5)P₂ small, unilamellar vesicles (see methods) and measuring the activity of the isolated enzyme and in the presence of 200 nM G $\beta\gamma$, 200 nM G α (GDP) and/or 200 nM G α (GTP γ S). Each point is taken from the average of 4 data sets where each sample was measured in triplicate and standard deviation is shown.

Figure 8.

Comparison of the crystal structure of the deactivated heterotrimer shown in Figure 2 (*left*) with a model of the $G\alpha_i(GTP\gamma S) - G\beta\gamma$ complex generated by GRAMM (*right*). Also noted in the activated model is Arg32⁵¹ in CPK, and the residues of $G\beta\gamma$ that are involved in PLC β 2 activation⁴⁰ (i.e. G β 86–015 green) in ball and stick form. Images were generated using DSVIEWER Pro 6.0.

Increase in the fluorescence intensity of CPM-Gai mutants in the deactivated and activated states after the addition of unlabeled G $\beta\gamma$ subunits, where n=3–6 and S.D. is given.

TABLE 1

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
GTP S	3C	29C	106C	217C	273C	305C
% increase	4.1±1.2	11.4± 10.2	4.4±2.4	132±0.3	-11.1±5.6	7.9±0.3