Determination of the Contact Energies between a Regulator of G Protein Signaling and G Protein Subunits and Phospholipase Cβ_1†

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ABSTRACT: Cell signaling proteins may form functional complexes that are capable of rapid signal turnover. These contacts may be stabilized by either scaffolding proteins or multiple interactions between members of the complex. In this study, we have determined the affinities between a regulator of G protein signaling protein, RGS4, and three members of the G protein−phospholipase Cβ (PLC-β) signaling cascade which may allow for rapid deactivation of intracellular Ca^{2+} release and activation of protein kinase C. Specifically, using fluorescence methods, we have determined the interaction energies between the RGS4, PLC-β, G-$\beta\gamma$, and both deactivated (GDP-bound) and activated (GTPγS-bound) Gα_q. We find that RGS4 not only binds to activated Gα_q, as predicted, but also to G$\beta\gamma$ and PLCβ_1. These interactions occur through protein−protein contacts since the intrinsic membrane affinity of RGS4 was found to be very weak in the absence of the protein partner PLCβ_1 or a lipid regulator, phosphatidylinositol-3,4,5 trisphosphate. Ternary complexes between Gα_q, G$\beta\gamma$ and phospholipase Cβ_1 will form, but only at relatively high protein concentrations. We propose that these interactions allow RGS4 to remain anchored to the signaling complex even in the quiescent state and allow rapid transfer to activated Gα_q to shut down the signal. Comparison of the relative affinities between these interacting proteins will ultimately allow us to determine whether certain complexes can form and where signals will be directed.

Heterotrimeric G proteins are membrane-bound proteins consisting of α, β, and γ subunits (for reviews, see refs *1* and *2*). Upon activation, GTP-bound α subunits lose their affinity for G$\beta\gamma$ subunits but increase their affinity for protein effectors (*3*, *4*). This subsequent interaction alters effector activity and ultimately causes cellular changes. Additionally, G$\beta\gamma$ subunits change the activity of a specific set of effectors (*5*). There are four families of Gα proteins, and the main effector of the Gα_q family is mammalian inositide-specific phospholipase C-β (PLC-β). PLC-β catalyzes the hydrolysis of phosphatidyloinositol 4,5-bisphosphate (PIP_2) to produce two second messengers, 1,4,5-inositol trisphosphate (IP_3), which causes the release of Ca^{2+} from intracellular stores, and diacylglycerol (DAG), which promotes the activation of protein kinase C (see ref *6* for recent review).

PLCs have a modular structure (*7*) consisting of an N-terminal pleckstrin homology domain, which has been shown to confer binding and activation by G$\beta\gamma$ subunits (*8*, *9*), four elongation factor (EF) hands, a catalytic domain with a long insertion loop, a C2 domain which has been shown to specifically bind Gα_q subunits (*10*), and a 400 residue tail which is needed for activation by Gα_q (*11*−*13*). Interestingly, residues in this tail have been shown to also accelerate the rate of GTP hydrolysis of α_q thereby increasing the rate of deactivation of α_q and in turn the rate of deactivation of the PLC-β (*14*). By deactivating its G protein activator, PLC-βs are able to turn off their own signal.

Adding to the list of players in the inositol-signal transduction is a newly discovered class of proteins called regulators of G protein signaling (RGS)[1] (*15*). Thus far, the RGS proteins are known to bind to the α subunits of G proteins and increase the rate of GTP hydrolysis by stabilizing the transition state of the complex (*4*). For example, RGS4, which specifically acts on Gα_i and Gα_q family members, increases the GTPase activity of Gα_q 1000-fold (*16*). There are many members of the RGS family, and each family member has an ∼130 residue homologous core region that is responsible for GAP activity. This core, termed the RGS domain, binds strongly to Gα in the GDP−AlF_4^- bound state (*17*), and the structure of the RGS4−Gα_{i1}(GDP)−AlF_4^- suggests that the core stabilizes the GTP to GDP transition state lowering the energetic requirement for hydrolysis (*4*). This stabilization allows RGSs to turn off the effector signal without diminishing the signal strength.

On each side of the RGS core are flanking regions which presumably dictate the specificity of RGS family members to Gα families or other binding targets. These flanking

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[1] RGS, regulators of G protein signaling; PLC, mammalian inositide-specific phospholipase C; GAP, GTPase activation; PI(3,4,5)P_3, dipalmitoyl-phosphatidylinositol 3,4,5- trisphosphate; Ins(1,4,5)P_3, -D-myo-inositol (1,4,5) trisphosphate; Laurdan, 6-lauroyl-2 -dimethylaminonaphthalene; Dabcyl SE, 4-dimethylaminophenylazophenyl-4-sulfonyl chloride succinylester; C, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine.

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regions were unresolved in the crystal structure, and the role that they play in RGS function is unclear. The N-terminus of RGS4, which contains several basic and hydrophobic residues, appears to be responsible for targeting to the plasma membrane since removal of the first 33 residues significantly reduces the membrane localization in yeast (*18*). Another study suggests that the N-terminus targets RGS4 to particular G protein coupled receptors occupying a position that can prevent coupling between $G\alpha_q$ and PLCβ_1 (*19*). Although it is clear that RGS4 must be localized near membrane receptors, it is not clear whether it is localized by an intrinsic affinity for membranes or by interactions with receptors, G protein subunits, or effectors. Evidence of membrane localization of RGS4 comes from a recent study showing that phosphatidylinositol 3,4,5-trisphosphate inhibits the GAP activity of RGS4 presumably by directly interacting with the positively charged residues on the surfaces of helices 4 and 5 (*20*). This inhibition may be overcome by displacement of RGS4 from the membrane by Ca^{2+}/calmodulin, thereby allowing RGS4 to bind and deactivate Gα subunits (*20*).

The idea that RGS4 and other components in the inositol-signaling pathway are localized in protein complexes is intriguing since this would allow for rapid signal flow (*21*−*24*). There is precedent for these complexes. Association between seven transmembrane receptors and $G\alpha_q$ has been observed in cells (*25*). Notably, in *Drosophila* a signaling complex involving receptor, PLC-β and protein kinase C and a scaffold protein has been identified (*26*). In a reconstituted in vitro system, we have found that Gα(GDP) will bind to the activated PLC-β_2−G$\beta\gamma$ complex and rapidly turn off the activation without physically disrupting the complex (*27*). In studies of PLCβ activation, Ross and co-workers have presented kinetic data to argue that $G\alpha_q$ must remain bound to receptor during turnover (*14*). Taken together, these studies point to a model in which receptor, the G protein heterotrimer, effector, and RGS4 can be colocalized in signaling complexes. However, not all studies support this model. For example, the presence of RGS4 interferes with $G\alpha_q$ activation of PLCβ_1 leading the authors of those studies to speculate that the interaction sites between these proteins are shared (*28*). Thus, it is possible that protein complexes will form under some circumstances but not others.

In this study, we begin to define the biophysical conditions under which RGS4, PLCβ_1, and G$\alpha\beta\gamma$ will associate on membrane surfaces using fluorescence methods. As mentioned, we have previously defined the interaction energies between $G\alpha_q$, G$\beta\gamma$ and PLCβ_{1-3} on membrane surfaces (*3*). From these studies, we know the local concentrations at which association between the individual species occur. Here, we extend this work to include the conditions for association of RGS4 to components of this pathway. We find that RGS4 not only associates with activated Gα subunits, but has secondary interaction with the other components of the signaling system as well. The results of these studies lead to a model in which RGS4 is corralled in the complexes by weaker secondary interactions that allow for rapid transfer of RGS4 contacts to $G\alpha_q$ upon activation.

MATERIALS AND METHODS

Sample Preparation. Untagged, recombinant PLCβ_1, $G\alpha_q$, and His_6-G$\beta\gamma$ subunits were prepared in Sf9 cells as previously described (*3, 29*). Preparation of RGS4(1−33) has been described (*19*). Large, unilamellar vesicles (LUVs), 100 nm in diameter, were prepared by extrusion. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) except for dipalmitoyl PI-3,4,5-trisphosphate, which was synthesized using the method of Reddy et al. (*30*). The bacterial cell strain pREP4/His_6−RGS4 in BL21/DE3 cells was a generous gift from A. G. Gilman and was prepared as described (*28*) in pure form as determined by western blot analysis using an RGS4 antibody (Santa Cruz Biochemicals) and MALDI-MS analysis. Since it does not affect the GAP activity of the protein, the N-terminal was not removed in these studies. Labeling of the proteins with amine-reactive probes has also been described (*3*). GAP activity of labeled and unlabeled RGS4 was checked using bacterially expressed recombinant $G\alpha_{i1}$ a single turnover assay as previously described (*17*).

Protein Labeling and Reconstitution. All probes were purchased from Molecular Probes, Inc. (Eugene, OR). $G\alpha_q$ and G$\beta\gamma$ subunits were covalently labeled with amine-reactive probes which do not affect their ability to activate PLCβ_1 or PLCβ_2, respectively. RGS4 and PLCβ_1 were similarly labeled as described (*27*). The protein:probe labeling ratio was determined by BCA analysis and absorption spectroscopy using the extinction coefficients provided by the probe manufacturer and was ∼1:1 (mol/mol) for all preparations. Labeled G-α_q was reconstituted into lipid vesicles by adding the detergent-solubilized protein to a large excess of preformed extruded vesicles.

Fluorescence Measurements and Data Analysis. Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL) using a 3 mm cuvette with magnetic stirrer. Adequate stirring was assessed at the beginning of the study by ensuring that upon addition of a dilutant, the emission spectrum was identical after inversion. Also, at the beginning of each study, we verified that the sample was at thermal equilibrium by showing that the initial scan was stable over several scans. Coumarin-labeled proteins were excited at 380 nm and scanned from 420 to 560 nm. Laurodan probes were excited at 350 nm and scanned from 380 to 560 nm. Coumarin−Dabcyl SE resonance energy transfer was determined by the loss of coumarin fluorescence caused by addition of the nonfluorescent Dabcyl-labeled acceptor protein. Signals were corrected for dilution and compared to loss of fluorescence caused by addition of buffer alone.

To determine the affinities of the protein complexes, energy transfer data were analyzed as binding isotherms assuming that the maximum loss of fluorescence represents the maximum extent of complex formation and that the stoichiometry of this complex is 1:1 (mol/mol). Titration curves were the fit to a bimolecular association constant.

Membrane binding was determined using a variety of fluorescence based methods (see ref *28*). To obtain the partition coefficient (K_p) for membrane, we used the large decrease (∼20%) in the intrinsic fluorescence of RGS4 presumably due to quenching of internal Trp/Try residues by the ionic groups on the membrane surface. We note that the K_p values reported here correspond to apparent partition coefficients since we assume that RGS4 partitions on membranes through nonspecific, electrostatic interactions rather than forming a chemical complex between a particular lipid(s) or nucleotide base(s).

$$K_p \text{ (i.e., Kapp)} = ([P]_b/[L])/[P]_f$$

K_p is defined as the mole fraction of substrate-bound protein, ($[P]_b/[L]$), divided by the concentration of free protein $[P]_f$. $[P]_b$ is the concentration of substrate-bound protein and [L] is the concentration of substrate. Each trial as well as the average, was fit to a hyperbola using SigmaPlot (Jandel Inc.).

RESULTS

Binding of RGS4 to Activated and Deactivated $G\alpha_q$ Subunits. Although it has been well established that RGS4 binds strongly to $G\alpha_q$, we needed to characterize the conditions of this interaction for this study. We thus measured the association energy between RGS4 and $G\alpha_q$ in its activated (i.e., GTPγS-bound) and deactivated (i.e., GDP-bound) forms using fluorescence resonance energy transfer. In this method, $G\alpha_q$ was labeled on a primary amine with a fluorescent probe that is capable of transferring its excited-state energy to an acceptor when the probes are within close proximity. Although many energy transfer probe pairs are known, we used coumarin-SE (abbreviated C-) as the donor because of its high quantum yield and dabsyl-SE (D−) as the acceptor because this probe is not fluorescent and its use eliminates the need for correcting for acceptor contribution to the donor emission spectrum. Experimentally, as the two labeled proteins associate, the emission intensity of the coumarin probe decreases due to transfer to dabcyl. By monitoring the loss in C−$G\alpha_q$ fluorescence as a function of D−RGS4 concentration, an apparent bimolecular dissociation constant can be determined.

Recombinant $G\alpha_q$ subunits were prepared from Sf9 cells, labeled with coumarin, activated with non-hydrodrolyzable GTPγS, and reconstituted on large, unilamellar vesicles of PC:PS:PE (1:1:1) as described (*3*). We have previously found that labeling C−$G\alpha_q$ at a 1:1 probe:protein does not affect GTPase activity or its ability to activate PLCβ_1. Titrations were then conducted by adding incremental amounts of D−RGS4 in buffer to a solution of membrane-bound C−α_q-(GTPγS) and recording the spectrum. These data were then corrected using data from titrations where buffer was added instead of D−RGS4. At 10 nM C-$G\alpha_q$(GTPγS) complete binding was observed when a stoichimetric increment of D−RGS4 was added. Reducing the concentration of C−$G\alpha_q$-(GTPγS) to1 nM gave identical behavior which indicates that we were operating at concentrations above the dissociation constant and were observing stoichiometric binding rather than true equilibrium binding. These results show that the K_d for this complex is below 1 nM and out of range of accurate determination using this method.

The crystal structure of the RGS4 complex with activated $G\alpha_i$ shows that the major interaction sites are in the $G\alpha$ switch regions (*4*). Most likely, this is also the case for interaction for $G\alpha_q$(GTPγS). Therefore, deactivation of C−$G\alpha_q$ should weaken its interaction with RGS4, and this idea is supported by fluorescence data (Figure 1). Although the association is weakened as compared to C−$G\alpha_q$(GTPγS), it is still close to the detectable limit by fluorescence methods and the apparent dissociation constant is estimated to be 0.2 ± 0.1 nM. To determine whether we were viewing equilibrium binding, we repeated the study at a 10-fold higher C−$G\alpha_q$(GDP) which shifted the curve appropriately but did not allow better resolution of the apparent K_d.

Binding of RGS4 to Other Protein Partners. The idea that RGS4 is part of a signaling complex implies that it has

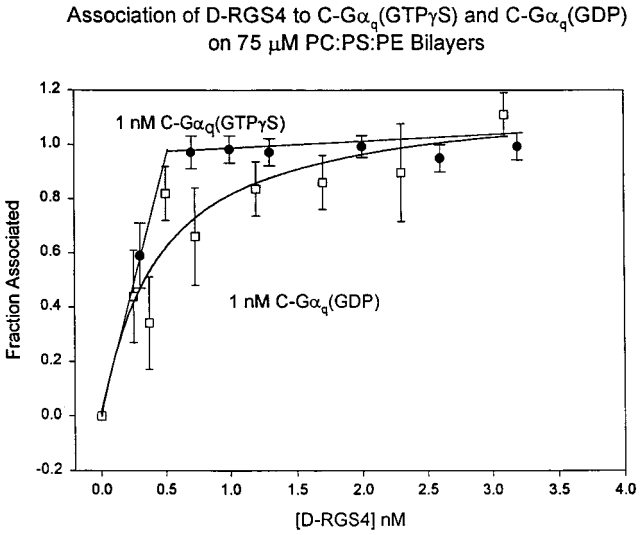

FIGURE 1: Fraction of complex formed between RGS4 and activated (●) ($n = 11$) and deactivated (□) $G\alpha_q$ ($n = 6$) reconstituted on lipid surfaces as measured by fluorescence resonance energy transfer (see method). The fraction formed was determined by the ∼15% loss in coumarin (C-) fluorescence as its emission energy is transferred to nonfluorescent dabcyl (D−) probes. The line through the $G\alpha_q$(GTPγS) data shows the curve for protein interactions that are too strong to be accurately quantified by this method. The curve through the $G\alpha_q$(GDP) data is the fitted binding isotherm which gives a $K_d = 0.2 \pm 0.1$ nM.

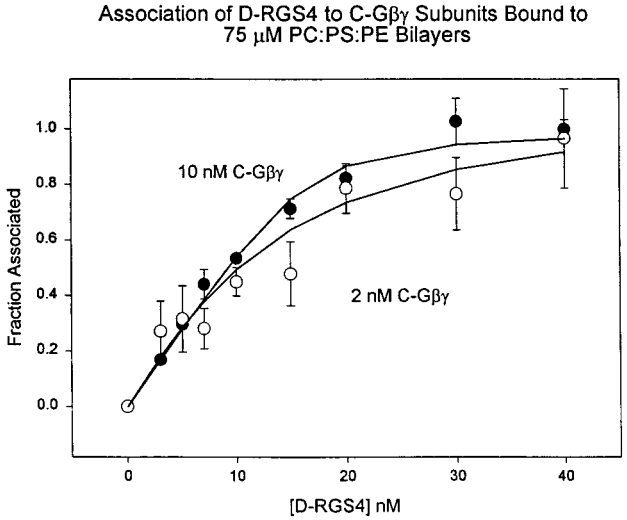

FIGURE 2: Plot showing the concentration dependence of the fraction of complex formed, as determined by the ∼18% loss in coumarin fluorescence due to resonance energy transfer, between RGS4 and membrane-bound G$\beta\gamma$ subunits at a higher (10 nM, ●) and lower (2 nM, ○) where $n = 3$. The lines are the fitted binding isotherms, that both give similar values for Kd within error (see text).

secondary interaction sites for other proteins in the inositol-signaling network. Therefore, we tested the possibility that RGS4 has multiple protein partners by measuring its ability to bind to recombinant C−G$\beta_1\gamma_2$. Binding studies were again done by titrating D−RGS4 into a solution of C−G$\beta\gamma$ reconstituted on lipid membranes and substituting dialysis buffer as a control. The results, shown in Figure 2, indicate a much weaker binding to these G protein subunits, i.e., a 10-fold higher range of D−RGS4 was needed to achieve binding. The titration curves in Figure 2 shows an appropriate

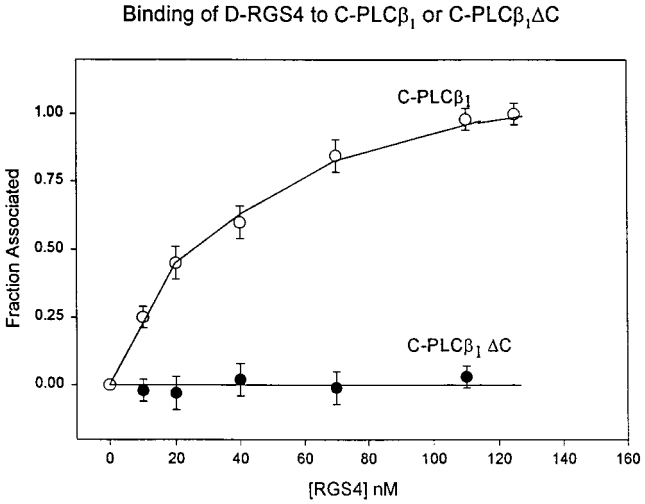

FIGURE 3: Plot showing the association, as determined by fluorescence resonance energy transfer that results in a ~16% loss in donor fluorescence, of RGS4 to PLCβ_1 (○) (n = 6) and a C-terminal truncated mutant (●) (n = 3). Identical results were obtained in the presence of 75 μM POPC:POPE:POPS (1:1:1) (data not shown).

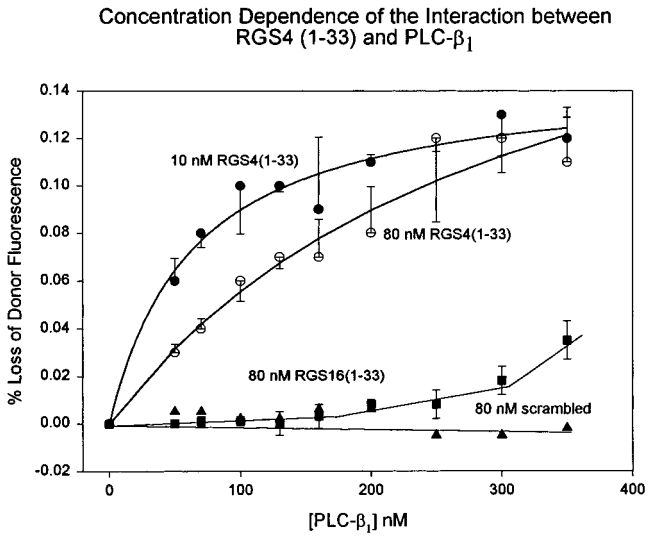

FIGURE 4: Concentration dependence of the interaction between the N-terminus of RGS4 and PLCβ_1 as seen in the plot of the loss in donor fluorescence at two different RGS4(1−33) (n = 3) concentrations, 10 nM (●) and 80 nM (○) relative to 80 nM of scrambled peptide (▲). The weak interaction between RGS16(1−33), (■) (n = 3) and PLCβ_1 is also shown.

shift when the initial C−G$\beta\gamma$ concentration is changed from 10 to 2 nM. If the data in Figure 2 reflect equilibrium binding, then their K_d values should be similar whereas if the data in Figure 2 reflect stoichiometric binding, then the apparent K values would identical to the initial concentrations of C−G$\beta\gamma$ (i.e., 10 and 2 nM). We find that the dissociation constants are the same within error (5.3 ± 1.7 and 11.5 ± 4.5 nM, for 10 and 2 nM, respectively), indicating that we are viewing equilibrium binding.

We then measured the association of RGS4 to membrane-bound PLCβ_1 by fluorescence energy transfer (Figure 3) using either buffer of trypsin-treated D−RGS4 as controls We find that RGS4 binds to PLCβ_1 with an apparent binding constant of 27± 10 nM. Although PLCβ_1 binds very strongly to membranes, it is soluble in aqueous solution. Thus, we were able to compare the effect of lipid on protein association. No changes in the apparent dissociation constant was observed indicating that the site of interaction is not the membrane binding face of PLCβ_1.

Identification of the Interaction Region between RGS4 and PLCβ_1. Gα_q interacts with PLCβ_1 on the regions immediately following the catalytic domain in the linear sequence (*3*, *10*). To determine whether the RGS4 binding site of PLCβ_1 is also localized on the C-terminal region, we measured the binding of RGS4 to a catalytically active, C-terminal deletion mutant of PLCβ_1. No interactions can be seen between these proteins (Figure 3). Thus, either RGS4 binds to the C-terminal tail or removal of this tail results in misfolding of the interaction site.

Since receptor specificity of RGS proteins have been linked to its N-terminal domain, we measured the PLCβ_1 binding of a synthetic peptide consisting of residues 1−33 of RGS4 in the absence of lipid membranes. In this series of experiments, the peptide was labeled with coumarin and dabsyl-PLCβ_1 was the titration partner. Control studies using a coumarin-labeled synthetic peptide with a scrambled sequence showed no changes in fluorescence intensity. The results, presented in Figure 4, show that the peptides bind with affinities of 54 ± 13 nM for the 10 nM trials and 75 ± 13 nM for the 80 nM trials. Despite strong sequence homology (*32*), a peptide consisting of residues 1−33 of RGS16 showed a much weaker interaction with PLCβ_1 (Figure 4).

Table 1: Summary of the Apparent K_ds of RGS4 to Protein Partners

	RGS4
Gα_q(GTPγS)	<0.1 nM
Gα_q(GDP)	0.2 nM
G$\beta\gamma$	8.4 nM
PLCβ_1	27 nM
PLC$\beta_1\Delta C$	no binding
{PLCβ_1- Gα_q(GTPγS)}	300

Formation of Ternary Complexes. We explored the possibility that ternary complexes between RGS4, PLCβ_1, and Gα_q(GTPγS) can form. First, we formed complexes between C−RGS4 and Gα_q(GTPγS) on membrane surfaces at concentrations well above the estimated dissociation constant (i.e., 10 nM of each) and titrated in D−PLCβ_1. Although D−PLCβ_1 bound to the complex, the binding was far weaker than the binding of PLCβ_1 to Gα_q(GTPγS) or for RGS4 (see Table 1). An example of the titration data, shown in Figure 5, shows that saturation is not reached even at high PLCβ_1 concentrations. From these data, we estimate the apparent dissociation to be 300 ± 80 nM.

The data in Figure 5 could correspond to formation of a weak ternary complex as well as to the binary association between D−PLCβ_1 and C−RGS4 in the presence of competing Gα_q(GTPγS). Thus, we conducted several follow-up studies to determine which is the predominant case. In one series of studies we labeled each protein with Oregon Green and assessed the amount of fluorescence homotransfer that occurs when all three proteins are in the solution (see refs *33* and *3* for background and methodology) at 140−210 nM total protein. The formation of binary complexes results in a reduction in fluorescence anisotropy of 10−15% for this probe. The addition of the third component caused a further (i.e., 7%) reduction in anisotropy, indicating that some of

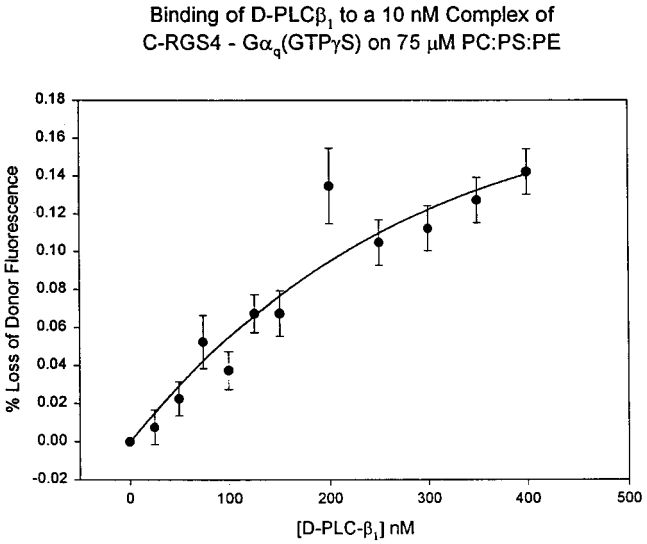

FIGURE 5: Binding of D−PLCβ_1 and the C−RGS4-Gα_q(GTPγS) complex ($n = 5$).

the proteins are aggregating into a ternary complex. In a second series of studies, we added a large excess (400 nM) of unlabeled RGS4 to a 1 nM solution of C−Gα_q(GTPγS)−D−PLCβ_1. If R654 contributed to disparate heterodimes at this RGS4 concentration, we would expect displacement of C−Gα_q(GTPγS) from D−PLCβ_1 by RGS4 and reversal of the 16% decrease in Gα_q(GTPγS) fluorescence due to energy transfer. However, no significant changes in fluorescence were observed. In a third series of studies, we measured the ability of unlabeled PLCβ_1 to dissociate C−Gα_q−D−RGS4 both at 1 nM concentration reconstituted on lipid bilayers. Addition of 100 nM PLCβ_1 increased the donor intensity by 40% and addition of 250 nM PLCβ_1 increased the intensity 10-fold indicating that PLCβ_1 is inducing some changes in the local environment around the coumarin probe. Since this increase is far greater than the change one would expect by a simple displacement of the energy transfer pair. This result indicates that PLCβ_1 is interacting with the Gα_q-(GTPγS)−RGS4 complex. However, the nature and extent of this complex formation is not clear.

RGS4 Binds Weakly to Membranes, But Can Be Recruited by PI(3,4,5)P$_3$ and PLCβ_1. To analyze the protein interactions observed in the above studies, we needed to know whether RGS4 is soluble in the aqueous phase or whether it is membrane bound and laterally associates with PLCβ_1 and Gα_q under our conditions (see discussion). We thus measured the membrane partition coefficient of RGS4 alone and in the presence of two membrane-localized partners.

Membrane binding was assessed by several types of fluorescence methods: changes in intrinsic fluorescence as the protein associates to the membrane surface, changes in the fluorescence of coumarin-labeled RGS4, energy transfer between RGS4 Trp donors and membranes doped with a fluorescent probe, and membranes doped with a detergent-like fluorescent probe (Laurdan) that is sensitive to changes in the polarity of the membrane surface. We found that the method that was most sensitive to membrane binding was the ~20% decrease in Trp/Try fluorescence upon membrane binding probably due to quenching of these fluorophores by the ionic headgroups of the lipid.

By following the change in intrinsic fluorescence upon membrane binding, we found that binding to large, unilamellar vesicles composed of PC:PS:PE (1:1:1) was weak ($K_p \approx 1$ mM). However, membrane affinity could be increased at least three ways. First, since the binding of RGS4 to membranes has a strong electrical component, (*34*) we found that the partition coefficient changes to $K_p \approx 300$ μM if we used membranes composed entirely of negatively charged lipids (i.e., POPS). This increase from a weak binding affinity due to negatively charged lipids is supported by sedimentation studies using sucrose-loaded vesicles (A. Arbouzova and S. McLaughlin, unpublished results). Second, the presence of 33% PI(3,4,5)P$_3$ with 67% PC increases the binding affinity to K_p 30 ± 10 μM (Figure 6). Third, incorporation of PLCβ_1 increased the affinity to PC:PS:PE (1:1:1) bilayers to $K_p = 50 \pm 18$ μM indicating that RGS4 can also bind to membranes through protein−protein contacts in addition to protein−lipid.

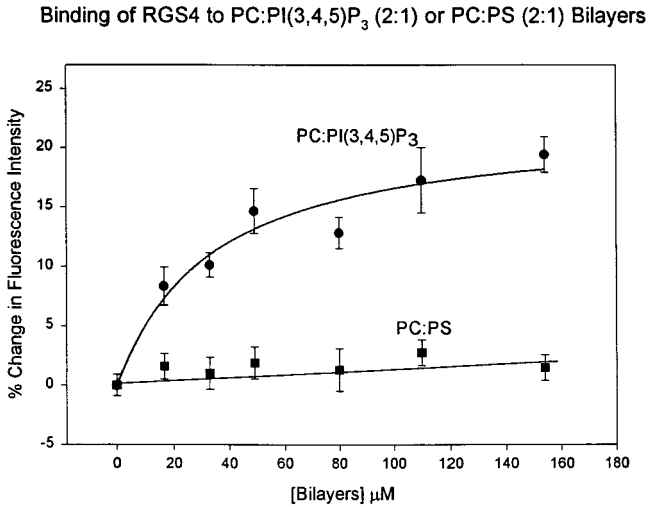

FIGURE 6: Comparison of the membrane association, monitored by the ~20% decrease in intrinsic fluorescence, of RGS4 to POPC:PI(3,4,5)P$_3$ (2:1) or POPC:POPS (2:1) where $n = 3$.

DISCUSSION

In this study, we have determined the apparent affinities between RGS4 and G protein subunits and the G protein effector, PLCβ. Before analyzing the energies associated with these protein−protein interactions, it is important to know whether RGS4 is concentrated on the membrane surface or freely diffusing in solution. Thus, we measured the affinity of RGS4 to model membranes and found that RGS4 bound only weakly to bilayers containing physiological concentrations of negatively charged lipids (see *33*). Therefore, at the low lipid concentrations used in this study, we will treat our protein association data assuming that RGS4 is soluble and freely diffusing in solution (see below).[2]

Our membrane-binding results are in accord with Berstein and co-workers (*34*), who worked at higher (i.e., 1 mM) lipid concentrations to show that RGS4 binds to membranes through an N-terminal amphipathic helix which contributes to its preference for negatively charged lipids. We have found here that the membrane affinity of RGS4 is greatly increased

[2] RGS4 has a higher GAP activity in a vesicle system rather than a detergent (see ref *31*). While the cause of this may be the concentrating effects of the membrane, it is possible that membrane association of Gα_q may allow for more productive binding of RGS4. Alternately, GAP activity may be sensitive to detergents.

by the presence of PI(3,4,5)P_3 to an extent greater than would be expected by charge alone. Specific interactions between RGS4 and PI(3,4,5)P_3 have been previously reported (*20*), and these interactions have the effect of inhibiting the RGS4 GAP activity possibly by occluding RGS4 residues that also interact with Gα subunits. This PI(3,4,5)P_3−RGS4 association has been proposed to be part of a feedback mechanism that initially keeps RGS4 sequestered after PI-3-kinase stimulation at the early phase of a G protein signal that produces a high local concentration of PI(3,4,5)P_3. As Ca^{2+} levels in the cell increase, RGS4 can be displaced from the membrane by Ca^{2+}/calmodulin to deactivate Gα subunits (see ref *34*).

We also found that RGS4 can be recruited to the membrane by the incorporation of a protein-binding partner due to strong protein interactions (see below). This result implies that if PIP(3,4,5)P_3 was also present, then recruitment would be multiplicative unless the two sites on RGS4 were the same. Unfortunately, this idea was not directly testable due to the strong background contribution of PLCβ_1 to the fluorescence assay. Localization of RGS4 by a protein partner has been suggested by studies showing that most of the cytosolic RGS4 can be recruited to the membrane surface by overexpression of a GTPase-deficient $G\alpha_{i2}$ by a region outside the RGS domain (*36*).

The protein partners of RGS4 studied here are all membrane bound. Since we conducted studies under conditions where soluble RGS4 bound to proteins that were dilute on the membrane surface, we can analyze the dissociation constants assuming a biomolecular association without the need to invoke models that account for membrane association. These are listed in Table 1. Note that our estimated picomolar affinity between RGS4 and activated $G\alpha_q$ are in accord with the subnamolar affinity determined for RGS4 and activated $G\alpha_{i1}$ by surface plasma resonance techniques (*17*). We find that the affinity between RGS4 and activated $G\alpha_q$ was too strong to be quantified by fluorescence energy transfer, but deactivation of $G\alpha_q$ put it in a detectable range. Even so, the association between RGS4 and $G\alpha_q$(GDP) is remarkably strong and this strong residual interaction may keep RGS4 colocalized to the protein complex in the basal state. However, it is expected that other proteins, most notably Gβγ and $G\alpha_q$-specific receptors, would compete with RGS4 for deactivated $G\alpha_q$. A comparison of the crystal structure of the $G\alpha_{i1}$ (GDP-AF_4^-)−RGS4 (*4*) complex with the structure of $G\alpha_{i1}$(GDP)−Gβγ (*37*) shows that both RGS4 and Gβγ interact with Gα switch regions and the complexes formed will be dictated by their relative interaction energies. A direct comparison of the affinities between $G\alpha_q$(GDP)−RGS4 and $G\alpha_q$(GDP)−βγ derived from fluorescence studies is not possible since the G protein subunits are confined to the membrane surface whereas RGS4 is not. Thus, depending on the available area in which the G protein subunits can laterally associate, their effective concentration is expected to be much higher than the freely diffusing RGS4. From our previous studies that translated the apparent dissociation constant between membrane-bound $G\alpha_q$(GDP) and Gβγ into a K_d that was independent of lipid (*27*), we find that, at local lipid concentrations of ∼20 μM, Gβγ should compete with RGS4 for deactivated $G\alpha_q$(GDP).

Interestingly, we find that although Gβγ may displace RGS4 from deactivated $G\alpha_q$, it could also serve as a binding partner for RGS4 (Figure 2 and Table 1). The RGS4 affinity for Gβγ is much weaker than for $G\alpha_q$, but this secondary interaction may serve to keep RGS4 localized in the signaling complex and possibly in a optimal orientation for $G\alpha_q$ rebinding. It may also allow productive palmitoylation on the N-terminus but inhibit palmitoylation at sites in the RGS domain which eliminate its GAP activity (*38*). It is noteworthy that some RGS families have Gγ-like domains that allow for strong interaction with Gβ subunits (see ref *31*).

We also found that RGS4 will interact with the $G\alpha_q$ protein effector PLCβ_1 which also has GAP activity. This interaction may offer another site which localizes RGS4 in the signaling complex. Unlike RGS4, PLCβ_1 binds strongly to membranes (*29*) and will interact laterally with $G\alpha_q$ subunits, but not with Gβγ subunits. Comparing the RGS4−G α_q affinities obtained here to previously determined PLCβ_1−$G\alpha_q$ affinities (*3*), we find that the primary PLCβ_1 interaction will be expected to be competitive with RGS4 interaction to activated and deactivated $G\alpha_q$ at local lipid concentrations below 100 μM.

Our results suggest that RGS4, $G\alpha_q$(GTPγS), and PLCβ_1 may form weakly associating ternary complexes. We find a 10-fold decrease in the affinity of RGS4 to PLCβ_1 in the presence of $G\alpha_q$(GTPγS). Since the RGS4 interaction with PLCβ_1 appears to be mediated by the C-terminal tail (Figure 3), which also partially binds $G\alpha_q$, then the reduction in affinity may be due to occlusion in this region or binding-induced conformational changes. The suggestion that ternary complexes form at higher concentrations of protein is in accord with previous studies by Helper and co-workers, who worked at lower protein concentrations and found that RGS4 blocks PLCβ_1 activation by $G\alpha_q$(GTPγS) (*39*). It is possible that at high local concentrations of proteins, the close proximity of the two GAPs allows them to rapidly switch off and on Gα and greatly increase the rate in which the signal is turned over.

The inherently weak membrane affinity in the absence of PI(3,4,5)P_3 and protein partners raises the possibility that RGS4 can be cytosolic and diffuse between different signaling complexes on the plasma membrane depending on the availability of interaction sites, and regulation of RGS4 action may occur by its membrane recruitment. However, the strong interaction of RGS4 with G protein subunits, with G protein effectors, coupled with previous kinetic studies suggesting RGS4 localization with G protein receptors (*19*) leads to the idea that these proteins maybe complexed in a signaling domain through contact of secondary sites. Moreso, the strength of these association suggests that scaffolding proteins may not be required. Evidence for the localization of RGS4 to a signaling complex comes from studies showing that RGS4 selectively inhibits $G\alpha_q$ responses from particular G-protein receptors indicating that RGS4 is localized to particular receptor types and that localization occurs through the first 33 residues (*19*). These studies are in accord with our data indicating that, although RGS4 has weak membrane binding ability, it has many partners besides activated Gα that can keep it localized to particular areas in cells. In addition, the suggestion that the N-terminus of RGS4 plays a role in PLCβ_1 effector association, along with the suggestion that this region plays a key role in receptor interaction implies that this region of RGS4 can transfer its protein partner depending on the local conditions.

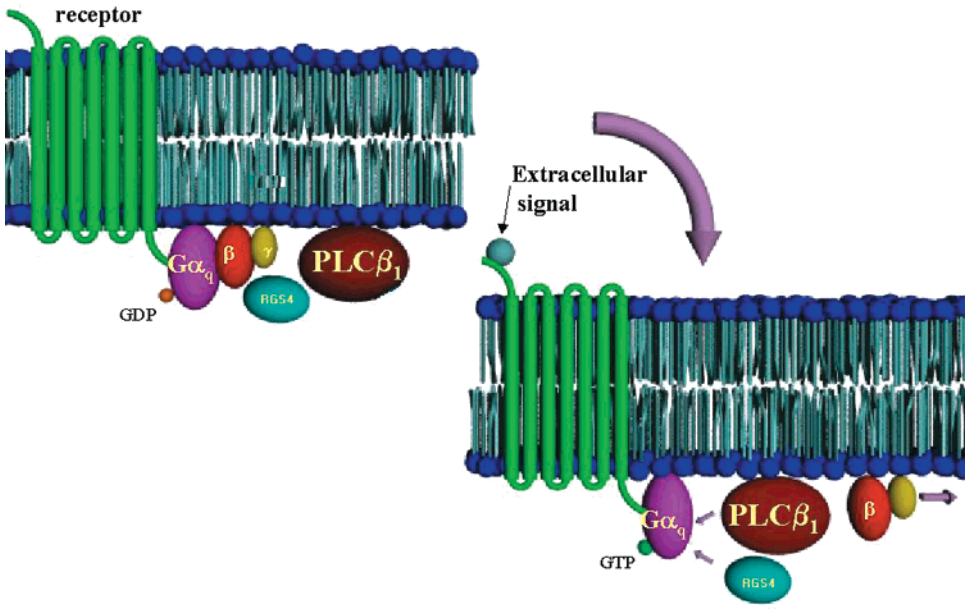

FIGURE 7: Model of possible RGS4 interactions with PLCβ_1 and G protein subunits that may accompany stimulation (see text for details).

The results obtained here can be summarized in the cartoon shown in Figure 7. In the unstimulated state, Gα_q(GDP) will be associated with G$\beta\gamma$ at local lipid concentrations of $\leq$20 μM. Compared to G$\beta\gamma$, PLCβ_1 has a much weaker affinity for Gα_q(GDP), and the binding of PLCβ_1 to the deactivated heterotrimer and to G$\beta\gamma$ is very weak. Thus, unless the local protein concentrations are high, PLCβ_1 would be expected to either be dissociated from the G protein heterotimer or only loosely associated. Also, RGS4, which can diffuse through the cytoplasm, can associate with membrane-bound PLCβ_1 which in turn facilitates its palmitoylation and keeps it in close proximity to Gα_q. Upon activation of Gα_q, PLCβ_1 can displace G$\beta\gamma$ to become activated, but since the effector-bound RGS4 may be situated very close to Gα_q, it can compete with PLCβ_1 for the full interaction site. It is possible that RGS4 competes with PLCβ_1 for the initial binding to Gα_q, which would diminish the strength of the signal. Kinetic studies by Ross and co-workers in a reconstituted system show that RGS4 affects the signal duration rather than amplitude (*16*).

The cartoon in Figure 7 is a greatly simplified model of the interactions that may occur in a signaling complex, but it may be used as a basis to better define the factors that enhance or antagonize these associations. While it is tempting to define these interactions in terms of composites of free energies as derived from the dissociation constants, the observation that the same interaction sites can be partially occluded from other protein partners means that the energies are not simply additive, and this adds another level of complexity to understanding the interactions in signaling domains.

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