GAIP and RGS4 Are GTPase-Activating Proteins for the G_i Subfamily of G Protein α Subunits

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Summary

A novel class of regulators of G protein signaling (RGS) proteins has been identified recently. Genetic evidence suggests that RGS proteins inhibit G protein–mediated signaling at the level of the receptor–G protein interaction or the G protein α subunit itself. We have found that two RGS family members, GAIP and RGS4, are GTPase-activating proteins (GAPs), accelerating the rate of GTP hydrolysis by $G_{i\alpha1}$ at least 40-fold. All G_i subfamily members assayed were substrates for these GAPs; $G_{s\alpha}$ was not. RGS4 activates the GTPase activity of certain $G_{i\alpha1}$ mutants (e.g., R178C), but not others (e.g., Q204L). The GAP activity of RGS proteins is consistent with their proposed role as negative regulators of G protein–mediated signaling.

Introduction

Heterotrimeric guanine nucleotide–binding regulatory proteins transduce a wide variety of receptor-initiated signals across the plasma membrane in reactions that are controlled by nucleotide-driven conformational changes (Gilman, 1987; Kaziro et al., 1991; Neer and Smith, 1996; Hamm and Gilchrist, 1996). Two particularly crucial events in these schemes are, first, the receptor-catalyzed exchange of GDP for GTP, which activates the pathway by promoting dissociation of the GTP–bound α subunit of the G protein from a complex of its β and γ subunits; and second, inactivation by hydrolysis of bound GTP to GDP by the α subunit. Although the intrinsic rate of hydrolysis of GTP by α is slow (k_{cat} is usually 2–5/min), this reaction is fast compared with the basal (uncatalyzed) rate of nucleotide exchange, and the predominant form of the G protein in the absence of an appropriate agonist is thus the inactive GDP–bound form. An agonist–receptor complex shifts the balance to the GTP–bound form of α by accelerating nucleotide exchange.

In addition to their intrinsic GTPase activity, two extrinsic control mechanisms suppress downstream signaling by various GTP–binding proteins. One group of regulators, typified among heterotrimeric G proteins by the $\beta\gamma$ subunit complex, inhibits nucleotide exchange; these proteins are termed guanine nucleotide dissociation inhibitors. The second group of extrinsic regulators are GTPase-activating proteins (GAPs). By accelerating the rate of the intrinsic GTPase reaction, a GAP shortens the time that α spends in the active GTP–bound conformation and thus presumably inhibits signal transduction.

GAPs were originally identified for protein synthesis elongation factors such as EF-Tu (Kaziro, 1978) and for low molecular mass monomeric GTPases such as ras (Trahey and McCormick, 1987; McCormick, 1989) and Rho (Garrett et al., 1991). Although GAPs for heterotrimeric G protein α subunits have been identified, they have not been as forthcoming (Ross, 1995). The best characterized G protein GAP is phospholipase C-β, which is both a GAP for $G_{q\alpha}$ and the effector that is activated by that G protein (Berstein et al., 1992; Biddlecome et al., 1996). The GTPase activity of the α subunit of transducin is also stimulated by its effector, the γ subunit of a retinal cyclic GMP–specific phosphodiesterase (Arshavsky and Bownds, 1992), as well as by a second unidentified protein (Angleson and Wensel, 1993; Pages et al., 1993; Arshavsky et al., 1994).

A novel family of putative regulators of G protein signaling (RGS) has been identified recently, predominantly by genetic techniques (Roush, 1996). Evidence suggests that these proteins suppress G protein signaling by actions at the level of the α subunit or the interaction between receptor and G protein. Notably, mutations in the *SST2* (supersensitivity to pheromone) gene in Saccharomyces cerevisiae result in unchecked G protein–mediated signaling in response to pheromone (Chan and Otte, 1982a, 1982b; Dietzel and Kurjan, 1987; Weiner et al., 1993). Dominant gain-of-function *SST2* mutations block pheromone signaling but do not suppress events initiated by overexpression of $\beta\gamma$ (the downstream signaling component in this system), emphasizing the negative function of Sst2p and its presumed site of interaction with receptor, the G protein heterotrimer, or the α subunit (Dohlman et al., 1995). Koelle and Horvitz (1996) note that the *egl-10* gene in Caenorhabditis elegans encodes a protein similar to Sst2p and that this gene product opposes signaling induced by activation of the G protein GOA-1. A total of 15 mammalian genes have been identified that are similar to *SST2* and *egl-10*, in that they share a conserved newly identified and named RGS domain. Koelle and Horvitz describe partial sequences of nine new RGS proteins by polymerase chain reaction (PCR) amplification of rat brain cDNA (Koelle and Horvitz, 1996). RGS genes (2) had been cloned previously: RGS1 (BL34) in activated B lymphocytes (Newton et al., 1993; Hong et al., 1993) and RGS2 (GOS8) in monocytes (Siderovski et al., 1994). An additional two genes, RGS13 and RGS14, have been identified as expressed sequence tags (Druey et al., 1996). Finally, De Vries et al. (1995) have detected a protein that they term GAIP (G Alpha Interacting Protein) by yeast two-hybrid screening, using $G_{i\alpha3}$ as bait. This protein appears to interact strongly with $G_{i\alpha3}$, weakly with $G_{i\alpha2}$, and not at all with $G_{q\alpha}$. These authors also note the relationship between GAIP, Sstp2, and other RGS proteins.

We have initiated investigation of the mechanism of action of this family of apparent negative regulators of G protein–mediated signaling by biochemical reconstitution in vitro, where the relevant protein–protein interactions can be studied in detail. We have expressed two proteins to date, GAIP and RGS4. The latter protein

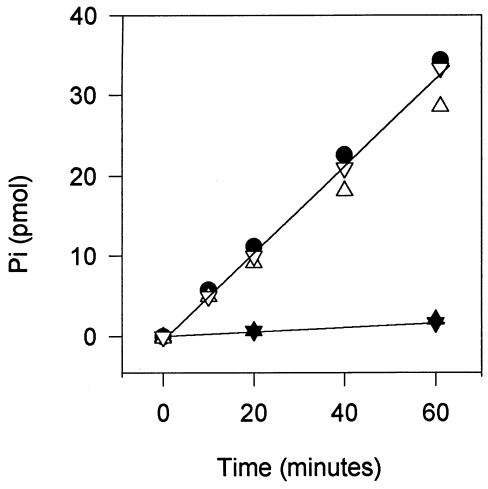

Figure 1. RGS4 and GAIP Do Not Affect Steady-State Hydrolysis of GTP by $G_{i\alpha1}$

$G_{i\alpha1}$ alone (250 nM; closed circle) or $G_{i\alpha1}$ with either 2.2 μM GAIP (open triangle) or 1.5 μM RGS4 (inverted open triangle) were incubated at 30°C with 6 mM $MgSO_4$ and 5 μM [γ-^{32}P]GTP in 50 mM NaHEPES (pH 8.0), 1 mM EDTA, 2 mM dithiothreitol, and 0.05% C12E10. GAIP (2.2 μM; closed triangle) or RGS4 (1.5 μM; inverted closed triangle) were also incubated as described in the absence of $G_{i\alpha1}$. Duplicate aliquots were withdrawn at the indicated times and transferred to the charcoal-quenching solution. Zero-time values were subtracted from all experimental points. The results represent averages of similar duplicates and are representative of three experiments.

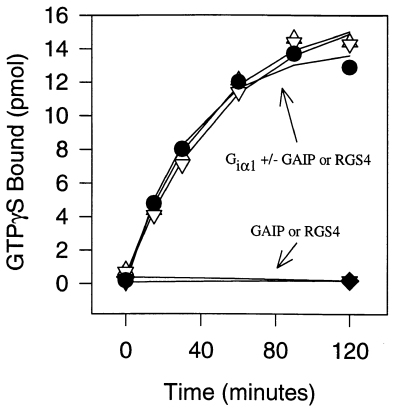

Figure 2. RGS4 and GAIP Do Not Affect the Time Course of GTPγS Binding to $G_{i\alpha1}$

Incubation conditions were identical to those in Figure 1, except for the substitution of 5 μM [^{35}S]GTPγS for GTP. At the indicated times, duplicate aliquots were withdrawn and filtered. The results represent averages of similar duplicates and are representative of two experiments. Reaction mixtures contained the following proteins: $G_{i\alpha1}$ (closed circle), $G_{i\alpha1}$ plus RGS4 (inverted open triangle), $G_{i\alpha1}$ plus GAIP (open triangle), RGS4 (inverted closed triangle), GAIP (closed triangle).

has been identified in a screen of rat brain cDNAs whose expression suppresses the phenotype associated with deletion of *SST2* in yeast (Druey et al., 1996). RGS4 transcripts were found only in brain. Our data indicate that both GAIP and RGS4 are potent GAPs for members of the $G_{i\alpha}$ subfamily of G protein α subunits.

Results

Expression of GAIP and RGS4

GAIP and RGS4 were both expressed in Escherichia coli as full-length proteins with hexa-histidine tags at their amino-termini. GAIP was purified by Ni-NTA and Mono Q column chromatography, while RGS4 was purified by Ni-NTA and hydroxylapatite column chromatography. The yield of GAIP was approximately 0.5 mg/l of bacterial culture, while that of RGS4 was 2 mg/l of culture. Both proteins were approximately 80% pure, as judged by Coomassie brilliant blue staining of sodium dodecylsulfate polyacrylamide gels (data not shown). Both preparations were devoid of detectable GTPγS binding activity and GTPase activity. Results shown below have recently been verified with homogenous preparations of RGS4.

RGS Proteins Do Not Affect Dissociation of GDP

The steady-state rate of hydrolysis of GTP by G protein α subunits is not limited by the catalytic step but rather by the rate of dissociation of product, GDP. Thus, steady-state GTPase assays measure the rate of GDP dissociation. The rate of GTP hydrolysis by 250 nM $G_{i\alpha1}$ was 0.04/min in the absence or presence of either 1.5 μM RGS4 or 2.2 μM GAIP (Figure 1). Similarly, the rate of pseudoirreversible binding of [^{35}S]GTPγS to $G_{i\alpha1}$ is also limited by dissociation of GDP, and this rate (0.03/min) was also unaffected by GAIP or RGS4 (Figure 2). Thus, there is no evidence that RGS proteins function as guanine nucleotide dissociation inhibitors, at least with the G_i subfamily of α subunits. As mentioned above, this role is played by the βγ subunit complex.

GAIP and RGS4 Accelerate GTP Hydrolysis by $G_{i\alpha1}$

The intrinsic rate of hydrolysis of GTP by G protein α subunits must be measured under conditions where dissociation of GDP is not rate limiting. This can be accomplished by reconstitution of G protein heterotrimers with receptors (to facilitate GDP dissociation) or by first binding GTP to the α subunit under conditions in which the nucleotide cannot be hydrolyzed; one round of hydrolysis of the prebound nucleotide can then be initiated. Binding of GTP to G_α proteins is not dependent on Mg^{2+}, while hydrolysis of the nucleotide requires nanomolar concentrations of the divalent cation. [γ^{32}P]GTP was thus bound to $G_{i\alpha1}$ in the presence of 5 mM EDTA, and the release of $^{32}P_i$ was monitored after addition of Mg^{2+} to initiate catalysis and unlabeled GTP to prevent rebinding of labeled substrate. Since only one round of hydrolysis is observed, relatively high concentrations of protein are required. The reaction mixtures thus contained 250 nM $G_{i\alpha1}$ and a 5- to 10-fold excess of either GAIP (Figure 3A) or RGS4 (Figure 3B). In control reactions, which contained equal amounts of boiled GAIP or RGS4, the k_{cat} for hydrolysis of GTP was 2/min, similar to the published value for $G_{i\alpha1}$ alone (Linder et al., 1990). In the presence of either RGS protein, the reaction was complete at the earliest timepoint (7 s), precluding estimation of the magnitude of stimulation of the GTPase reaction. Incubation of RGS4 with a 2-fold molar excess of the G protein $\beta_1\gamma_2$ subunit (3 μM final concentration) had no effect (data not shown). Control extracts of E.

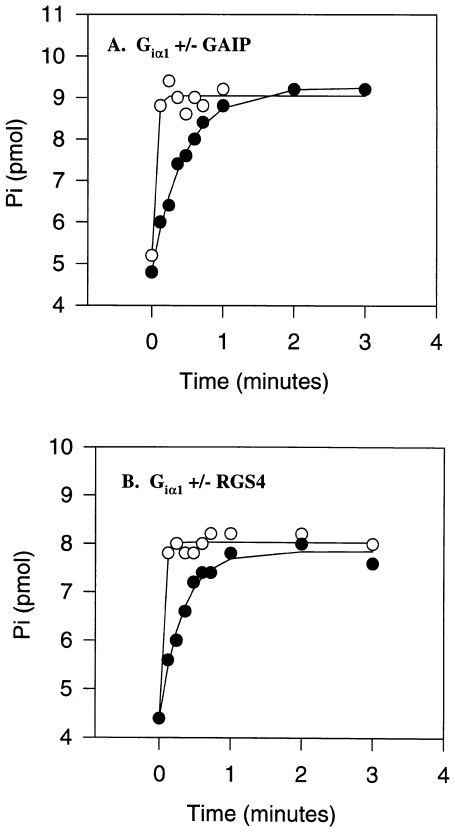

Figure 3. RGS Proteins Accelerate the Hydrolysis of GTP by $G_{i\alpha1}$

[γ-^{32}P]GTP (1 μM) was allowed to bind to $G_{i\alpha1}$ for 15 min at 30°C in the presence of 5 mM EDTA. The temperature was then lowered to 20°C. A solution containing Mg^{2+} and the indicated RGS protein was added to initiate GTP hydrolysis, and aliquots were taken at the indicated times.

(A) Incubation contained 250 nM $G_{i\alpha1}$ plus 2.2 μM GAIP (open circle) or $G_{i\alpha1}$ plus boiled GAIP (closed circle).

(B) Incubations contained 250 nM $G_{i\alpha1}$ plus 1.5 μM RGS4 (open circle) or $G_{i\alpha1}$ plus boiled RGS4 (closed circle). The basic experiment has been replicated on multiple occasions.

coli did not accelerate GTP hydrolysis by $G_{i\alpha1}$ (data not shown).

Effect of Mg^{2+} and Temperature

As mentioned, Mg^{2+} is required for GTP hydrolysis (Higashijima et al., 1987). By binding to a defined site on the protein, the divalent cation coordinates the β and γ phosphates of the nucleotide and stabilizes the hydrolytic transition state (Coleman et al., 1994; Sondek et al., 1994). We were thus surprised to find that addition of RGS4 to the GTP–$G_{i\alpha1}$ complex permitted rapid nucleotide hydrolysis in the presence of 5 mM EDTA (Figure 4A). Again, hydrolysis was complete at the first timepoint examined. The control reaction contained boiled RGS4 protein, eliminating the explanation that Mg^{2+} contaminates the RGS4 preparation. In the presence of the RGS GAP, the GTPase reaction may be independent of Mg^{2+}; more likely, the affinity of the GTP–$G_{i\alpha1}$–RGS4 complex for Mg^{2+} is extremely high (see below).

In an attempt to slow the rate of the GAP–stimulated GTPase reaction to permit more precise quantitation, reactions were performed at 4°C (Figure 4B). Although the control rate for the GTPase reaction was reduced to 0.35/min, the GAP–stimulated reaction was again complete after 7 s. Based on these data, the lower limit for the magnitude of acceleration of the GTPase reaction by RGS4 is 40-fold. By comparison, Biddlecome et al. (1996) found that phospholipase C-β1 accelerates the rate of hydrolysis of GTP by $G_{q\alpha}$ by at least 90-fold.

RGS Proteins Are GAPs for $G_{i\alpha}$ Family Members But Not $G_{s\alpha}$

The functional specificity of interaction of RGS proteins with G_α proteins is a question of obvious importance. The 20 known G protein α subunits are generally organized in four subfamilies, based on their amino acid sequences and functional properties (Simon et al., 1991): G_s, G_i, G_q, and G_{12}. The effects of both GAIP and RGS4 on five G protein α subunits are shown in Figure 5. Both RGS proteins stimulated hydrolysis of GTP by all four members of the G_i subfamily tested, $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha}$ (Figures 5A–5D). Despite the fact that these reactions were performed at 4°C, they were all complete by 7 s, precluding quantitative comparison of the relative effects of the GAPs within this group. All of the $G_{i\alpha}$ proteins tested in this experiment were myristoylated at their amino-termini, and we were thus unable to distinguish between effects of RGS4 or GAIP on myristoylated and nonmyristoylated $G_{i\alpha1}$ (comparison with Figure 3). RGS4 also appeared to stimulate the GTPase activity of $G_{z\alpha}$ (another G_i subfamily member), although this protein is very difficult to load with GTP and the signal-to-noise ratio was thus very low (data not shown). However, neither RGS4 nor GAIP influenced the GTPase activity of

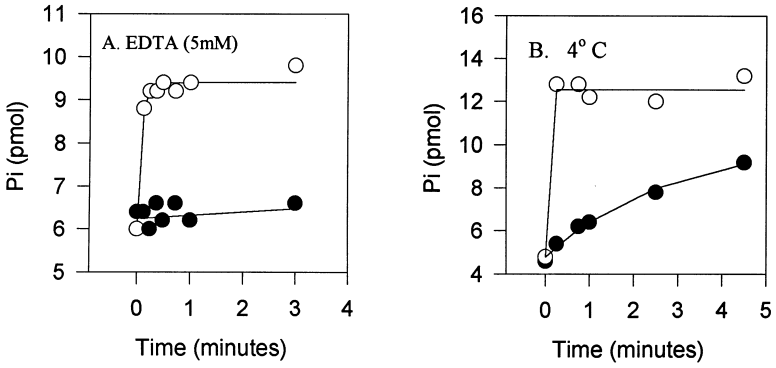

Figure 4. Effect of Mg^{2+} and Temperature on the GAP Activity of RGS4

(A) Measurement of k_{cat} in the presence of 5 mM EDTA. The conditions were the same as those in Figure 3, except that Mg^{2+} was not added at zero time. Incubations contained 250 nM $G_{i\alpha1}$ plus 1.5 μM RGS4 (open circle) or $G_{i\alpha1}$ plus boiled RGS4 (closed circle). This experiment has been replicated three times.

(B) Measurement of k_{cat} at 4°C. The conditions were the same as those in Figure 3, except that GTP hydrolysis was initiated after lowering the temperature to 4°C. Incubations contained 250 nM $G_{i\alpha1}$ plus 1.5 μM RGS4 (open circle) or $G_{i\alpha1}$ plus boiled RGS4 (closed circle). This experiment has been replicated many times; see also Figure 5.

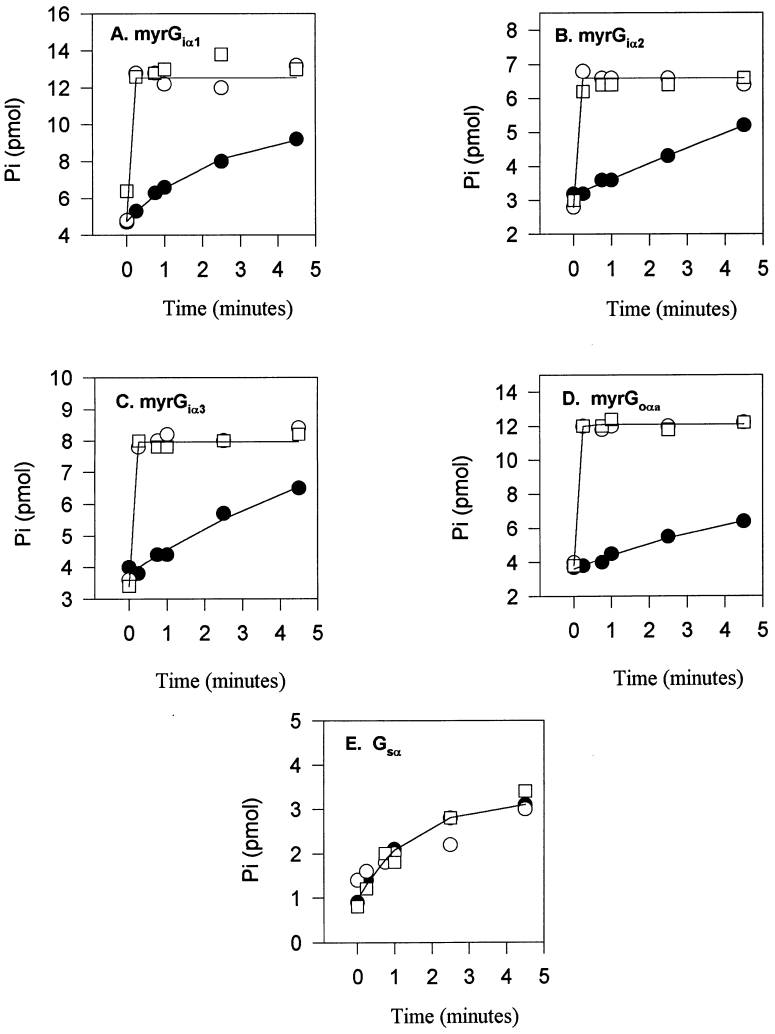

Figure 5. RGS Proteins Are GAPs for G_i Subfamily Members But Not G_s

The conditions were the same as those in Figure 3, except that all k_{cat} measurements were performed at 4°C. In each panel, incubations contain the indicated G_α protein (250 nM) alone (closed circle), the indicated G_α protein (250 nM) plus 2.2 μM GAIP (open square), or the indicated G_α protein (250 nM) plus 1.5 μM RGS4 (open circle). The G_i subfamily proteins were all myristoylated. Experiments with $G_{i\alpha1}$, $G_{o\alpha}$, and $G_{s\alpha}$ have been replicated many times; those with $G_{i\alpha2}$ and $G_{i\alpha3}$ have been replicated twice.

$G_{s\alpha}$, demonstrating specificity among subfamilies of G protein α subunits (Figure 5E). Technical difficulties precluded performance of the type of experiment shown here with $G_{q\alpha}$ or $G_{12\alpha}$, since binding of GTP is difficult to achieve in the absence of a receptor. These proteins will probably have to be reconstituted in vitro with appropriate receptors in phospholipoid vesicles to examine their interactions with RGS4, GAIP, and other members of the RGS family. More quantitative estimation of the affinities or efficacies of interactions between $G_{i\alpha}$ family members and the RGS GAPs will require detailed kinetic analysis, which is in progress. However, it is obvious that the affinity of RGS4 and GAIP for these $G_{i\alpha}$ proteins is relatively high.

The Effect of RGS4 on $G_{i\alpha1}$ Mutants

To approach the mechanism of action of RGS4 and GAIP, we tested their effects on $G_{i\alpha1}$ proteins with missense mutations in residues involved in guanine nucleotide hydrolysis (Arg^{178} and Gln^{204}) and Mg^{2+} binding (Ser^{47}; see Coleman et al., 1994). Mutation of Arg^{178} of $G_{i\alpha1}$ to Cys or of Gln^{204} to Leu abolishes GTP hydrolysis (Graziano and Gilman, 1989). Arg^{178} stabilizes negative charge on the phosphate leaving group in the transition state of the hydrolytic reaction complex, while Gln^{204} stabilizes the same transition state and orients the attacking water molecule (Coleman et al., 1994; Sondek et al., 1994). Mutation of the corresponding Gln residue in ras is oncogenic, as is mutation of either of these cognate residues in pituitary $G_{s\alpha}$ (Der et al., 1986; Landis et al., 1989). This Arg residue in $G_{s\alpha}$ and $G_{t\alpha}$ is also the site of ADP-ribosylation by cholera toxin, a modification that inhibits GTPase activity (Cassel and Selinger, 1977; Van Dop et al., 1984).

Of interest, the defunct GTPase activity of R178C $G_{i\alpha1}$ was partially restored by RGS4 (Figure 6A), although the rate achieved (2/min) only approximated that of the wild-type protein in the absence of the GAP. Intermediate rates of catalysis were observed in the presence of lower concentrations of RGS4 (data not shown). However, we cannot yet determine if the affinity of RGS4 for R178C $G_{i\alpha1}$ is reduced or if the maximal value of k_{cat} is lowered. There was no hydrolysis of GTP by Q204L $G_{i\alpha1}$ in the presence or absence of RGS4 (Figure 6B).

The S47N (or equivalent) mutants of G protein α subunits have been hypothesized to act as dominant negatives (Slepak et al., 1993), as does the corresponding mutation in ras (Feig and Cooper, 1988). This Ser residue

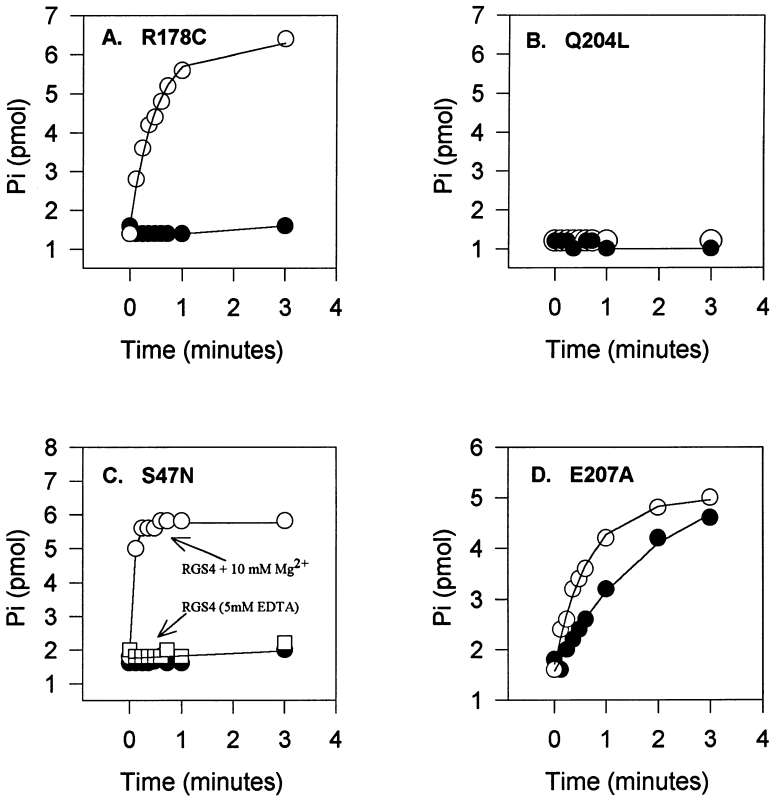

Figure 6. The Effect of RGS4 on $G_{i\alpha1}$ Mutants

The indicated mutant $G_{i\alpha1}$ proteins (150–200 nM) were incubated as described in the legend to Figure 3. In each panel, GTP hydrolysis was monitored in the presence of the mutant α subunit alone (closed circle) or with 1.5 μM RGS4 (open circle). In (C), an additional reaction was performed without added Mg^{2+} (open square). These experiments have been replicated three times.

provides one of the major ligands for Mg^{2+} binding, and missense mutation of this residue lowers the apparent affinity of GTP–$G_{i\alpha1}$ for Mg^{2+} by 100,000-fold, to an appoximate apparent K_d of 1 mM (D. M. B. and A. G. G., unpublished data). At cellular Mg^{2+} concentrations of 100–500 μM, the mutated Mg^{2+} binding site is not saturated, preventing the conformational changes required for dissociation of α from $\beta\gamma$ (Lee et al., 1992). We have estimated the k_{cat} for hydrolysis of GTP by S47N $G_{i\alpha1}$ to be at least 0.1/min in the presence of 10 mM Mg^{2+}.

If an RGS GAP obviates the requirement for Mg^{2+} for the GTPase reaction, we hypothesize that S47N $G_{i\alpha1}$ would hydrolyze GTP in the presence of RGS4 and 5 mM EDTA, as did the wild-type protein (see Figure 4A). Although the effect of RGS4 on GTP hydrolysis by S47N $G_{i\alpha1}$ was marked in the presence of 10 mM Mg^{2+}, this GAP–stimulated GTPase reaction was eliminated by 5 mM EDTA (Figure 6C; compare with Figure 4A). We thus suggest that Mg^{2+} is still required for the GTPase activity of wild-type $G_{i\alpha1}$ in the presence of an RGS protein and that the affinity of the GAP–GTPase complex for Mg^{2+} is extraordinarily high.

Glu^{207} or its equivalent residue is conserved in all G protein α subunits. Because of its proximity to the hydrolytic water molecule in the G_α crystal structure, Noel et al. (1993) have suggested that this residue might act as a general base, facilitating deprotonation and increasing nucleophilicity of the water molecule. However, Kleuss et al. (1994) demonstrate that E207A $G_{i\alpha1}$ hydrolyzes GTP relatively normally, ruling out participation of Glu^{207} in the unstimulated GTPase reaction. Surprisingly, 1.5 μM RGS4 had only a modest effect on the GTPase activity of E207A $G_{i\alpha1}$, increasing k_{cat} from 0.5/min to approximately 1.5/min (Figure 6D). However, hydrolysis was nearly complete by 7 s, when the concentration of RGS4 was raised to 15 μM (data not shown). Again, unfortunately, we cannot ascertain if RGS4 has a reduced affinity for E207A $G_{i\alpha1}$ or if its maximal effect on GTPase activity is reduced.

Discussion

Genetic evidence from fungi and nematodes has led to the hypothesis that RGS proteins inhibit heterotrimeric G protein–based signaling by disrupting the receptor–G protein interaction or by some action at the level of the G protein α subunit itself. Additional observations that GAIP interacts physically with $G_{i\alpha3}$ (De Vries et al., 1995) and that transfected RGS proteins can interfere with G protein–mediated signaling (Dohlman et al., 1995; Druey et al., 1996) buttress this hypothesis. We have thus sought the biochemical mechanism of this effect and have discovered that both GAIP and RGS4 accelerate the rate of hydrolysis of GTP by $G_{i\alpha1}$ by at least 40-fold. All members of the G_i subfamily of G protein α subunits tested are substrates for both GAPs; $G_{s\alpha}$ is a substrate for neither.

RGS4 and GAIP are 36% identical (54% similar); within the confines of the RGS domain, they are 44% identical (62% similar). Comparison of the RGS domains of the 15 mammalian genes identified to date indicates that GAIP and RGS4 are not among the most closely related. What, then, are we to make of the fact of their similar (or indistinguishable) substrate specificity? There are

several possible explanations. First, the assay that we have employed to date has not permitted careful examination of the affinities of GAIP or RGS4 for its substrates. Differences among these proteins may yet emerge when the kinetics of GTP hydrolysis are examined in more detail. Second, the apparent lack of specificity for members of the G_i subfamily may be an in vitro artifact. Similar problems have been encountered in examining the specificity of GAPs for the low molecular mass GTPase, Rho. Although Rho GAP stimulates the GTPase activity of both Rho and Rac in vitro, the protein appears specific for Rho in vivo (Lancaster et al., 1994; Ridley et al., 1993). Other proteins may interact with either the GAP or its substrate to impose greater specificity in vivo. Third, it is possible that all of the identified RGS proteins may, in fact, be GAPs for the entire G_i subfamily of α subunits. This hypothesis implies an extraordinary level of complexity, at which differential cellular expression and additional determinants of specificity, imposed by such factors as transcription regulation and posttranslational modification, will presumably come into play.

The mechanism of stimulation of the GTPase activity of G protein α subunits will also be of great interest. The effects of RGS proteins on the $G_{i\alpha1}$ mutants described above provide interesting clues. RGS4 restores much of the hydrolytic capacity that is lost by mutation of Arg^{178}, a residue that stabilizes negative charge on the γ phosphate leaving group (Coleman et al., 1994; Sondek et al., 1994). Similarly, ras, which lacks a residue that corresponds to Arg^{178}, has an exceedingly slow basal rate of GTP hydrolysis, but this rate is accelerated greatly by ras GAP (Trahey and McCormick, 1987). Perhaps both ras GAP and RGS proteins provide, in some way, additional stabilization for the transition state of hydrolysis. Transition-state stabilization may also account for the increased affinity for Mg^{2+} that is implied by the data of Figure 4A, coupled with the effect of RGS4 and EDTA on the GTPase activity of S47N $G_{i\alpha1}$ (Figure 6C).

RGS4 does not stimulate GTP hydrolysis by the Q204L mutant of $G_{i\alpha1}$; similarly, the Q61L mutant of ras is unaffected by ras GAP, even though the mutant ras has a high affinity for its GAP (Adari et al., 1988; Gideon et al., 1992). The function of the Gln residue, which appears essential for orienting and polarizing the hydrolytic water, thus appears indispensible. However, it is possible that mutation at this site prevents remodeling of the catalytic domains associated with the actions of the respective GAPs. Of interest, the GTPase activity of the corresponding mutant of $G_{q\alpha}$ (Q209L), while severely compromised, is enhanced roughly 20-fold by phospholipase C-β1 (J. Hepler, G. Biddlecome, E. M. Ross, and A. G. G., unpublished data). Thus, the mechanism of stimulation of GTPase activity by RGS proteins and phospholipase C-β may differ significantly.

As mentioned above, Glu^{207} in $G_{i\alpha1}$ is conserved in all G protein α subunits. Although it is not required for hydrolysis of GTP (Kleuss et al., 1994), its mutation dramatically impairs the activity of RGS4. We are tempted to speculate that the RGS protein causes remodeling of the active site, switching GTP hydrolysis by α from a "Gln mechanism," for orientation and polarization of the hydrolytic water, to a "Glu mechanism," where a general base is used to extract a proton from the hydrolytic water. While the results of mutation of Glu^{207} are consistent with this hypothesis, the lack of effect of RGS4 on Q204L $G_{i\alpha1}$ is not, unless the Q204L mutation is imposing structural rather than chemical restraints on the activity of RGS4. Alternatively, Glu^{207} may form an essential part of the binding site for RGS proteins. The crystal structure of $G_{i\alpha1}$ has been solved in various conformations. Cocrystallization of RGS4 and $G_{i\alpha1}$ is an obvious goal.

Experimental Procedures

Cloning of GAIP and RGS4

cDNA was synthesized from normal human lung polyA+ mRNA (Clontech) and was used to amplify a full-length cDNA encoding GAIP, using PCR. The first strand cDNA synthesis was completed using the Superscript system (Gibco/BRL). The PCR protocol was modified from that of Wilkie and Simon (1991). Incubations for PCR were at 94°C for 1 min, 58°C for 1 min, and 72°C for 2.5 min for 30 cycles, followed by a 5 min incubation at 72°C; dimethyl sulfoxide (5%) was included because of the high T_m of the GAIP cDNA. The sense PCR primer began with an NcoI site, followed by a glycine and six histidine (underlined) codons: 5′-AATAATCCATGGGACACC ATCACCATCACCACGGCATGCCCACCCCGCATGAGG-3′. The antisense PCR primer was 5′-GAGTAGCGAAGCTTGGGGTCTGTGCTG CTGGGGGCGGCCTA-3′. The PCR product was cloned into the NcoI and HindIII sites of the plasmid pQE60 (Qiagen). A cDNA encoding RGS4 was cloned in a similar manner, starting with rat brain mRNA. The sense PCR primer began with an NcoI site, followed by a glycine and six histidine (underlined) codons: 5′-CCGCCATGGGACACCAT CACCATCACCACGGCATGTGCAAAGGACTCGCTGG-3′. The antisense PCR primer was 5′-CCGGGATCCTTGGCATTT-CGGTTCTCT GCC-3′. The PCR product was cloned into the NcoI and BamHI sites of plasmid pQE60. The DNA sequences for both GAIP and RGS4 were identical to those published (Koelle and Horvitz, 1996; De Vries et al., 1995; Druey et al., 1996).

Purification of Recombinant Proteins

G protein α subunits were expressed and purified as described elsewhere (Lee et al., 1994). Unless specifically stated, experiments with $G_{i\alpha1}$ and its mutants were performed with nonmyristoylated protein. $G_{o\alpha}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ were myristoylated. Vectors encoding amino-terminal hexahistidine-tagged RGS proteins were transformed into E. coli strain JM109. An overnight culture of bacteria (10 ml) containing the appropriate plasmid was diluted into 1 l of enriched medium as described by Lee et al. (1994). At $OD_{600} = 0.55$, 10 μM IPTG was added, and incubation was continued for 3.5 hr at 37°C (for RGS4) or for 3 hr at 30°C (for GAIP). The cells were pelleted, flash-frozen, and lysed by thawing in 30 ml of 50 mM Tris–HCl (pH 8.0), 20 mM β-mercaptoethanol, and 100 μM phenylmethylsulfonylfluoride (TBP). Lysozyme (6 mg) was added to complete cell lysis. The lysates were clarified by centrifugation and then applied to 1 ml Ni-NTA (Qiagen) columns, equilibrated with TBP. Columns were washed with 20–30 ml of TBP containing 100 mM NaCl, followed by a second wash with 5 ml of TBP containing 5 mM imidazole. The protein was then eluted with 7 ml of TBP containing 150 mM imidazole.

The eluate containing GAIP was diluted into 35 ml of 50 mM Tris–HCl (pH 8.0) and 2 mM dithiothreitol (TD) and injected onto a 1 ml fast protein liquid chromatography Mono Q anion exchange column (Pharmacia). The column was washed with 20 ml of TD and eluted with a continuous 20 ml gradient of TD containing NaCl (0–500 mM). The eluate containing RGS4 was also diluted into 30 ml of TD and injected onto a 5 ml fast protein liquid chromatography column of Macroprep ceramic hydroxylapatite (BioRad). This column was washed with 35 ml of TD, and the protein was eluted with a continuous gradient of potassium phosphate (pH 7.0; 0–1 M). Column fractions were analyzed by polyacrylamide gel electrophoresis, and peak fractions containing RGS proteins were exchanged into 50 mM NaHEPES (pH 8.0), 1 mM EDTA, and 2 mM dithiothreitol.

Nucleotide Binding and Hydrolysis

All assays were performed in buffer containing 50 mM NaHEPES (pH 8.0), 2 mM dithiothreitol, 0.05% C12E10, and either 1mM EDTA (GTPγS binding and steady-state GTPase assays) or 5 mM EDTA (k_{cat} measurements). Temperatures, protein concentrations, and other additions are described in the figure legends. GTPγS binding was quantified as described by Sternweis and Robishaw (1984). Steady-state GTPase assays were performed as described previously (Higashijima et al., 1987). Measurements of k_{cat} for hydrolysis of GTP were modified from the procedure of Linder et al. (1990). Briefly, G protein α subunits were loaded with [γ-^{32}P]GTP (1 μM) in the presence of 5 mM EDTA for 15–30 min at 30°C; the temperature was then lowered to 20°C or 4°C (as indicated) for 5 min. Before initiation (15 s) of GTP hydrolysis, a 50 μl aliquot of the reaction mixture was removed and added to 750 μl of 5% (w/v) Norit in 50 mM NaH_2PO_4; this was the zero timepoint. The reaction mixture was then added to a tube containing unlabeled GTP (150 μM final concentration), $MgSO_4$ (15 mM total final concentration), and the RGS protein or buffer. Aliquots (50 μl) were removed at the indicated times and processed as described above. After centrifugation at 1500 rpm for 5 min, 400 μl aliquots of supernatant containing $^{32}P_i$ were counted by liquid scintillation spectrometry. Boiled RGS proteins were included as controls where indicated.

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