

Specificity of G Protein-RGS Protein Recognition Is Regulated by Affinity Adapters

Report

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Summary

RGS proteins regulate the duration of cell signaling by modulating the lifetime of activated G proteins. The specificity of RGS-G protein mutual recognition is critical for meeting unique timing requirements of numerous G protein-mediated pathways. Our study of two splice isoforms of RGS9 expressed in different types of neurons revealed a novel mechanism whereby this specificity is determined by specialized protein domains or subunits acting as affinity adapters. The long RGS9 isoform contains a C-terminal domain that provides high-affinity interaction with its target G protein. The lack of this domain in the short RGS9 isoform is compensated by the action of a G protein effector subunit that is structurally similar to this C-terminal domain. This allows the short isoform to specifically target the complex between the G protein and its effector. Thus, the specific timing needs of different signaling pathways can be accommodated by affinity adapters positioned at various pathway components.

Introduction

RGS proteins are ubiquitous negative regulators of G protein signaling, which act by stimulating the rate of GTP hydrolysis on G protein α subunits (Berman and Gilman, 1998; Burchett, 2000; Ross and Wilkie, 2000; Neubig and Siderovski, 2002; Arshavsky et al., 2002). The abundance of signaling cascades where RGS and G proteins are involved raises the issue of how specificity in their mutual recognition is achieved. Most of the progress in this direction has been gained by establishing the patterns of preferential RGS-G protein interactions in studies that used purified proteins or proteins expressed in cell culture (reviewed in Burchett, 2000; Ross and Wilkie, 2000; Neubig and Siderovski, 2002; Arshavsky et al., 2002). However, this information alone is not sufficient for understanding the specificity of RGS protein action within a specific G protein cascade. In order to ensure that the timing of the entire signaling event provided by a given pathway is physiologically appropriate, RGS proteins may also be required to discriminate between the free activated G protein α subunits and their complexes with effectors or other regulatory proteins (Arshavsky and Pugh, 1998).

An example of a signaling pathway where an RGS protein interacts specifically with the G protein-effector complex is the visual transduction cascade in vertebrate

rods and cones (see Burns and Baylor, 2001; Arshavsky et al., 2002, for recent reviews). In this cascade, photoexcited rhodopsin activates G protein transducin (G_{α_t}), which then stimulates the activity of the effector enzyme, cGMP phosphodiesterase (PDE), by binding to the PDE γ -subunit and releasing the inhibitory constraint that PDE γ imposes on the PDE catalytic subunits. The lifetime of the activated G_{α_t} in this pathway is regulated by the short splice isoform of RGS9, RGS9-1 (He et al., 1998; Chen et al., 2000). Critical to this regulation is the ability of RGS9-1 to interact selectively with G_{α_t} , bound to PDE γ (Tsang et al., 1998), which prevents signal termination before the effector is activated by G_{α_t} . This specificity is achieved through the ability of PDE γ to increase the affinity between RGS9-1 and G_{α_t} by over 20-fold (Skiba et al., 2000), thus targeting RGS9-1 to G_{α_t} .

Alternative splicing of RGS9 yields another isoform, RGS9-2, where 18 C-terminal amino acid residues of RGS9-1 are replaced by a longer sequence of 209 amino acid residues (Granneman et al., 1998; Rahman et al., 1999). RGS9-2 is expressed in the striatal part of the brain (Thomas et al., 1998; Granneman et al., 1998; Rahman et al., 1999), where it is likely to regulate the lifetime of activated G_o and/or G_i proteins, both of which belong to the same G protein subfamily as G_t (Granneman et al., 1998; Rahman et al., 1999; Koo et al., 2000; Hooks et al., 2003). PDE γ is not expressed in the brain, which brings up the question of how RGS9-2 can specifically recognize its target(s) in the absence of PDE γ .

In this study, we demonstrate that the C-terminal domain of RGS9-2 plays a crucial role in setting RGS9-2 G protein recognition specificity. It targets RGS9-2 to its specific G protein α subunit, G_{α_o} , by increasing their mutual binding affinity, exactly as PDE γ does for the binding between RGS9-1 and transducin. Furthermore, the C-terminal domain of RGS9-2 shares a significant degree of overall structural organization and sequence homology with PDE γ . Taken together, these observations reveal a novel mechanism of setting the specificity in RGS protein action. In this mechanism, small protein domains or subunits, such as PDE γ or RGS9-2 C terminus, act as affinity adapters between RGS proteins and their physiological G protein targets. These adapters could be positioned at different components of the signaling pathways to accommodate for their specific timing needs.

Results

Catalytic Properties of RGS9 Splice Isoforms

There is a consensus in the literature that both splice isoforms of RGS9 are suited to work with the members of G_i subfamily of G proteins. Transducin (G_{α_t}) has been established as the physiological target of RGS9-1 in an array of biochemical, physiological, and genetic studies in photoreceptors (He et al., 1998; Chen et al., 2000). The attribution of G_o and/or G_i as specific targets of RGS9-2 has come mostly from studies in cell culture (Granneman et al., 1998; Rahman et al., 1999). Until now,

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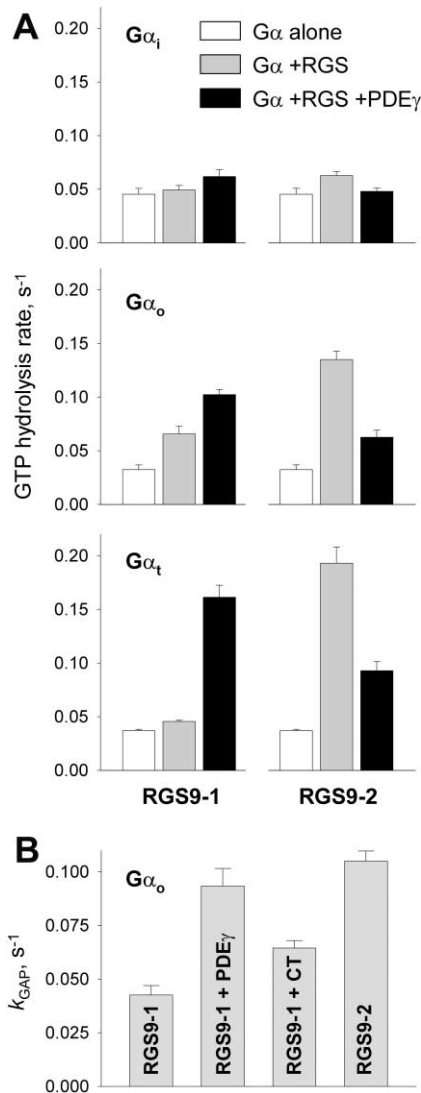


Figure 1. Catalytic Properties of RGS9 Splice Isoforms Determined in the Single Turnover GTPase Assays

(A) The regulation of the GTPase activity of G α class members in the presence or absence of PDE γ .

(B) The C terminus of RGS9-2 potentiates the ability of RGS9 to stimulate the GTPase activity of G α_o , both in *cis* and in *trans*. k_{GAP} is calculated as the difference between the rates of G protein GTPase activity in the presence of RGS9 and the basal GTPase activity of the same G protein (Krumins and Gilman, 2002).

the latter conclusion has not been tested in direct biochemical experiments. Therefore, we conducted a kinetic analysis of G α_t , G α_o , and G α_i GTPase regulation by recombinant RGS9-2 and compared its catalytic properties with those of RGS9-1 (Figure 1A).

In our studies, we used recombinant RGS9 isoforms complexed with their constitutive subunit, the type 5 G protein β subunit, G β_5 (Snow et al., 1998; Makino et al., 1999; Kooor et al., 2000; Witherow et al., 2000). We have found that RGS9-2 stimulates the GTPase activity of G α_o much better than that of G α_t , indicating that G α_o is the most likely physiological partner of RGS9-2. This is consistent with the observation that the striatum is

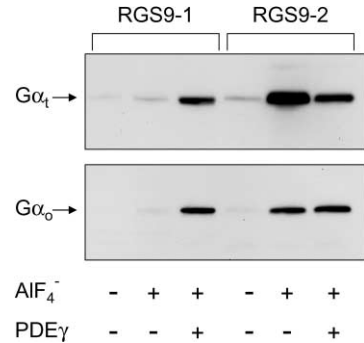


Figure 2. The Binding of G α_t and G α_o to RGS9 Splice Isoforms Attached to the Ni-NTA Agarose Beads

Both α subunits were activated by AlF₄⁻, which mimics the transition conformation for GTP hydrolysis most favorable for binding to RGS proteins (Tesmer et al., 1997). The amounts of G α_t and G α_o retaining on the beads was determined by Western blotting using antibodies specific for each subunit.

enriched with G α_o (Worley et al., 1986). Interestingly, RGS9-2 was also active with G α_t , although these proteins have not been reported to be localized in the same cells (Rahman et al., 1999). With all three G proteins tested, the activity of RGS9-2 was higher than the activity of RGS9-1. PDE γ stimulated the activity of RGS9-1 not only with transducin but also with G α_o and G α_t . In all three cases, the G protein GTPase activity level observed with RGS9-2 alone was approximately the same as the level observed with the combination of RGS9-1 and PDE γ . Although G α_o and G α_t are not expressed in the same cells with PDE γ in vivo, this observation is consistent with biochemical data showing that PDE γ can interact with these G protein α subunits in vitro (Otto-Bruc et al., 1994). In contrast to RGS9-1, the activity of RGS9-2 was always inhibited by PDE γ , likely reflecting the competition between RGS9-2 and PDE γ for binding to G protein α subunits (see below). Taken together, these data indicate that RGS9-2 alone has the same catalytic properties as the combination of RGS9-1 with PDE γ .

C-Terminal Domain of RGS9-2 Is a Functional Homolog of PDE γ

Because the only difference between RGS9-1 and RGS9-2 resides in the composition of their C termini, we conclude that the C-terminal domain of RGS9-2 plays the same functional role in facilitating RGS9-2 interactions with G protein α subunits as PDE γ does for RGS9-1. This conclusion is further supported by the observation that the C terminus of RGS9-2 expressed as an individual protein (CT) was able to enhance the ability of RGS9-1 to stimulate the GTPase activity of G α_o (Figure 1B). The effect of CT was about one half of the effect caused by PDE γ when both proteins were used at saturating concentrations.

Direct evidence that the C-terminal domain of RGS9-2 increases the affinity between RGS9-2 and its G protein α -subunit targets, as PDE γ does for RGS9-1, was obtained in pull-down assays conducted with both RGS9 isoforms (Figure 2). High-affinity binding of RGS9-2 to G α_t or G α_o did not require the presence of PDE γ , whereas any appreciable G α interaction with RGS9-1

was observed only when PDE γ was present (cf. Skiba et al., 1999, 2000, 2001). We also found that an excess of PDE γ decreased G α_t binding to RGS9-2, indicating that PDE γ can compete with RGS9-2 for binding to G α_t . No reliable PDE γ inhibition was observed with G α_o . This is different from the inhibition seen in GTPase assays (Figure 1A) and likely reflects a higher affinity of RGS9-2 for the AIF $_4^-$ -activated G α_o used in pull-down assays than for GTP-activated G α_o used in GTPase assays. This affinity difference results in a more efficient RGS9-2 competition with PDE γ for binding to G α_o in pull-down assays than in GTPase assays. The likely reason why PDE γ still competes with RGS9-2 for G α_t in pull-down assays is that the absolute affinity of PDE γ for G α_t is much higher than its affinity for G α_o (Otto-Bruc et al., 1994). In summary, the results obtained in pull-down assays indicate that PDE γ and the C-terminal domain of RGS9-2 act by increasing the affinity between the RGS9 splice isoforms and their corresponding G protein α subunit partners. For this reason, we call them "affinity adapters."

Additional control experiments were performed in order to prove that functional properties of RGS9-1 are different from those of RGS9-2 because RGS9-1 lacks the PDE γ -like domain and not because it has its own unique C-terminal extension. This was tested with an RGS9 mutant completely lacking its C-terminal sequence beyond the RGS homology domain (DIGR mutant from Skiba et al., 2001). This mutant displayed an ability to cooperate positively with PDE γ and with the C-terminal domain of RGS9-2 upon stimulating the GTPase activity of G α_o (see Supplemental Figure S1 at <http://www.neuron.org/cgi/content/full/38/6/857/DC1>) and G α_t (Skiba et al., 2001). It also cooperated with PDE γ in binding both G α_t and G α_o in pull-down assays (see Supplemental Figure S2).

C-Terminal Domain of RGS9-2 Shares Common Structural Organization with PDE γ

Remarkably, the functional homology between PDE γ and the C-terminal domain of RGS9-2 is paralleled by a significant degree of similarity in their structural organization (Figure 3A). Both of these molecules have strongly positively charged N-terminal regions with an abrupt transition to strongly negatively charged C-terminal sequences. In both, the transition point is located close to the sites of exon boundaries. The cationic regions, although different in length, are both rich in prolines and arginines and contain conserved SH3 recognition motifs. The anionic regions are similar in length and contain a highly homologous sequence of approximately 12 amino acids, which includes a stretch of six identical amino acids.

The structural similarity between PDE γ and the C terminus of RGS9-2 prompted us to assess the relative contributions of cationic and anionic regions from the C terminus of RGS9-2 in regulating the GTPase activity of G α_o . It is well established that both cationic and anionic regions of PDE γ contribute to its high-affinity binding to G α_t (reviewed in Pfister et al., 1993), with several hydrophobic amino acids of the anionic region, including Trp70, being crucial for the ability of PDE γ to facilitate RGS9-1-G α_t interactions (Slepak et al., 1995). We there-

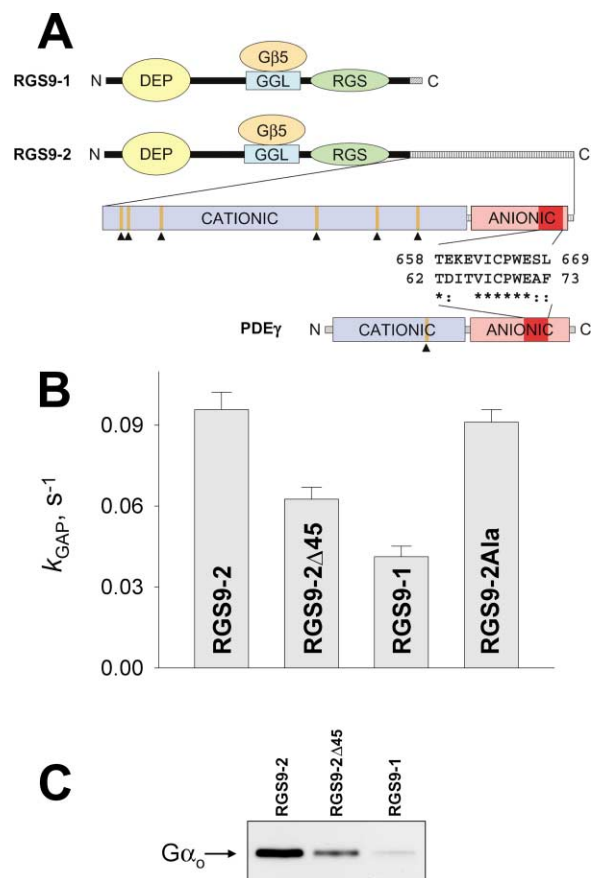


Figure 3. The C-Terminal Domain of RGS9-2 Is a Structural and Functional Analog of PDE γ

(A) Schematic representation of domain compositions of RGS9 splice isoforms and PDE γ . Abbreviations: DEP, Dishevelled/EGL-10/Pleckstrin homology domain; GGL, G protein γ subunit-like domain; RGS, RGS homology domain. SH3 recognition motifs are marked by arrowheads.

(B) The stimulation of G α_o GTPase activity by RGS9 isoforms and mutants in single turnover assays. RGS9-2 Δ 45 is the mutant lacking the entire anionic region of the C terminus; RGS9-2A1a is the mutant where residues 662–667 were replaced by alanines.

(C) The binding of G α_o to RGS9 variants attached to the Ni-NTA agarose beads.

fore compared the catalytic activity of RGS9-2 with the activity of the RGS9-2 mutant in which the anionic region was deleted (RGS9-2 Δ 45) and with RGS9-1, which naturally lacks both regions (Figure 3B). The rate of the G α_o GTPase activity observed with the RGS9-2 Δ 45 mutant was found to be intermediate between the rates observed with the two splice variants of RGS9. The data from pull-down assays shown in Figure 3C indicate that this reduction in activity results from a reduction in the affinity between RGS9-2 Δ 45 and G α_o . The affinity of the RGS9-2 Δ 45 mutant for G α_o in these assays was found to be intermediate between the affinities of the RGS9 splice variants (Figure 3C). Taken together, the data from Figures 3B and 3C indicate that the cationic and anionic regions of the RGS9-2 C terminus make approximately equal contributions to its ability to serve as an affinity adaptor between RGS9-2 and G α_o .

We also tested whether the six amino acid residues

of RGS9-2 (662–667), which are identical to the residues 66–71 of PDE γ , are important for activating G α_o GTPase by RGS9-2. However, the activity of RGS9-2 did not decrease when all six residues were substituted for alanines (Figure 3B). These results indicate that the overall structural organization of the affinity adaptor domains in two different pathways is important for their function, whereas the roles of individual amino acid residues within these domains are not necessarily functionally conserved.

Discussion

An unsolved puzzle in the field of signal transduction is how the physiological timing of signaling events is conferred through the interactions between activated G protein α subunits and their negative regulators, RGS proteins. To solve this problem, it is crucial to understand how the specificity of G protein-RGS protein mutual recognition is achieved on the molecular level. The results obtained in this study indicate that this specificity can be attained by the action of protein domains or subunits serving as affinity adapters between RGS proteins and their G protein α subunit partners.

Remarkably, the positioning of affinity adapters on different molecules in various signaling pathways provides flexibility in accommodating for these pathways' specific functional requirements. Two examples shown in Figure 4 illustrate the consequences of placing these adapters either on the G protein effectors or on the RGS proteins themselves in two different neuronal pathways. Making the affinity adaptor a part of the effector in the visual signaling pathway allows efficient targeting of RGS9-1 to the activated G protein-effector complex (G α_t -PDE), thus providing timely signal termination. Meanwhile, the low affinity between RGS9-1 and free G α_t prevents G α_t discharge before it activates PDE (Figure 4A; see Arshavsky and Pugh, 1998, for a more detailed discussion).

Alternatively, placing the affinity adaptor on an RGS protein, as in the case of RGS9-2, allows specific RGS targeting of free activated G α subunits. This appears beneficial for pathways that utilize G protein $\beta\gamma$ subunits for signal transmission (Clapham and Neer, 1997; Dascal, 1997). In these pathways, RGS proteins determine the duration of signaling events by stimulating GTP hydrolysis on free G α subunits, which is followed by a reassociation of G α -GDP with G $\beta\gamma$ that leads to the termination of physiological response (Arshavsky and Pugh, 1998; Ross and Wilkie, 2000). Figure 4B illustrates the putative striatal pathway where the role of the affinity adaptor is played by the C terminus of RGS9-2. The following evidence makes the existence of such a pathway likely. First, all of its components (G α_o , RGS9-2, and G protein-gated inwardly rectifying K $^+$ [GIRK] channels) are present in striatal neurons (Worley et al., 1986; Greif et al., 1995; Thomas et al., 1998; Granneman et al., 1998; Rahman et al., 1999; Pruss et al., 2003). Second, the $\beta\gamma$ subunits of G α_o /G α_i proteins have been shown to couple the activation of m2 muscarinic, D2 dopamine, and μ -opioid receptors to the modulation of GIRK channels' activity in model systems (see Dascal, 1997, for a review and Granneman et al., 1998; Zhang et al., 2002; and Blanchet

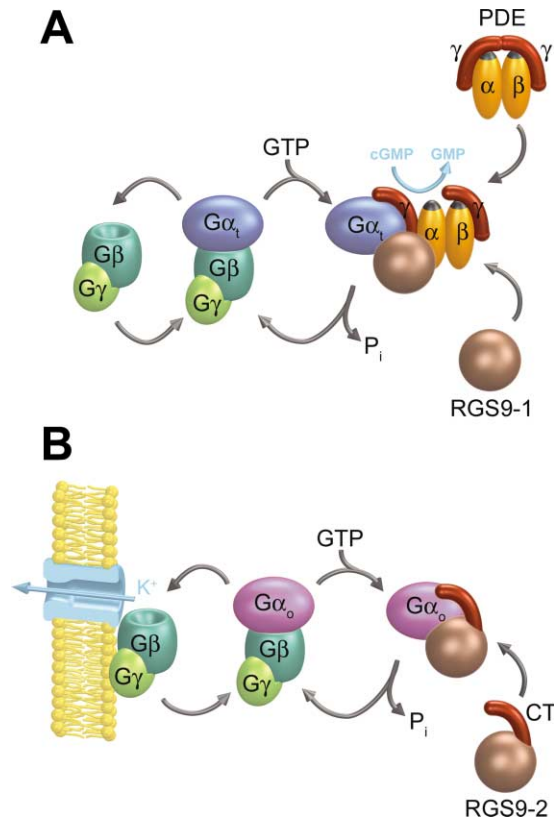


Figure 4. The Role of Affinity Adapters in Targeting RGS9 Splice Isoforms to G Protein α Subunits in Different Neuronal Pathways (A) Visual transduction pathway. (B) Putative pathway of G α_o -dependent control of G protein-gated inwardly rectifying K $^+$ channels in striatum. The affinity adapters are highlighted in dark red (see text for explanations).

and Luscher, 2002, for more recent updates). Third, RGS9-2 accelerates the kinetics of GIRK channels activated by μ -opioid or D2 dopamine receptors (Granneman et al., 1998; Kovoor et al., 2000; Garzon et al., 2001).

Another intriguing aspect of the similarities between PDE γ and the C terminus of RGS9-2 revealed in this study is that these similarities hint at the possibility of a common evolutionary origin of these proteins. This idea is consistent with close localization of RGS9 and rod PDE γ genes on the same chromosome (within loci 17q24-17q25 in the human genome). One could speculate that either the PDE γ gene originated from a duplication of a portion of the RGS9 gene, or alternatively, that the 3' portion of the RGS9 gene originated from a duplication of the PDE γ gene (cf. Meyer, 2003). For example, the visual signaling cascade might have evolved from a duplication of the 3' portion of the RGS9 gene, resulting in the emergence of a PDE γ prototype. This prototype would already be able to mediate G protein-RGS protein interactions and perhaps eventually acquired the ability to regulate the activity of the effector, PDE.

Experimental Procedures

DNA Constructs and Proteins

The coding region of RGS9-2 was amplified by PCR from cDNA containing the mouse RGS9-2 gene (a gift from Dr. S. Gold) using

specific upstream and downstream primers containing BamHI and EcoRI sites, respectively. The PCR product was cloned into a modified version of the baculovirus transfer vector pVL1392 digested at the corresponding restriction sites (Skiba et al., 2001). The resulting construct encoded RGS9-2 preceded by an amino acid sequence containing a His₆-tag and thrombin cleavage site. Site-directed substitutions of RGS9-2 amino acids 662–667 for alanines were introduced by PCR using a BamHI containing the upstream primer and the mutagenic primer 5'-CCCTTCCGCCAGGCTCGCCGCGGCGGC GGCGGCTTCTT-3' followed by reamplification using a 3' flanking primer containing an EcoRI site. Deletion of 45 C-terminal amino acids of RGS9-2 (631–675) was performed by PCR using primer 5'-GATGAATTCAGATCTGGAAAAAGTTGGCTAC-3' also containing an EcoRI site and the upstream BamHI-containing primer. The region encoding the C-terminal part of RGS9-2 (aa 467–675) was amplified by PCR using primer 5'-TGACGGATCCCCAGTCCAGCACTT GGC-3' containing BamHI and the downstream EcoRI primer. The resulting PCR products were treated with BamHI and EcoRI endonucleases and cloned into the pVL1392 plasmid in place of the wild-type RGS9-2 gene. The construction of the pVL1392 plasmids encoding RGS9-1, DIGR mutant of RGS9 (amino acids 1–431), and the splice variants of Gβ5 (Gβ5S and Gβ5L) was described previously (Skiba et al., 2001).

The RGS9 and Gβ5 constructs were used to generate recombinant baculoviruses using the custom service of BD Pharmingen (San Diego, CA). The proteins were expressed in the Sf-9 insect cell line. Cells at the 1.7×10^6 cells/ml density were cotransfected with recombinant baculoviruses carrying the genes for RGS9 and Gβ5 or CT alone and grown for 48 hr post infection. RGS9/Gβ5 protein complexes were purified as described previously (Skiba et al., 2001). RGS9-1 and RGS9-2 were coexpressed with their corresponding physiological partners, Gβ5L and Gβ5S, respectively. Neither RGS isoform could be expressed in soluble form without Gβ5. Control experiments did not reveal any difference in the catalytic properties of RGS9-2 complexed to either Gβ5 isoform.

Urea-treated bovine rod outer segment membranes (uROS) were obtained as described (Nekrasova et al., 1997). The plasmid encoding His₆-Gα_o was a gift from Dr. N.O. Artemyev; the plasmid encoding His₆-Gα_i was a gift from Dr. N.P. Skiba; both recombinant proteins were purified from *E. coli* as described (Lee et al., 1994). Myristoylated Gα_o was purchased from Calbiochem. Transducin and its Gα_i and Gβ₁γ₁ subunits were purified from bovine retinas as described (Ting et al., 1993). Recombinant PDE_γ was obtained as described (Slepek et al., 1995).

GTPase Assays

GTPase activities of G_β, G_α, and G_i were determined using the single turnover technique (Skiba et al., 1999, 2000). Gα subunits (1 μM) were combined with 1 μM β₁γ₁ and incubated at room temperature with bleached uROS containing 20 μM rhodopsin as a source of activated receptor (photoexcited rhodopsin). The reactions were started by combining 20 μl of the G protein/uROS mixture with 10 μl of 0.6 μM [γ -³²P]GTP (~10⁵ dpm/sample), supplemented with 1 μM RGS9-Gβ5 when necessary. PDE_γ in these assays was substituted by 50 μM of its PDE_γ⁶³⁻⁶⁷ peptide previously shown to be equipotent to PDE_γ in regulating transducin GTPase (Skiba et al., 2000). In the experiments addressing the effect of the C terminus of RGS9-2 on G_i and G_α, GTPase activity recombinant CT was used at 3 μM concentration, shown to be saturating for stimulating the GTPase activity of G_o by RGS9-1. The reaction was stopped by the addition of 100 μl of 6% perchloric acid, and the ³²P formation was measured in the supernatant with activated charcoal. Control experiments with each G protein type (see Arshavsky et al., 1994) indicated that the observed GTP hydrolysis rates were not limited by GDP/GTP exchange rates on α subunits. To emphasize the catalytic properties of RGS9, we calculated the parameter of k_{GAP} , defined as the difference between the rates of G protein GTPase activity in the presence of an RGS protein and the basal GTPase activity of the same G protein (Krumins and Gilman, 2002).

Pull-Down Assays

Pull-down assays were conducted as previously described (Skiba et al., 2001) with minor modifications. Ten microliters of Ni-NTA

agarose beads were equilibrated with a binding buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM MgCl₂, 10 μM GDP, 0.25% lauryl sucrose, and 50 μg/ml bovine serum albumin. The beads were then incubated on ice for 20 min with 50 μl of 5 μM His₆-tagged RGS9-Gβ5 complexes and then washed with 500 μl of the binding buffer. The washed beads were resuspended in 50 μl binding buffer containing 250 nM of either Gα_i or Gα_o, 10 mM NaF and 30 μM AlCl₃ (yielding AlF₄⁻) and/or PDE_γ (1 μM final) were added when necessary. The samples were incubated on ice for 10 min with occasional shaking. The beads were spun down and washed twice with 1 ml of the binding buffer supplemented with 20 mM imidazole. NaF, AlCl₃, and PDE_γ were also present in samples initially containing these components. Bound Gα subunits were eluted from the beads with 50 μl SDS-PAGE sample buffer. 10 μl aliquots of the eluates were then subjected to SDS-PAGE. The bound Gα subunits were detected using commercial polyclonal antibodies against each subunit from Santa Cruz Biotechnology.

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