Evidence for a Second, High Affinity Gβγ Binding Site on Ga11(GDP) Subunits

It is well known that Ga11(GDP) binds strongly to Gβγ subunits to form the Ga11(GDP)-Gβγ heterotrimer, and that activation to Ga11(GTP) results in conformational changes that reduces its affinity for Gβγ subunits. Previous studies of G protein subunit interactions have used stoichiometric amounts of the proteins. Here, we have found that Ga11(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 Ga11 and Gβγ subunits. Also, we find that phospholipase Cβ2, an effector of Gβγ, does not compete with the second binding site implying that effectors can be bound to the Ga11(GDP)-(Gβγ)2 complex. Biophysical measurements and molecular docking studies suggest that this second site is distant from the primary one. A synthetic peptide having a sequence identical to the putative second binding site on Gai1 subunit competes with binding of the second Gβγ subunit. Injection of this peptide into cultured cells expressing eYFP-Gai1(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.

The plasma membranes of cells are organized as a series of protein-rich and lipid-rich domains (1–3). Many of the protein-rich domains, in particular those organized by caveolin proteins, 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 (for review see Refs. 9–12). The ligand-bound receptor will then catalyze the exchange of GTP for GDP on the Ga subunit in the G protein heterotrimer. In the basal state, Ga(GDP) binds strongly to Gβγ, but in the GTP-bound state this affinity is reduced, allowing Ga(GTP) and Gβγ subunits to individually bind to a host of specific intracellular enzymes and change their catalytic activity.

Although 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 Ga(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 four families of Ga subunits that each contain several members, and, additionally, there are many subtypes of Gβγ subunits (18). It is possible that differences in dissociation behavior reflect differences in affinity between G protein subunit subtypes (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 Cβ1 (PLCβ1), an important effector of Gaq (23), is bound to Gaq prior to activation and throughout the activation cycle (6) implying that Gaq(GDP) interacts with PLCβ1 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 membranes of the Gaq-Gβγ/PLCβ1/RGS4 signaling system have secondary, weaker 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.

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4 The abbreviations used are: PLCβ1, phospholipase Cβ1; GTPγS, guanosine 5′-3-O-(thio)triphosphate; CPM, 7-diethylamino-3-(4′-maleimidophenyl)-4-methylcoumarin; eYFP, enhanced yellow fluorescent protein; eCFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; D-, Dabcyl/SE; FCS, fluorescence correlation spectroscopy.]
MATERIALS AND METHODS

Protein Expression and Purification—His6-Gα11 proteins were expressed in *Escherichia coli* and purified as previously described. The plasmids of these proteins were kindly provided by Dr. Heidi Hamm (Dept. of Pharmacology, Vanderbilt University). Her laboratory has shown that the single Cys mutants behave identically to wild type (see Refs. 25, 26 for full description of their properties as well as their expression and purification). Goα1(GDP) was activated by incubation at 30 °C for 30 min, with an activation buffer (50 mM Hepes, 100 mM (NH₄)₂SO₄, 150 mM MgSO₄, 100 mM EDTA, and 100 μM GTPγS) (25, 27).

His6-Gβ1γ2 was expressed in SF9 cells through baculovirus infection (28). This method allows for post synthetic modifications. The geranylgeranylated chain on the Gγ2 subunit was assessed on LK5D linear-k silica gel TLC plates. His6-PLCβ2 was expressed using a baculovirus-SF9 expression system.

Protein Labeling—Goα1 proteins were labeled with the thiol-reactive probe, 7-diethylamino-3-(4-sulfobenzyl)-4-methylcoumarin (CPM) at a 1:1 probe to protein ratio as determined by absorption spectroscopy. Unreacted probe was removed by dialysis (3× for 30 min) against a 100-fold excess of buffer containing dithiothreitol. Gβγ was labeled at pH 8.0 with DabcylSE or Alexa488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester (Invitrogen), which reacts with primary amines to give a labeling ratio of 1.5 probe:protein as determined by absorption spectroscopy. This low level of labeling is due to acylation 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-Gbg by fluorescence correlation spectroscopy.

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

FCS Measurements—FCS measurements were performed on an Alba dual-channel confocal fluorescence correlation instrument (ISS), equipped with an argon ion laser (Melles Griot) 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. The experimental autocorrelation function, G(r), was fit using the three-dimensional diffusion model, \( G(r) = 1/N (1 + r/TD)^{-1}(1 + \tau/\lambda)D_{0}^{-0.5} \), in which rTD is the residence time of a molecule in FCS observation volume, N is the average number of particles, and a is the structural parameter describing the observed volume. For photon counting histogram analysis, histograms were generated from the raw data using software developed by Enrico Gratton (Laboratory of Fluorescence Dynamics at University of California, Irvine) and were subsequently fit to an equation describing Gaussian beam waist photon counting histogram (PCH) distribution involving two components. The brightness ratio of the 1st and 2nd components was fixed at 2 (see Ref. 29 for details), and the fraction of components were calculated from the relative population ratio of the two species.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/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 24–48 h to achieve 80–90% confluence, the media was then replaced. 5 μg of eYFP-Gox11 and eCFP-Gβ1 and 10 μg of HA-Gγ7 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 dropwise. The cells were then incubated at 37 °C, and the media were replaced after 8–14 h. The cells were allowed to recover for 8–14 h and split into 35-mm glass bottom Mattek dishes and imaged 48–72 h later. Membrane preparations from cells transfected with eYFP-Gox11 and eCFP-Gβ1 activate PLCβ effectors at a ~50% higher level than membrane prepared from cells transfected with empty vector.

Microinjection—Transfected cells were grown in Mattek dishes for 48 h at 70–80% to achieve confluence. Prior to microinjecting, the media was changed to phenol-free Leibovitz-15. The sample microinjection solutions consisted of 130 nM peptide with 0.2% deoxycholic acid in 20 mM 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 N12 with a FemtoJet pump from Eppendorf to microinject the solutions into cytoplasm. We typically set the injection pressure \( P_{i} = 30 \) hPa, and kept the compensation pressure \( P_{c} = 15 \) hPa. The injection time was \( t = 0.4 \) s. Typically, we injected about 10–25 cells within a 10- to 20-min 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 Confocor 2 apparatus (Jena, Germany) and collected images. FRET analysis of the images were collected and analyzed as previously described (24).

RESULTS

FRET Studies Suggests that Goα1 Binds Multiple Gβγ Subunits—In a previous study, Hamm and coworkers developed a series of single Cys mutants of Goα1 to monitor conformational changes that occur upon activation (25). We have used these mutants to measure the affinity between Gβγ and Goα1(GDP) on 200 μM lipid bilayers to be \( K_{D} \approx 1 \) nM (24). To determine the changes in orientation between Goα1, and Gβγ that occur upon activation, we labeled each of these single Cys Goα1 mutants with an environmentally sensitive fluorescent probe (CPM) and monitored the ability of these mutants to transfer excited state energy (i.e. FRET) to Gβγ subunits labeled on the N terminus with the non-fluorescent FRET acceptor,
DabcylSE (D-), at a 0.3:1 probe to protein and note that this low ratio is due to significant fraction of acetylated protein as determined by mass spectrometry. We also note that there is uncertainty of the labeled species due to the difficulty in assessing the small G/H9253. Therefore, we are using FRET as a general indicator of binding.

Because Dabcyl is a non-fluorescent FRET acceptor, transfer from CPM will be seen as a decrease in donor fluorescence. The Ro for these probes, which corresponds to the distance at which 50% of donor fluorescence is lost to transfer, is 20 Å (30). Keeping in mind that the amount of FRET follows the 6th power of the distance, then from the crystal structure (31), only Cys-106 will be close enough to the N terminus of G/H9253 to participate in FRET barring a large amount of free rotation from the N terminus of G/H9253. Unexpectedly, the titration curves for all of the G/H9251i1 mutants showed a biphasic behavior (Fig. 1). At low G/H9253 concentrations, an increase in C-G/H9251i1 donor intensity was observed. Above 5 nM, the decreases in C-G/H9251i1 intensities are not seen when unlabeled G/H925 subunits are used. We have previously reported this increase in CPM-G/H9251i1(GDP) intensity with the addition of unlabeled G/H925 and interpret it to be due to protection of the CPM from solvent quenching as G/H925 subunits bind to G/H9251 forming the heterotrimer. If the data obtained for the unlabeled G/H925 is subtracted from D-G/H925, then this increase is eliminated. Thus, the initial portions of the titration curves are interpreted to represent the primary G/H925 binding site of G/H9251i1(GDP).

Unexpectedly, at higher G/H925 concentrations, a decrease in intensity, indicative of FRET, was seen. This decrease was observed for all mutants and saturated at ~80 nM G/H925. We interpret the loss in C-G/H9251i1 intensity to be due to FRET to D-G/H925. We find that the extent of this decrease varied for each mutant (see Fig. 4), 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/H9251 mutants ranging from 21 to 34 nM giving an average of $K_d = 23 \pm 5$ nM (see

![FIGURE 1. A, raw data of a single set of titrations comparing the change in fluorescence intensity in arbitrary units of CPM-labeled Cys-217 with the addition of unlabeled G/H925 (open circles) or D-G/H925 (closed circles), and then with the addition of PLC/2. For this plot, the intensity values of the control sample were offset to better display the rise of the samples at low G/H925 concentrations. B, similar study showing the intensity changes of CPM-labeled Cys-3 upon the addition of G/H925 or D-G/H925, and then with the addition of PLC/2, where the data are an average of five trials with ± S.D. C, the results using activated CPM-labeled Cys-3.](image-url)
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The apparent second site observed using FRET (clearly observe a weaker association that is on the same order as because the location of the probes are not interfacial and apparent affinity is due to the presence of fluorescent tags, associated with little or no FRET. We do not believe that this weaker with one exception, all of the probe positions should be associ-

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 different single Cys sites (i.e. within 20 Å). Association of a second Gβγ subunit to the heterotrimer at higher concentrations was confirmed by chemical cross-linking (see supplemental material).

We repeated the FRET titrations using the activated forms of the Cys-29 and Cys-106 mutants. We could not detect binding at low Gβγ concentrations in accord with the reduced affinity that accompanies activation (e.g. Fig. 1C). We did, however, clearly observe a weaker association that is on the same order as the apparent second site observed using FRET (Kd(app) = 45 ± 5 nm for Cys-29 and 62 ± 11 nm for Cys-106). These results suggest that, in the activated state, the high affinity site is replaced by a second, weaker site. Cross-linking studies also suggest that only one Gβγ binding site is operative when Ga is activated (see supplemental material).

Brightness Analysis Shows That Ga11 Can Bind to Multiple Gβγ Subunits—To verify the existence of a second site, we labeled Gβγ with Alexa488 and measured the molecular brightness of the molecules in solutions containing different stoichiometric amounts of Ga11 (Fig. 2) (32) on an FCS instrument. Measurements were performed without lipid vesicles, which would interfere with diffusion measurements. In the absence of Ga11, 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.

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cantly change. However, as the amount of added Ga11 increases, the

FIGURE 2. Dependence of molecular brightness of 80 nm A-Gβγ at different concentrations (closed circles) and Ga11(GTPγS) (open circles) where each point is an average of 18-23 measurements and ± S.D. is shown. Inset, plot of the ln of the Ga11(GDP) versus concentration shown to better distinguish the behavior of the molecular brightness at low concentrations of Ga11(GDP).

supplemental material). These apparent Kd values are ~20-fold weaker than those measured for Ga11(GDP)-Gβγ by other methods. Addition of excess PLCβ2, which binds strongly to Gβγ subunits, caused a partial recovery of the CPM donor intensity (Fig. 1) suggesting that the binding of the second Gβγ subunit is reversible.

The results of the FRET titrations are surprising because all mutants display a significant amount of FRET that begins above the concentration at which the Ga11-Gβγ heterotrimer should have formed (i.e. apparent Kd ~ 1 mM (24)), and also because, with one exception, all of the probe positions should be associated with little or no FRET. We do not believe that this weaker apparent affinity is due to the presence of fluorescent tags, because the location of the probes are not interfacial and because 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 different single Cys sites (i.e. within 20 Å). Association of a second Gβγ subunit to the heterotrimer at higher concentrations was confirmed by chemical cross-linking (see supplemental material).

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the above studies using activated G\textalpha\textsubscript{i1}. We find that the brightness is constant through a wide range of G\textalpha\textsubscript{i1} concentrations (Fig. 2) suggesting that, in the activated state, only one G\textbeta\gamma binding site is available.

**FRET Studies between G\textbeta\gamma Subunits Support a G\textbeta\gamma-G\textalpha\textsubscript{i1}-G\textbeta\gamma Complex**—To support the idea that G\textalpha\textsubscript{i1} is capable of binding two G\textbeta\gamma subunits, we carried out a FRET study in which we added CPM-labeled G\textbeta\gamma to a stoichiometric amount of D-G\textbeta\gamma and measured the ability of the two proteins to FRET when unlabeled G\textalpha\textsubscript{i1} is added. We find that G\textalpha\textsubscript{i1} produces FRET between the G\textbeta\gamma subunits. In Fig. 3 we show results from a similar study in which D-G\textbeta\gamma was added to the preformed G\textalpha\textsubscript{i1(GDP)}-G\textbeta\gamma heterotrimer where G\textbeta\gamma was labeled with CPM. A decrease in fluorescence intensity is seen suggesting that FRET between the G\textbeta\gamma subunits is occurring.

The **Second G\textbeta\gamma Site Does Not Occlude PLC\textbeta2 Effector Binding**—PLC\textbeta2 is strongly activated by G\textalpha\textsubscript{i1} but not G\textalpha\textsubscript{i1} subunits, and it is also activated by G\textbeta\gamma subunits (for review see Refs. 33, 34). We determined whether PLC\textbeta2 could displace the second G\textbeta\gamma from the G\textalpha\textsubscript{i1(GDP)}-G\textbeta\gamma heterotrimer by adding PLC\textbeta2 to the C-G\textalpha\textsubscript{i1}-D-G\textbeta\gamma complex and measuring the change in the amount of FRET. Addition of 80 nM PLC\textbeta2 to solutions containing 10 nM G\textalpha\textsubscript{i1} and 80 nM G\textbeta\gamma did not affect the amount of FRET most likely due to binding of the enzyme to unbound G\textbeta\gamma subunits (Figs. 1 and 4). However, addition of 236 nM PLC\textbeta2 resulted in a partial loss in FRET for Cys-106, Cys-217, and Cys-305, which had a high degree of FRET, and a complete loss in FRET for Cys-3, Cys-29, and Cys-273, which had a lower degree of FRET. Thus, either the PLC\textbeta2 directly displaced the second G\textbeta\gamma site or addition of excess PLC\textbeta2 caused dissociation of G\textbeta\gamma by a shift in equilibrium. To distinguish between these mechanisms, we added D-G\textbeta\gamma to C-G\textalpha\textsubscript{i1} in the presence of PLC\textbeta2 where the PLC\textbeta2 was at a concentration high enough to be completely bound to G\textbeta\gamma (i.e. 20 nM). We found that PLC\textbeta2 did not affect binding implying that the site of interaction between the second G\textbeta\gamma and G\textalpha\textsubscript{i1(GDP)} does not overlap with the effector binding site (data not shown).

**Identification of the Second Interaction Site**—To identify the nature of the second binding site, we used GRAMM (Dr. I. Vasker, University of Kansas) to dock G\textbeta\gamma to the G\textalpha\textsubscript{i1} crystal structure of Sprang and coworkers (31). Of the 20 lowest energy conformations, approximately half involve direct G\textbeta\gamma-G\textbeta\gamma 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 G\textbeta 86–105, which comprises the PLC\textbeta2 activation site (35–37), and the possible membrane orientation of the proteins (38, 39).

We then carried out a study to distinguish between the remaining models based on differences in the solvent accessibilities of the single Cys side chains in the G\textalpha\textsubscript{i1} mutants when the first and second G\textbeta\gamma subunits are 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 Invitrogen product literature) and thus, Cys accessibility can be judged by the amount of fluorescence. We then added CPM to a solution containing membrane-bound G\textalpha\textsubscript{i1(GDP)} and G\textbeta\gamma subunits whose Cys residues were blocked by pretreatment with iodoacetamide. We compared the amount of fluorescence at 0 and 30 min for the Cys-106, Cys-217, and Cys-273 of G\textalpha\textsubscript{i1} at G\textbeta\gamma concentrations where the first site should be primarily occupied (10 nM G\textbeta\gamma) and at concentrations the second site would be occupied (80 nM G\textbeta\gamma). Cys-3 and Cys-29 were not tested due to their close proximity to the first site and the membrane interface. We find that Cys-217 and Cys-273 were more (~38 ± 5%) shielded and less solvent-accessible (i.e. exhibited less CPM fluorescence) at 80 nM G\textbeta\gamma as compared with 10 nM. However, Cys-106 shows the same amount of CPM fluorescence at both concentrations suggesting that it is exposed when the second G\textbeta\gamma is bound. This finding narrows down the potential models to a set of three similar models (the lowest energy one is shown in Fig. 5). This model also correlates well with the FRET measurements.
According to this model, the most prominent contact made between \( \Gamma_1 \) and the second \( \Gamma_2 \) subunit involves residues 267–271 of \( \Gamma_2 \). We prepared a peptide that has this sequence and should compete with binding of the second \( \Gamma_2 \). We note that because this contact region is small, the test peptide may not be as effective for competition with the second binding site as compared with larger peptides (e.g. Ref. 40). Two types of assays were carried out. In the first, we added \( \Delta-G_\Gamma_2 \) to \( \Delta-G_\Gamma_1 \) until FRET was clearly observed indicating the formation of \( \Delta-G_\Gamma_2-(\Delta-G_\Gamma_1)-G_\Gamma_2 \) complex. We then added peptide to the complex and found a complete reversal of FRET suggesting dissociation of the \( \Delta-G_\Gamma_2 \) subunit (Fig. 6, top). In a second series of studies, we titrated \( \Delta-G_\Gamma_2 \) into a solution containing \( \Delta-G_\Gamma_1 \) in the absence and presence of 10 \( \mu \)M peptide. We found that the peptide shifts the binding of the second \( \Delta-G_\Gamma_2 \) to higher concentrations from \( K_{\text{d(app)}} = 15.4 \pm 2.7 \) nm to \( 32 \pm 9.6 \) nm and reduced the extent of binding as judged by the amount of FRET (Fig. 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, \( \Gamma_1 \) only binds one \( \Gamma_2 \) subunit, and our titration curves show this binding has an affinity on par with the second \( \Gamma_2 \) site seen for \( \Gamma_1 \) in the deactivated state. To determine whether the second \( \Gamma_2 \) binding site in the deactivated state overlaps with the single binding site in the activated state, we carried out a titration study of \( \Delta-G_\Gamma_2 \) to \( \Delta-G_\Gamma_1 \) (GTP\( \gamma \)) in the presence of 10 \( \mu \)M peptide. We found that the presence of peptide had little affect on the binding of \( \Delta-G_\Gamma_2 \) to \( \Delta-G_\Gamma_1 \) suggesting that the loop encompassing the second binding sites has little or no overlap with the \( \Gamma_1 \) (GTP) site. However, it is still possible that, even though there is no direct overlap, binding of the full-length proteins could be competitive.

Addition of Peptide Reduces \( \Gamma_1 \)-\( \Gamma_2 \) in Cells—To determine if the second site has functional significance, we determined whether the peptide could disrupt \( \Gamma_2 \)-\( \Gamma_1 \) association in HEK293 cells. These studies were carried out by transfecting HEK293 cells with the FRET pair, eCFP-\( \Gamma_1 \), and eYFP-\( \Gamma_1 \). We note that we added a tracer, the red dye (Cy5), whose emission is out of range of the CFP/YFP channels, to
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.

**DISCUSSION**

In this study, we have shown that Gα11 subunits contain a second binding site for Gβγ subunits. Molecular modeling coupled with fluorescence and accessibility studies suggests that this site encompasses residues 267–271 of Gβ. Activation of Gα11 eliminates the high affinity site but promotes the formation of a low affinity site that does not share the identical interface with the second site of the deactivated protein. The Gβγ affinity of the second 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 Ref. 41). In the deactivated heterotrimer, Gα11(GDP) contacts Gβγ subunits at several points, including one that encompasses the first ~30 residues of Gα11(GDP), resulting in a high affinity interaction (31). Exchange of GDP for GTP produces conformational changes in three key regions that result in a loss in affinity of ~10-fold (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 that may stabilize the heterotrimer (e.g. Refs. 4, 7, 15). Additionally, we have found that activation of Gα11 changes its orientation with respect to Gβγ subunits allowing exposure of PLCβ2 interaction sites without dissociation of the heterotrimer (24).

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 Gβγ association site has not yet been observed despite the strong affinity. In the studies carried out here, association between the subunits was promoted by the...
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effective reduction in dimensionality by carrying out the measurements on membrane-bound proteins (see Ref. 28). It is thus important to consider the affinities reported here as apparent affinities, and the true affinities are expected to be weaker. A lower affinity was qualitatively observed in the brightness measurements, which were carried out in the absence of membranes.

Elementary docking studies of the second site suggest that there are several contact points along the surface of Gβγ 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 G protein receptor kinase or the peptide binding region of adenylyl cyclase (42, 43). This region is not involved in PLCβ2 binding (36, 44). We note, however, that the second site involves multiple interactions over four Gβγ blades, two of which are close to the PLCβ2 binding site.

The observation that the affinity of the second Gβγ for deactivated Gα11 is in range of the affinity seen for the Gβγ binding site in activated Gα11 leads to the speculation that the sites overlap. If this were the case, then Gα11 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 Gα11, but not from activated Gα11. Because Gα11 subunits undergo large conformational changes upon activation, it is likely that the Gβγ 267–271 to Gα11 interaction is weakened or replaced by other contacts.

While it is unclear whether some, if any, of the contacts between the second Gβγ site and Gα11(GDP) are utilized by activated Gα11, our studies using cultured cells show that microinjecting a peptide whose sequence corresponds to the 267–271 disrupts Gα11(GDP)-Gβγ association. This observation suggests that this second site may serve 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 set a window of the concentration range of Gβγ that will allow the subunits to be released in cells and subsequently contact distant effectors.

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