# [48] Measuring RGS Protein Interactions with $G_q\alpha$

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## Introduction

Many hormones, neurotransmitters, and sensory inputs rely on heterotrimeric guanine-nucleotide regulatory proteins (G proteins) to exert their actions at target cells and tissues.[1-3] G proteins act by directly coupling cell surface receptors to the regulation of specific effector proteins at the plasma membrane. All G proteins are composed of three subunits designated as $G\alpha$ (39–46 kDa), $G\beta$ (35–39 kDa), and $G\gamma$ (8–11 kDa), and, to date, at least 21 unique $G\alpha$, 6 $G\beta$, and 12 $G\gamma$ subunits have been identified.[1-3] The identity of the individual $G\alpha$ subunit defines the G protein trimer, and $G\alpha$ subunits have been classified into four major subfamilies ($G_s$, $G_i$, $G_q$, and $G_{12}$) based on amino acid sequence identities and functional similarities.[1-3] At target cells, hormone or neurotransmitter receptor activation results in stimulation of phospholipase C, which hydrolyzes phosphatidylinositols to generate two important second messengers, inositol (1,4,5)trisphosphate [Ins(1,4,5)$P_3$] and diacylglycerol. Ins(1,4,5)$P_3$ mobilizes internal $Ca^{2+}$ stores whereas diacylglycerol activates certain forms of protein kinase C. Members of the $G_q$ subfamily of G proteins ($G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, and $G_{15/16}\alpha$) directly couple cell surface receptors to activation of the $\beta$ isoforms of phospholipase C-$\beta$ (PLC-$\beta$) and inositol lipid signaling.

G proteins serve as molecular switches in cell signaling. Activated receptors promote GTP binding to $G\alpha$ subunits, and the duration of a particular G-protein-directed signaling event is dictated by the lifetime of the $G\alpha$–GTP species. $G\alpha$ subunits are GTPases, and G protein signaling is terminated by $G\alpha$-catalyzed hydrolysis of bound GTP. As such, $G\alpha$–GTPase activity represents an important cellular control point for modulating hormone and neurotransmitter signaling events. Recent findings indicate that $G\alpha$ GTPase activity and linked signaling events are regulated by a newly identified class of signaling proteins, the regulators of G protein signaling (RGS proteins). RGS proteins comprise a large family (more than 30 mammalian forms) of highly diverse, multifunctional signaling proteins which share a conserved 120 amino acid domain (RGS domain).[4-9]

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RGS domains bind directly to activated $G\alpha$ subunits to act as GTPase-activating proteins (GAP) and/or effector antagonists to modulate hormone receptor and G-protein-directed signaling. Nearly all RGS proteins studied to date serve as GAPs for $G_i\alpha$ family members and/or $G_q\alpha$.[4–9] RGS4 was the first family member recognized to be an inhibitor of $G_q\alpha$-directed signaling.[10,11] Whereas RGS4 can act as a GAP for both $Gi\alpha$ family members and $G_q\alpha$, RGS2 was the first family member identified to be a selective inhibitor of $G_q\alpha$ function.[12–14] More recent studies have identified many other RGS proteins that also can regulate $G_q\alpha$ signaling functions (see Refs. 7, 8, and references therein), although not all block $G_q\alpha$ signaling by accelerating $G\alpha$–GTPase activity. For example, the well-studied G-protein-coupled receptor kinase GRK2 contains an RGS domain that binds tightly to $G_q\alpha$ to block its interactions with PLC-$\beta$ without affecting $G_q\alpha$–GTPase activity.[15] In this regard, GRK2 and other RGS proteins[10,12,14] can act as effector antagonists.

Because of the broad importance of $G_q\alpha$-regulated inositol lipid signaling in cell physiology[16] and a new appreciation of the role for certain RGS proteins as regulators of $G_q$ function,[4–9] considerable research interest has now focused on understanding and measuring RGS interactions with $G_q\alpha$. Numerous studies have examined RGS modulation of $G_q$-directed inositol lipid and $Ca^{2+}$ signaling in various intact cell systems (see Refs. 7, 8, and references therein). In these cases, RGS/$G_q\alpha$ interactions were observed as a consequence of introducing cDNA and overexpression of recombinant RGS proteins in target cells. This approach has proven to be very useful for qualitatively confirming the negative regulatory effects of various RGS proteins on $G_q$ signaling pathways. However, these methods do not allow for the quantitative measurement of direct RGS/$G_q\alpha$ interactions. This chapter will describe currently available methods for quantitatively measuring direct RGS interactions with $G_q\alpha$ *in vitro*. Three methods will be discussed in detail: (1) RGS stimulation of $G_q\alpha$–GTPase activity; (2) RGS inhibition of $G_q\alpha$-directed stimulation of phospholipase C-$\beta$; and (3) RGS inhibition of hormone receptor and $G_q/11\alpha$-directed stimulation of PLC$\beta$ in broken cell membrane preparations. Other available methods for measuring RGS/$G_q\alpha$ interactions that offer special advantages will also be discussed, but only briefly because of the general unavailability of required materials.

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## Special Materials

### *Purified Recombinant RGS Proteins*

Recombinant, hexahistidine($His_6$)-tagged RGS4, RGS2, and RGS-GAIP are synthesized in *Escherichia coli* and purified by Ni-NTA affinity chromatography as previously described.[12,17]

### *Purified Recombinant Hexahistidine Tagged $G_q\alpha$*

$G_q\alpha$ is not an abundant protein in native tissues and, as such, is very difficult to purify from native tissue sources such as bovine brain or liver. However, reasonable amounts (0.1–1 mg) of active recombinant $G_q\alpha$ protein can be obtained by using baculoviral expression systems and overexpression in Sf9 insect cells. In most cases, $G_q\alpha$ must be coexpressed with recombinant $G\beta\gamma$ subunits to generate properly folded, detergent-soluble $G\alpha$ subunit that is active. Procedures for the purification of recombinant $G_q\alpha$ from Sf9 (*Spodoptera frugiperda* ovary) cell membranes using conventional chromatography have been described.[18] However, $G_q\alpha$ engineered to contain a hexahistidine tag at the carboxy terminus ($G_q\alpha : CH_6$) can be coexpressed as a functional heterotrimer with either $G\beta_1$ or $G\beta_2$ in combination with $G\gamma_2$, and readily purified to homogeneity as free $G_q\alpha$:$CH_6$ by sequential Ni-NTA affinity and anion-exchange chromatography steps as previously described.[19] Addition of the hexahistidine tag does not affect recombinant $G_q\alpha : CH_6$ interactions with $G\beta\gamma$,[19] PLC-$\beta_1$,[19] and RGS proteins.[10,12]

### *Purified Recombinant Wild-Type $G_q\alpha$ and $G_q\alpha$R183C*

Both of these proteins can be purified essentially as described originally by Biddlecome *et al.*,[20] with some minor modifications.[21] Briefly, either $G_q\alpha$ or $G_q\alpha$R183C is coexpressed in Sf9 cells with recombinant, hexahistidine tagged forms of $G\beta_2$ and $G\gamma_2$ ($His_6$-$G\beta_2$/$His_6$-$G\gamma_2$). Following the extraction of membranes with cholate, the solubilized trimer is diluted into lubrol and bound to Ni-NTA, followed by extensive salt washing of the resin. Subsequently, the column is brought to room temperature and washed with a cholate/lubrol buffer

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containing GTP$\gamma$S, which elutes unwanted endogenous (insect) G$\alpha$ subunits from the resin, leaving recombinant $G_q\alpha$ bound to $His_6$-G$\beta_2$/$His_6$-G$\gamma_2$. Finally, $G_q\alpha$ or $G_q\alpha$R183C is eluted with aluminum, fluoride, and magnesium ions, which frees G$\alpha$ from the $His_6$-G$\beta$2/$His_6$-G$\gamma$2 bound to the Ni-NTA resin. For the final desalting of eluted protein, CHAPS is recommended over cholate, which may promote denaturation.[21] Under ideal conditions, this protocol should yield virtually pure (>95%) wild-type recombinant $G_q\alpha$. $G_q\alpha$R183C, which expresses poorly, should yield a preparation that is about 40% pure, which is adequate for single-turnover experiments but not recommended for reconstitution studies.

### *Baculoviruses Encoding Recombinant Wild-Type and Hexahistidine-Tagged G$\beta$ and G$\gamma$ Subunits*

Generation of baculoviruses and purification of recombinant forms of mammalian G$\beta_1$, G$\beta_2$, and G$\gamma_2$ subunits from Sf9 insect cells has been described in detail.[22] Baculovirus encoding hexahistidine-tagged forms of G$\beta_2$ and G$\gamma_2$ for use in the purification of $G_q\alpha$ has also been been described.[20]

### *Purified Phospholipase C-$\beta_1$*

Purified $G_q\alpha$ family members activate the $\beta$ isoforms of PLC (PLC-$\beta_1$, -$\beta_2$, and -$\beta_3$).[19,24,44] PLC-$\beta_1$ is abundant in brain cytosol and is readily obtained in a partially purified form using protocols that rely on sequential conventional chromatography steps.[23,25,26]

### *Antisera*

Antisera that specifically recognize RGS4, RGS2, $G_q\alpha$, and PLC-$\beta_1$ are commercially available (Santa Cruz Biotechnology, Santa Cruz, CA; Upstate Biologicals, Lake Placid, NY). Available anti-$G_q\alpha$ sera recognize both wild-type $G_q\alpha$ and $G_q\alpha$R183C. Note that placement of a hexahistidine tag at the carboxy terminus of $G_q\alpha$ can interfere with antibody recognition at this site.

## RGS Stimulation of $G_q\alpha$ GTPase Activity

### *General Considerations Regarding GTPase Assays*

G proteins traverse a cycle of GTP binding, hydrolysis, and GDP + $P_i$ dissociation. Agonist-bound receptors promote GDP dissociation and thus facilitate

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GTP binding and G protein activation. RGS proteins and other GAPs turn off activated G proteins by accelerating the rate of GTP hydrolysis. In the presence of activated receptor, this GAP effect serves to accelerate the steady-state turnover of GTP. However, GAPs have little or no effect on the steady-state GTPase activity of isolated $G\alpha$ since GDP dissociation is already rate-limiting under such conditions.[27] Therefore, GAP assays must be performed either under pre-steady-state conditions (i.e., GTP-bound to $G\alpha$) or in the presence of activated receptor.

The direct GAP effect of an RGS protein on $G\alpha$ can be detected as an increase in the rate of a single round of hydrolysis and $P_i$ release by $G\alpha$–GTP. The primary advantage of this pre-steady-state, bimolecular method is that it allows GAP effects to be quantified as direct fold-stimulation of the basal rate of GTP hydrolysis, and also permits an approximation of the affinity between RGS and free $G\alpha$–GTP.[28] To assay GAP activity under steady-state conditions is technically simpler but, as noted, requires receptor as well as $G\beta\gamma$, which themselves may influence RGS–$G\alpha$ interactions.[7,21] With $G_q$, both single-turnover and steady-state GTPase assays pose difficulties not encountered with $G_i$, $G_o$, or $G_s$.

### *RGS-Directed Stimulation of $G_q\alpha$R183C GTPase Activity*

*Special Considerations.* The direct GTPase activating effect of RGS proteins on GTP-bound $G_{i/o}\alpha$ subunits is readily detectable using purified $G\alpha$ subunits and RGS proteins in solution, as described elsewhere in this volume.[29] In contrast, analogous assays using wild-type $G_q\alpha$ are not practicable because of difficulties in isolating $G_q\alpha$–GTP. This stems primarily from the extremely slow dissociation of bound GDP, and probably also reflects the intrinsic instability of the nucleotide-free form of $G_q\alpha$.[30] These properties also account for the high nucleotide concentrations needed to observe $G_q$ binding. To facilitate the isolation of the GTP-bound species, the point mutant $G_q\alpha$R183C can be substituted for wild-type $G_q\alpha$. This mutant has a greatly decreased intrinsic rate of GTP hydrolysis,[21] as well as an increased rate of GDP dissociation. Slowly hydrolyzing G protein mutants in some cases are GAP-insensitive,[17]; however, as with $G_i\alpha$R178C, the arginine-to-cystine substitution does not prevent $G_q\alpha$R183C from hydrolyzing GTP more rapidly in response to GAPs.[21] The rate of GTP hydrolysis by $G_q\alpha$R183C is accelerated by phospholipase

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C-$\beta_1$,[21] RGS2,[13] RGS4,[31] RGS-GAIP, RGS1, and RGS3,[32] but not by rho-GEF,[33] GRK2,[15] RGS12,[34] RGS10, RGS11, or RGS14.[32]

*Preparation for Assay.* $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP is prepared by incubating 100 n*M* $G_q\alpha$R183C with 10 $\mu M$ [$\gamma$-$^{32}$P]GTP at 20° in 20 m*M* Na–HEPES (pH 7.5) containing 5.5 m*M* CHAPS, 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA, 900 $\mu M$ $MgSO_4$ (10 $\mu M$ final free $Mg^{2+}$), 30 m*M* $(NH_4)_2SO_4$, 2% (v/v) glycerol, and 0.1 mg/ml albumin for 2–3 hr, at which point approximately 30% of active $G_q\alpha$R183C will be bound to [$\gamma$-$^{32}$P]GTP. The reaction mixture is then diluted with an equal volume of ice-cold buffer A [50 m*M* Na–HEPES (pH 7.5), plus either 5.5 m*M* CHAPS or 0.3% cholate], and after 5 min on ice, $G_q\alpha$R183C–[$\gamma^{32}$-P]GTP is recovered by centrifugal gel filtration on Sephadex G-25 equilibrated in buffer A. This procedure removes 99.9% of free nucleotide and yields 20–30% recovery of $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP. A word is in order here concerning the stability and reactivity of $G_q\alpha$R183C in various detergents. We have found that Lubrol, CHAPS, Triton X-100, and dodecyl maltoside all yield approximately equal amounts of [$\gamma$-$^{32}$P]GTP and [$^{35}$S]GTP$\gamma$S binding to $G_q\alpha$R183C, whereas cholate clearly has a detrimental effect on [$\gamma$-$^{32}$P]GTP binding. Cholate was the only detergent tested that permitted the GAP effect of PLC-$\beta_1$ on $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP, whereas RGS4 GAP activity was evident to varying extents in the presence of cholate, CHAPS, Triton X-100, octylglucoside, dodecylmaltoside, deoxycholate, and Lubrol. Thus it may be necessary to optimize detergent conditions when assaying novel GAPs.

*Performing Assay.* To measure GTP hydrolysis, isolated $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP is diluted approximately 10-fold into 20 m*M* HEPES buffer (pH 7.5) containing the GAP under investigation plus 80 m*M* NaCl, 1 m*M* dithiothreitol, 1 m*M* EDTA, 900 $\mu M$ $MgSO_4$, 0.04 mg/ml albumin, and 2% glycerol plus appropriate detergent (e.g., 0.2–0.3% cholate). GTP hydrolysis is assessed by the release of free [$^{32}$P]$P_i$ into the buffer, which is isolated from Gq$\alpha$R183C–[$\gamma$-$^{32}$P]GTP and free [$\gamma$-$^{32}$P]GTP by the addition of a charcoal slurry followed by centrifugation, which leaves only free [$^{32}$P]$P_i$ in the supernatant.[20,21,28] Total $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP should be quantified by vacuum filtration over nitrocellulose filter discs at the outset of the assay.[20,21,28] In the absence of added GAP, the rates of both GTP hydrolysis and GTP dissociation are approximately 0.05 per minute

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at 20°. GTP dissociation thus can be appreciable over the time required to carry out an assay, and one should monitor binding as well as hydrolysis over the course of an experiment. It is also important to note that biphasic GTP hydrolysis is generally observed in the presence of GAP, suggesting that a fraction of the total $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP (typically 25–40%) is GAP-insensitive. This must be taken into account when fitting or calculating hydrolysis rates, and procedures for doing so have been described in detail.[21,28]

*Results.* In the experiment shown in Fig. 1, RGS4 increased the rate of GTP hydrolysis by $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP by a factor of about 100. In this experiment, the decrease in total $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP (open symbols) followed a similar time course to that of [$^{32}$P]$P_i$ generation (closed symbols), indicating

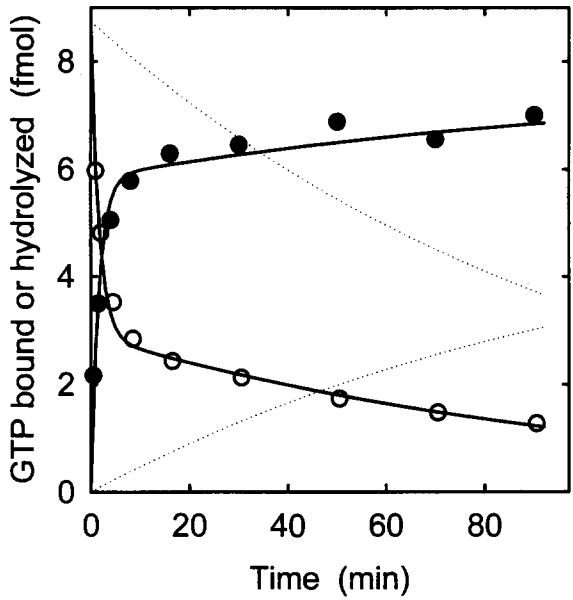


FIG. 1. Stimulation of $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP GTPase activity by RGS4. $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP was prepared and isolated as described and diluted with 9 volumes of assay buffer containing 0.2% cholate either without or with RGS4. At the times indicated, duplicate 20 $\mu$l aliquots were withdrawn and assayed for release of [$^{32}$P]$P_i$ or $G_q\alpha$-bound [$\gamma^{32}$P]GTP. All four sets of data were fitted simultaneously using a 2-component equation assuming a GAP-sensitive plus a GAP-insensitive fraction (see Ref. 21) to yield the fitted lines shown (dotted lines, no RGS4; solid lines, 120 n*M* RGS4). The solid and open symbols represent respectively the release of [$^{32}$P]$P_i$ and the amount of $G_q\alpha$-bound [$\gamma$-$^{32}$P]GTP in the presence of RGS4. Data in the absence of RGS4 have been omitted for clarity. In this experiment, 67% of the total $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP was sensitive to the GAP effect of RGS4. Basal rates of GTP hydrolysis and dissociation respectively were 0.0056 min$^{-1}$ and 0.0038 min$^{-1}$, and the rate of hydrolysis was stimulated approximately 100-fold by RGS4, to 0.61 min$^{-1}$. Reproduced with permission from P. Chidiac and E. M. Ross, *J. Biol. Chem.* **274,** 19639 (1999).

that RGS4 did not increase the rate of GTP dissociation. Note that both RGS4 curves are biphasic, indicating the presence of a GAP-insensitive population of $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP. Fitted curves corresponding to data acquired in the absence of RGS4 (dotted lines) show that loss of $G_q\alpha$R183C–[$\gamma$-$^{32}$P]GTP (which results from both dissociation and hydrolysis) occurs at about twice the rate at which [$^{32}P_i$] is produced, indicating that the basal rates of GTP dissociation and hydrolysis are similar.

*Receptor-Promoted Steady-State GTPase Assays*

Unlike studies with $G_i$, $G_o$, and $G_s$, attempts to assay receptor-promoted steady-state GTP turnover by $G_q$ in broken cell preparations have been largely unsuccessful. It is possible to clearly observe agonist-stimulated $G_q\alpha$ GTPase activity and the acceleration of that activity by GAPs using ml muscarinic receptors coreconstituted into phospholipid vesicles with heterotrimeric $G_q$.[20] This method requires that purified, detergent-solubilized receptor, $G_q\alpha$, and $G\beta\gamma$ be combined together, followed by gel filtration (or dialysis) to remove residual detergent. Although this technique presents a powerful means to study $G_q$–RGS interactions,[35] it is technically challenging in that each of the proteins used must first be purified in a nondenatured state, and typically over half of each protein is lost during the reconstitution process. For these reasons, this method is not readily approachable by most investigators.

A number of other experimental systems have been described that could be utilized for the study of receptor- and RGS-stimulated $G_q\alpha$–GTPase activity. Several investigators have developed novel assays for receptor-stimulated [$^{35}$S]GTP$\gamma$S binding to $G_q$ based on insect cell/baculovirus expression systems.[36–38] These techniques take advantage of the high receptor levels possible with baculovirus expression, combined in some instances with either the coexpression of $G_q$[38] or the addition of exogenous purified $G_q$.[37] Fusion proteins consisting of G protein coupled receptors linked directly to G$\alpha$ subunits have also been described[39,40] that allow direct study of receptor and G-protein-stimulated GTPase activity. Although none of receptors described thus far have been fused to $G_q\alpha$, there are no obvious barriers to this possibility and such a receptor/$G_q\alpha$ fusion protein could be used to study RGS interactions.[40] At least one experimental model system allows for the study of endogenous receptors linked to native $G_q$/11 and does not rely

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upon recombinant proteins. Turkey erythrocyte ghosts contain native $G_{11}$-linked receptors (e.g., P2y-purinergic) which stimulate GTP-supported phospholipase C activity[41] and can be used to study RGS protein interactions with $G_{11}$ and receptors.[42] While each of these experimental systems offers special advantages, they also require the use of special reagents and materials that make them generally unavailable to most laboratories. Nevertheless, it may be possible to adapt one or more of these methods to measure receptor- and RGS-stimulated $G_q\alpha$–GTPase activity in a partial reconstitution system.

## RGS Inhibition of $G_q\alpha$ Signaling Functions

### *RGS Inhibition of $G_q\alpha$-Stimulated Phospholipase C-$\beta_1$ Activity*

In addition to their defined roles as G$\alpha$–GAPs, RGS proteins also act as effector antagonists to block G protein signaling.[10,15,43] Consistent with this idea, RGS proteins form a stable complex with activated $G_q\alpha$ in *in vitro* reconstitution assays[10,12,15] and in cell membranes,[10] which prevents $G_q\alpha$/PLC-$\beta$ interactions and blocks inositol lipid signaling. Since surface residues on G$\alpha$ responsible for G$\alpha$/RGS interactions are predicted to differ from those involved with G$\alpha$/effector interactions,[43] RGS capacity to block PLC binding to $G_q\alpha$ is likely due to steric hindrance. Based on these interactions, we will describe a method for detecting RGS interactions with active $G_q\alpha$ by monitoring changes in $G_q\alpha$-directed PLC$\beta$ activity that is measured as hydrolysis of exogenously supplied [$^3$H]$PIP_2$ and formation of the water-soluble product, [$^3$H]Ins(1,4,5)$P_3$. A technical advantage of this assay over the measurement of G$\alpha$ GTPase activity is that the wild-type recombinant $G_q\alpha$ required for these assays is more readily isolated than the $G_q\alpha$R183C mutant, which, at present, must be purified by complex formation with hexahistidine-tagged $G\beta_2/G\gamma_2$ (discussed above). An important experimental advantage of this method is that it can detect RGS proteins that bind to but do not serve as stimulators of $G_q\alpha$ GTPase activity, as is the case with GRK2.[15] The primary disadvantages of this approach are that the it only detects indirect RGS effects on $G_q\alpha$ in the form of inhibition of measured PLC-$\beta$ activity, and it requires availability of PLC-$\beta_1$ and establishing protocols for measuring [$^3$H]$PIP_2$ hydrolysis.

*Special Considerations.* Under defined assay conditions[44] where PLC-$\beta$ and [$^3$H]$PIP_2$ substrate are held constant (1 ng/assay PLC$\beta$ and 50 $\mu M$ [$^3$H]$PIP_2$) and substrate consumption is within a linear range (3–5 min), the half-maximal

[41] J. L. Boyer, C. P. Downes, and T. K. Harden, *J. Biol. Chem.* **264,** 884 (1989).

[42] M. Cunningham, G. Waldo, S. Hollinger, J. R. Hepler, and T. K. Harden, *J. Biol. Chem.* **276,** 5438 (2001).

[43] J. J. G. Tesmer, D. M. Berman, A. G. Gilman, and S. R. Sprang, *Cell* **89,** 251 (1997).

[44] A. V. Smrcka, J. R. Hepler, K. O. Brown, and P. C. Sternweis, *Science* **251,** 804 (1991).

concentration of $G_q\alpha$ required to activate PLC-$\beta$ is approximately 30 n*M*, with maximal enzyme activity observed around 300 n*M*. Since RGS inhibition of PLC-$\beta$ binding to $G_q\alpha$ is a competitive reaction, it is necessary to use submaximal amounts of $G_q\alpha$ in the assay (1–3 n*M*) in order to completely inhibit $G_q\alpha$ function by RGS. Under these assay conditions, uninhibited $G_q\alpha$-stimulated $PIP_2$ consumption occurs at a much lower rate and product formation is linear for up to 60 min. As such, these assays need to be carried out for longer time periods (at least 30–40 min) in order to accumulate measurable amounts of $[^3H]Ins(1,4,5)P_3$.

*Preparation of $G_q\alpha$, RGS Proteins, and Buffers.* Proteins necessary for these assays include RGS (e.g., RGS2 or RGS4), $G_q\alpha$, and PLC-$\beta_1$. If possible, relatively pure proteins should be used to allow control of the total amounts and relative ratio of each protein in the assay. Proteins should be prepared as concentrated stocks (0.1 mg/ml or higher) to promote stability and to allow for flexibility in dilution. Two buffers are used for these assays: Buffer 1 [50 m*M* sodium HEPES, pH 7.2, 1 m*M* EDTA, 3 m*M* EGTA, 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol (DTT), 100 m*M* NaCl, and either 1% sodium cholate or 0.6% octylglucoside], and Buffer 2 (50 m*M* Na–HEPES, pH 7.2, 3 m*M* EGTA, 1 m*M* DTT, 80 m*M* KCl). Buffer 1 is used to prepare the $G_q\alpha$ and RGS proteins, whereas Buffer 2 is used to prepare PLC-$\beta_1$ mix, $[^3H]PIP_2$ phospholipid vesicles, and the $Ca^{2+}$mix. When all components are combined, the reactions are performed in a final total volume of 70 $\mu$l in a buffer consisting of 50 m*M* Na–HEPES (pH 7.2), 3 m*M* EGTA, 0.2 m*M* EDTA, 0.83 m*M* $MgCl_2$, 20 m*M* NaCl, 30 m*M* KCl, 1 m*M* dithiothreitol (DTT), 0.1 mg/ml ultrapure bovine albumin (Calbiochem, La Jolla, CA), 0.16% sodium cholate, and 1.5 m*M* $CaCl_2$ (to yield ~150–200 n*M* free $Ca^{2+}$). Detergent is included to preserve the solubility of the lipid-modified $G_q\alpha$. Although PLC-$\beta$ activity is inhibited by many detergents, activity is preserved in the presence of sodium cholate (Calbiochem, La Jolla, CA) or octyl glucoside (Calbiochem). $Ca^{2+}$ is an essential cofactor for PLC-$\beta$ activity, and $Ca^{2+}$ mix is prepared as a 9 m*M* solution of $CaCl_2$ in Buffer 2 and added as 10 $\mu$l/assay.

Prior to assay, solutions containing each of the reaction components are prepared separately and stored on ice. These include the following: (1) $G_q\alpha$ mix (10 $\mu$l/assay), (2) RGS mix (10 $\mu$l/assay), and (3) $Ca^{2+}$ mix (10 $\mu$l/assay). The remaining components, which include PLC-$\beta$ and $[^3H]PIP_2$ containing phospholipid vesicles, are prepared separately and are described below. Since the final reaction volume is 70 $\mu$l, RGS and $G_q\alpha$ are prepared at a concentration sevenfold higher than that desired in the final assay. RGS domains will form a stable complex with $G_q\alpha$ activated with either nonhydrolyzable analogs of GTP (e.g., GTP$\gamma$S) or with $AlF_4^-$, which traps G$\alpha$ in the active transition state during GTP hydrolysis. $G_q\alpha$ is activated with either 1 m*M* GTP$\gamma$S for 1 hr at 30° or with 10 m*M* NaF and 30 $\mu$*M* $AlCl_3$ for 15 min at room temperature in Buffer 1. RGS proteins are prepared as a stock concentration and then subjected to serial dilution in Buffer 1. Whereas

RGS4 is relatively stable in solution, RGS2 is sensitive to ionic strength and will form insoluble aggregates that precipitate if stored in buffers containing physiological salt concentrations.[12] As such, RGS2 is prepared and diluted in Buffer 1 containing 500 m*M* NaCl. The high salt conditions do not interfere with $G_q\alpha$ activation of PLC$\beta_1$.

*Preparation of $PIP_2$ Substrate Phospholipid Vesicles and PLC$\beta_1$ Mix.* In this method, RGS interactions with $G_q\alpha$ are detected as RGS capacity to inhibit $G_q\alpha$-directed PLC$\beta_1$ activity. PLC-$\beta$ activity is measured as hydrolysis of [$^3$H]$PIP_2$ and formation of the water-soluble product, [$^3$H]Ins(1,4,5)$P_3$. Activation of PLC-$\beta$ is achieved by reconstituting enzyme with activated Gq$\alpha$ and mixed phospholipid vesicles containing lipid substrate as described previously.[18] Radiolabeled $PIP_2$ substrate is prepared as a mixture of phosphatidylinositol 4,5-bisphosphate ($PIP_2$; Sigma, St. Louis, MO) and bovine brain phosphatidylethanolamine (PE; Sigma) in a ratio of 1:10 containing 5000 to 10,000 cpm/assay of added [$^3$H]$PIP_2$ (New England Nuclear, Boston, MA) as a radiolabel trace. Phospholipids (stored at −20° in chloroform under argon gas) are prepared first by drying under nitrogen gas at room temperature until organic solvent is removed and a lipid film remains at the bottom of the tube. The lipid film is then rehydrated, and vesicles are formed by brief sonication in Buffer 2 using a bath sonicator (Laboratory Supplies, Co.; Hicksville, NY). A probe sonicator may substituted for this purpose if available. The amount of $PIP_2$ necessary for the experiment is calculated based on preparing a stock solution at 175 $\mu M$ added as 20 $\mu$l/assay which yields 50 $\mu M$ (3000 pmol) final/reaction tube. The resulting lipid vesicles containing $PIP_2$, PE, and trace amounts of [$^3$H]$PIP_2$ (henceforth referred to as [$^3$H]$PIP_2$:PE mix) are stored on ice until ready for use. PLC-$\beta$ is prepared in Buffer 2 containing 1 mg/ml bovine serum albumin (BSA), which is included to stabilize enzyme activity. PLC-$\beta_1$ purified from bovine brain or as recombinant protein can be used at a final concentration of 1 ng/20 $\mu$l/reaction which will provide a low basal activity and a robust response to $G_q\alpha$. For simplicity, the [$^3$H]$PIP_2$:PE mix and PLC-$\beta$ mix are combined (20 $\mu$l each/reaction tube) and added as 40 $\mu$l/reaction tube immediately before the reaction is started (see below).

*Performing Assay and Processing Samples.* Round-bottom, polypropylene tubes (5 ml, 12 mm × 75 mm) are labeled and placed in a tube rack on ice. To each tube is added 10 $\mu$l Buffer 1 (for blanks and measurement of basal PLC$\beta$ activity) or Buffer 1 containing activated $G_q\alpha$. RGS proteins or an equivalent volume of Buffer 1 (10 $\mu$l/tube) are added and incubated with $G_q\alpha$ for at least 30 min on ice to allow for protein complex formation. After this time, $Ca^{2+}$ solution (10 $\mu$l) is added to all tubes, and the reaction mix is stored on ice. The assay is started by adding the PLC-$\beta$/[$^3$H]$PIP_2$ mix (40 $\mu$l) to each tube on ice, and samples are vortexed immediately. For this addition, it is helpful to use a repeat pipetter with Combitips (Eppendorf). Reactions are initiated by transferring tubes to a 30° water bath for the desired time. Under these defined assay conditions (50 $\mu M$ [$^3$H]$PIP_2$,

1 ng PLC-$\beta_1$, 1 n*M* $G_q\alpha$) substrate consumption is linear for up to 50–60 min, and reactions are typically allowed to proceed for 40 min. Reactions are terminated by rapid addition of 200 $\mu$l of 10% trichloroacetic acid (TCA), followed by 100 $\mu$l of bovine serum albumin (10 mg/ml). Samples are then transferred immediately to an ice bath and vortexed.

Precipitation of the albumin in acidic conditions will coprecipitate unhydrolyzed radiolabeled $PIP_2$. To isolate released [$^3$H]Ins(1,4,5)$P_3$, samples are centrifuged at 2000*g* for 10 min at 4°, and supernatant is recovered. Radioactivity in the supernatant is measured by liquid scintillation counting.

*Results.* In the example experiment provided (Fig. 2), the relative capacities of RGS2 and RGS4 to block $G_q\alpha$ activation of PLC$\beta_1$ were compared. The assays involved only three purified recombinant proteins ($G_q\alpha$, RGS, PLC-$\beta_1$) and

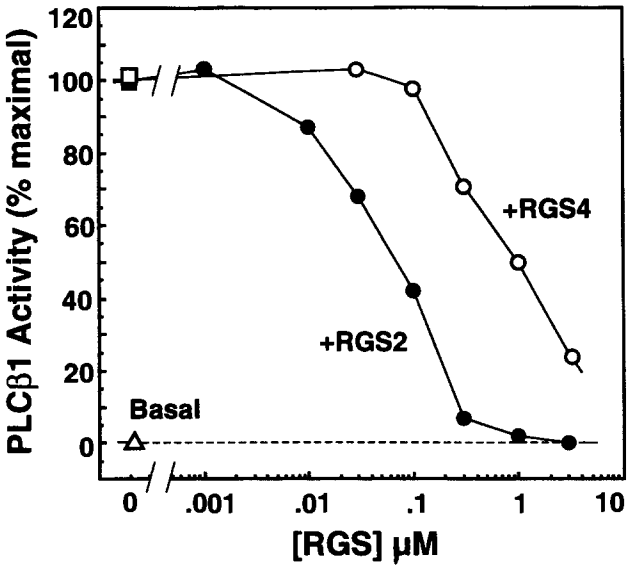


FIG. 2. RGS-directed inhibition of $G_q\alpha$-mediated phospholipase C$\beta_1$ (PLC$\beta_1$) activation. Purified recombinant $G_q\alpha$ was incubated with 1 m*M* GTP$\gamma$S for 1 hr at 30°. Activated $G_q\alpha$–GTP$\gamma$S (1 ng) was preincubated with various concentrations of RGS2 (closed circles), RGS4 (open circles), or no RGS proteins (squares), and then mixed with purified PLC$\beta_1$ and [$^3$H]phosphatidylinositol 4,5-bisphosphate-containing phospholipid vesicles. Synthesis of [$^3$H]inositol 1,4,5-trisphosphate ([$^3$H]Ins$P_3$) was measured and basal unstimulated PLC$\beta_1$ activity (open triangle; 170 pmol/min/ng PLC$\beta_1$) was subtracted from each value. Blank values (i.e., [$^3$H]Ins$P_3$ accumulation in the absence of PLC$\beta_1$) were 155 pmol/min/assay. Values are expressed as a percentage of the total [$^3$H]Ins$P_3$ accumulated over 20 min at 30° in the presence of $G_q\alpha$–GTP$\gamma$S (100% = 716 and 571 pmol/min/ng PLC$\beta_1$ for RGS4 and RGS2, respectively). Reproduced with permission from S. P. Heximer, N. Watson, M. E. Linder, K. J. Blumer, and J. R. Hepler, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 14389 (1997). Copyright 1997 National Academy of Sciences, U.S.A.

exogenously supplied substrate in the form of [$^3$H]$PIP_2$:PE phospholipid vesicles. RGS2 and RGS4 each bound to activated $G_q\alpha$ and blocked its capacity to stimulate PLC-$\beta_1$ activity over a broad concentration range (0.001–3 $\mu M$). Under the defined assay conditions, RGS2 inhibited the actions of 1 n$M$ $G_q\alpha$ with a $K_{0.5}$ value of approximately 30 n$M$ and was 10- to 30-fold more potent than RGS4 at blocking $G_q\alpha$/PLC-$\beta_1$ interactions (Fig. 2).

### *RGS Inhibition of Hormone Receptor and $G_q\alpha$-Stimulated Inositol Lipid Signaling in Broken Cell Preparations*

The negative regulatory effects of RGS proteins on $G_q\alpha$ signaling can also be detected in broken cell or crude cell membrane preparations. The principal advantage of this approach over defined reconstitution systems is that it allows for examination of RGS effects on receptor and $G_q$-directed signaling in the partially intact native environment provided by the cell membranes. While the amount of RGS protein can be controlled quantitatively, the relative amounts of receptor, G protein, and PLC-$\beta$ present are undefined and remain a function of the amount of washed membranes used in the assay. The approach is conceptually and technically similar to that described above with purified proteins, except that in this case endogenous PLC-$\beta$, $G_q\alpha$, and linked receptor are supplied by the cell membranes. Exogenous radiolabeled substrate is supplied as [$^3$H]$PIP_2$:PE phospholipid vesicles (as described above).[10,45] In theory, the method described in this section will work with membranes derived from any cell type. While $G_q$-directed inositol lipid signaling responses can be observed in many broken cell preparations, retention of linked hormone responses varies following cell lysis and should be determined empirically for each cell line. Among the broken cells that are responsive to hormone/neurotransmitter stimulation are NG-108 neuroblastoma × glioma cells which contain bradykinin receptors linked to Gq/11.[10,45] We will describe a method for measuring RGS mediated inhibition of bradykinin receptor and guanine nucleotide stimulation of PLC-$\beta$ activity in broken NG-108 cell preparations.

*Special Considerations.* Unlike the reconstitution assays described above, there is no step for preactivating native G proteins with guanine nucleotide. Instead, the assay relies on addition of hormone or spontaneous rates of nucelotide exchange to load nonhydrolyzable guanine nucleotide (GTP$\gamma$S) to endogenous $G_q\alpha$. Under these defined assay conditions, hormone plus guanine nucleotide responses occur at a much faster rate and are much more robust than those observed with guanine nucleotide alone due to receptor activation. GDP$\beta$S is included in the guanine nucleotide mix at a concentration in excess of GTP$\gamma$S (10 $\mu M$ and 3 $\mu M$,

[45] S. Gutowski, A. V. Smrcka, L. Nowak, D. Wu, M. I. Simon, and P. C. Sternweis, *J. Biol. Chem.* **266,** 20519 (1991).

respectively). This stable form of GDP binds with higher affinity than GTP$\gamma$S to G$\alpha$ that are uncoupled from receptors but with relatively lower affinity to G$\alpha$ that are coupled to activated receptors. As such, inclusion of GDP$\beta$S helps to maximize loading of GTP$\gamma$S to $G_q\alpha$ linked to activated receptors while, at the same time, minimizing nucleotide loading to uncoupled $G_q\alpha$ due to basal exchange. Ideal assay conditions would also permit GTP loading of $G_q\alpha$, which would allow measurement of RGS effects on GTPase activity. However, for unknown reasons, very few, if any, broken cell preparations of mammalian origin retain GTP-sensitive hormone activation of $G_q\alpha$ and PLC-$\beta$. An exception to this rule are specialized membrane preparations derived from turkey erythrocyte ghosts.[41] These preparations retain very robust GTP-sensitive hormone responses and, as discussed, could provide an alternative model system for studying RGS/G$\alpha$ interactions.[42]

*Preparation of NG-108 Cell Membranes.* NG108-15 cell membranes are prepared as described.[10,45] NG108-15 cells are grown to confluency on 3 to 5 150 mm culture plates at 37° in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 0.1 m*M* hypoxanthine, 0.4 m*M* aminopterin, 16 $\mu M$ thymidine, and appropriate antibiotics. Cells are rinsed with cold phosphate-buffered saline (pH 7.2) on ice and collected by scraping in the same buffer containing protease inhibitors. Recovered cells are placed into a nitrogen cavitation bomb (Parr Instruments, Moline, IL) under pressure (400–600 psi) for 30 min at 4°. Cells are lysed by rapid decompression, and lysates are recovered. Cell nuclei are removed by centrifugation at 500 *g* for 10 min at 4°. Total membranes are recovered from the supernatant by centrifugation and resuspended by homogenization (10 strokes on ice) in Buffer 3 (Buffer 1 without detergents), and protein concentrations are determined. At this point, concentrated membranes (preferably greater than 5 mg protein/ml) can be stored at 4° for immediate use, or frozen by liquid $N_2$ and stored at −80° until further use. Receptor responses will lose some activity (30–50%) following one or more freeze–thaw cycles. In practice, crude, unwashed membrane preparations provide a more robust inositol lipid signaling response. Reasons for this are uncertain, but may be due, at least in part, to the fact that extensive washings remove PLC-$\beta_1$ from plasma membranes, which is bound only by ionic interactions.

*Preparation of $PIP_2$ Substrate/Phospholipid Vesicles.* [$^3$H]$PIP_2$:PE vesicles are prepared by drying down a mixture of [$^3$H]$PIP_2$, $PIP_2$, and PE and resuspending the lipid film in Buffer 2 by sonication as described above. The final concentration of lipid is adjusted to provide 50 $\mu M$ $PIP_2$ and 500 $\mu M$ PE and trace amounts of [$^3$H]$PIP_2$ (5000–10,000 cpms) (i.e., [$^3$H]$PIP_2$:PE mix) in the final assay volume of 60 $\mu$l. Thus, a 3-fold concentrated [$^3$H]$PIP_2$:PE mix is prepared to add as 20 $\mu$l/reaction tube.

*Preparation of RGS Proteins, Hormone and Guanine Nucleotide Mix, and $Ca^{2+}$ Mix.* RGS proteins are prepared as concentrated stocks in Buffer 2 and subjected to serial dilutions as desired in the same buffer. Bradykinin alone (10 $\mu$l/assay),

GDP$\beta$S and GTP$\gamma$S as a mixture (10 $\mu$l/assay), or bradykinin plus guanine nucleotides as a mixture (10 $\mu$l/assay) are also prepared as concentrated stocks in Buffer 3. In each case, stocks are prepared at concentrations sixfold greater than that desired in the final reaction mix; each stock is added as 10 $\mu$l per reaction tube. Final desired concentrations for each component are 1 $\mu M$ for bradykinin, 3 $\mu M$ for GTP$\gamma$S, and 10 $\mu M$ GDP$\beta$S. $Ca^{2+}$ mix is prepared as a 9 m$M$ solution of $CaCl_2$ in Buffer 2 to be added as 10 $\mu$l per reaction tube.

*Performing Assay and Processing Samples.* Round-bottom, polypropylene tubes (5 ml, 12 mm × 75 mm) are labeled and placed in a tube rack on ice. To each tube is added 10 $\mu$l of membranes (5 $\mu$g/assay) and either 10 $\mu$l of Buffer 2 (for blank and basal PLC activity) or 10 $\mu$l of Buffer 2 containing RGS proteins. Membranes and RGS proteins are incubated together at 4° for 30 min. [$^3$H]$PIP_2$:PE phospholipid vesicles are added as 20 $\mu$l/assay followed by 10 $\mu$l of bradykinin and/or guanine nucleotides. Reactions are initiated by rapid addition of $CaCl_2$ mix (10 $\mu$l/tube), vortexing, and transferring the tube rack to a 30° water bath for the desired time. Under these assay conditions [$^3$H]$PIP_2$ consumption is linear for up to 50–60 min, and reactions typically proceed for 30 min. As described above, reactions are terminated by rapid addition of 200 $\mu$l of 10% trichloroacetic acid, followed by 100 $\mu$l of bovine serum albumin (10 mg/ml); samples are then transferred immediately to an ice bath and vortexed. For the start and stop addition, it is helpful to use a repeat pipetter with Combitips (Eppendorf).

As discussed above, precipitation of the albumin in acidic conditions will coprecipitate unhydrolyzed radiolabeled $PIP_2$, PE, and membranes. To isolate released [$^3$H]Ins(1,4,5)$P_3$, each of the samples is centrifuged at 2000$g$ for 10 min, and the supernatant is recovered. Radioactivity in the supernatant is measured by liquid scintillation counting.

*Results.* In the example experiment provided (Fig. 3), the relative capacities of RGS4 and RGS-GAIP to block bradykinin receptor and/or GTP$\gamma$S-mediated stimulation of PLC-$\beta$ are compared. The assays involved one purified recombinant RGS protein, crude cell membranes containing desired $G_{q/11}$-linked hormone receptors, and exogenously supplied substrate in the form of [$^3$H]$PIP_2$:PE phospholipid vesicles. RGS4 was a fully effective inhibitor of both hormone receptor-(bradykinin plus GTP$\gamma$S) and G-protein-directed (GTP$\gamma$S alone)stimulation of PLC-$\beta$ activity (Fig. 3A), with inhibition observed over a broad concentration range (0.001–1 $\mu M$) (Fig. 3B). Half-maximal concentration for RGS4 inhibition ($K_{0.5}$) was approximately 100 n$M$, with complete inhibition observed with 1$\mu M$ RGS4. By contrast, RGS–GAIP was a much less potent inhibitor of $G_q\alpha$ function, consistent with the idea that it is a physiological regulator of signaling by $G_i\alpha$ family members, but not $G_q\alpha$.[10,46] This method allows for

[46] L. DeVries, M. Mousli, A. Wursmer, and M. G. Farquhar, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 11916 (1995).

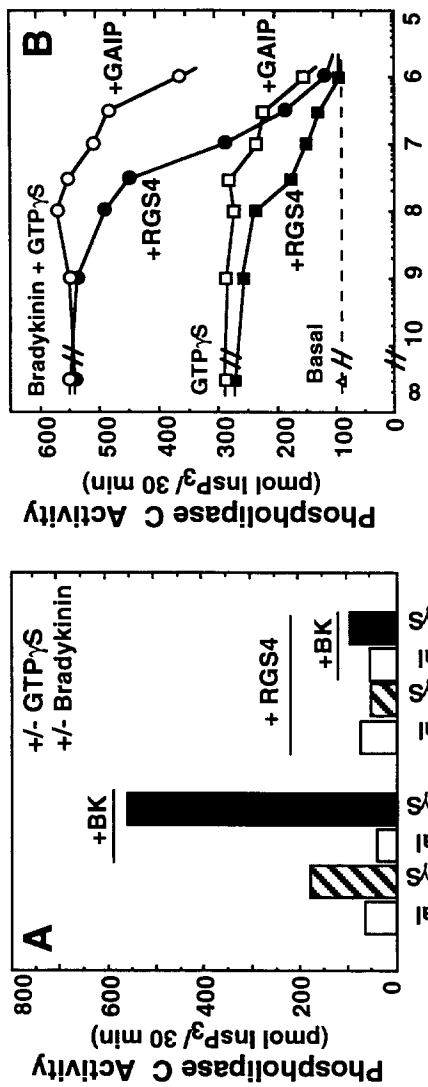


FIG. 3. RGS-directed inhibition of GTPγS and bradykinin-activated synthesis of inositol 1,4,5-trisphosphate by NG-108 cell membranes. (A) Total unwashed NG-108 membranes (5 μg protein/assay) were incubated with 10 μ*M* GDPβ*S* (basal) or 10 μ*M* GDPβ*S* and 3 μ*M* GTPγ*S* in the presence or absence of 1 μ*M* bradykinin (BK) and/or 1 μ*M* RGS4. The resulting accumulation of [$^3$H]inositol 1,4,5-trisphosphate ([$^3$H]Ins$P_3$) over 30 min at 30° was measured. (B) NG-108 cell membranes were incubated as described in (A) in the presence of bradykinin (BK) and GTPγ*S* (circles), or GTPγ*S* (squares), and the concentration of RGS4 (filled symbols) or RGS-GAIP (open symbols) was varied as indicated. Basal PLC-$\beta_1$ activity is indicated (open square). Reproduced with permission from J. R. Hepler, D. M. Berman, A. G. Gilman, and T. Kozasa, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 428 (1997). Copyright 1997 National Academy of Sciences, U.S.A.

measurement of RGS interactions with $G_q\alpha$ in a native cell membrane environment under the control of a linked receptor.

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# [49] Assays of Complex Formation between RGS Protein Gγ Subunit-like Domains and Gβ Subunits

*By* DAVID P. SIDEROVSKI, BRYAN E. SNOW, STEPHEN CHUNG, GREG M. BROTHERS, JOHN SONDEK, and LAURIE BETTS

## Introduction

Regulators of G-protein signaling (RGS) proteins accelerate the hydrolysis of GTP bound to heterotrimeric G protein α subunits[1,2] and are thus considered key negative regulators of G protein-coupled signaling pathways[3] by promoting formation of inactive G-protein heterotrimers (Gα–GDP/Gβ/Gγ complexes). Outside the hallmark RGS box, many RGS proteins have additional structural features that are thought to impart additional functions in signaling regulation.[4] We have identified[5] a subfamily of RGS proteins in which each member possesses both DEP (Dishevelled, EGL-10, Pleckstrin)[6] and GGL (G protein γ subunit-like)[5,7] domains N-terminal to the RGS box. This subfamily not only includes the mammalian RGS proteins RGS6, -7, -9, and -11, as depicted in Fig. 1, but also the *Caenorhabditis elegans* proteins EGL-10[8] and EAT-16[9], and the *Drosophila* RGS7 homolog.[10]

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