The GTPase-activating Protein RGS4 Stabilizes the Transition State for Nucleotide Hydrolysis*

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RGS proteins constitute a newly appreciated group of negative regulators of G protein signaling. Discovered by genetic screens in yeast, worms, and other organisms, two mammalian RGS proteins, RGS4 and GAIP, act as GTPase-activating proteins for members of the G family of G protein α subunits. We have purified recombinant RGS4 to homogeneity and demonstrate that it acts catalytically to stimulate GTP hydrolysis by Gi proteins. Furthermore, RGS4 stabilizes the transition state for GTP hydrolysis, as evidenced by its high affinity for the GDP-AlF4⁻-bound forms of Gαi and Gαs and its relatively low affinity for the GTPγS- and GDP-bound forms of these proteins. Consequently, RGS4 is most likely not a downstream effector for activated G α subunits. All members of the Gα subfamily of proteins tested are substrates for RGS4 (including Gαo and Gαz); the protein has lower affinity for Gαopt and it does not stimulate the GTPase activity of Gαs or G12α.

Heterotrimeric G proteinα α subunits cycle between inactive, GDP-bound and active, GTP-bound states, and the duration of activation is thus dependent on their intrinsic GTPase activity. Nucleotide hydrolysis by some G proteins is controlled extrinsically by activating proteins known as GAPs, and the G protein GAPs described previously are known effectors of the α subunit with which they interact (1). Thus, phospholipase C-β1 is activated by Gαq (1) and, in turn, increases the kcat for nucleotide hydrolysis by Gαq by roughly 100-fold (2, 3). Gαi interacts similarly with the γ subunit of a retinal cyclic GMP phosphodiesterase (4–7).

A family of negative regulators of G protein signaling, so-called RGS proteins, was identified recently as a result of genetic studies in yeast, worms, and other organisms (8–19).

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The abbreviations used are: G proteins, heterotrimeric guanine nucleotide-binding proteins; GAP, GTPase-activating protein; GTPγS, guanosine 5′-3-O-thio-triphosphate; C14EO, polyethylene glycol-10-lauryl ether.

We demonstrated that two of these proteins, RGS4 and GAIP, act as GAPs on several members of the Gα subfamily of α subunits (20). However, the mechanism of this effect and the possibility of other functional relationships between these GAPs and their target G proteins remained unclear. We demonstrate herein that purified recombinant RGS4 acts catalytically to accelerate the GTPase activity of Gα family protein members, that RGS4 has high affinity for the transition state complex of the G protein α subunit bound to GDP-AlF4⁻ (but low affinity for GTPγS-α and GDP-α), and that the rank order of affinities of RGS4 for transition state complexes of α subunits is Gαi family > Gαo ≫ Gαs. RGS4 has no detectable GAP activity toward Gαo or G12α.

EXPERIMENTAL PROCEDURES

Purification and Activation of Proteins—Gαs, Gαo, and Gαz were expressed and purified as described (21), as were Gαo and Gαz (22). Gαs and Gαz were myristoylated unless stated otherwise. Bovine retinal Gαs was the generous gift of Dr. Heidi Hamm (University of Illinois College of Medicine), while recombinant Gαo was supplied by Dr. John Hepler (this laboratory) (23). Gαo was activated with 100 μM GTP·S and 10 μM MgSO4 by incubation at 30 °C for 60 min. The indicated GDP-bound α subunits were activated with AlF4⁻ by incubation with 20 μM AlCl3, 10 μM NaF, and 10 μM MgSO4 for 10 min on ice.

RGS4 (hexahistidine-tagged at the amino terminus) was synthesized in Escherichia coli as described previously (20), except that cells were incubated for 12 h at 30 °C after induction. The bacterial lysate was purified on Ni-NTA resin (Qiagen) as described, except that 500 mM NaCl was included in the first wash. Ammonium sulfate (1.2 M final concentration) was added to the Ni-NTA column eluate, and this solution was applied to a 15-mL Phenyl-Sepharose FPLC column equilibrated with 50 mM Tris-HCl (pH 8), 2 mM dithiothreitol, and 1.2 M (NH4)2SO4. The column was washed with 100 ml of equilibration buffer and eluted with a 100-ml continuous gradient of 50 mM Tris-HCl and 1.2 to 0.84 M (NH4)2SO4. Fractions were analyzed electrophoretically, and protein was pooled and dialyzed into 50 mM NaHepes (pH 8), 1 mM EDTA, and 2 mM dithiothreitol prior to concentration and storage.

The GTPase activity of Gαs, Gαo, and G12α were monitored over 5 min by quantification of release of 32Pγ for GTP hydrolysis. Direct measurement of the kcat for GTP hydrolysis by Gαs and Gαz required an alternative protocol because of their slow rates of nucleotide exchange. G12α and Gαo were incubated with 5 μM [γ-32P]GTP (20–50 cpm/μmol) and 5 μM EDTA at 30 °C for 30 min. Samples were then gel-filtered at 4 °C through a Sephadex G-50 spin column (Pharmacia) equilibrated with 50 mM NaHepes (pH 8), 1 mM dithiothreitol, 5 mM EDTA, and 0.1% CH3CN to remove free GTP and Pγi. GTPase activity was measured at 15 °C in 50 μl of the same solution with addition of 8 μM MgSO4, 1 mM GTP (unlabeled), and the indicated concentration of RGS4.

RESULTS AND DISCUSSION

RGS4 Acts Catalytically—We reported previously that RGS4 and GAIP accelerate the rate of the GTPase reaction catalyzed by selected members of the Gα subfamily of α subunits by at least 40-fold; the concentrations of RGS proteins utilized in that study were in excess of those of the Gα protein substrates. Other GAPs have been shown to act catalytically (4, 24–26), and the same is true of RGS4. Thus, concentrations of RGS4 as low as 2.9 nM were sufficient to enhance the GTPase activity of 70 nM GTP·Gαo (Fig. 1A), and the initial slopes of the curves in
RGS4 Stabilizes the Transition State of G$_{o}$

Fig. 1. RGS4 acts catalytically to stimulate the GTPase activity of G$_{o}$. A, G$_{o}$ (200 nM; 10 pmol/sample) was incubated with 1 μM [γ-32P]GTP for 20 min at 20 °C and then transferred to an ice bath for 5 min. The indicated concentrations of RGS4 (plus Mg$^{2+}$) were added at zero time, and release of 32P$_{i}$ was determined over the indicated time course at 4 °C. (The estimated concentration of GTP-G$_{o}$ was 70 nM.) Inset, the initial rates of P$_{i}$ release are plotted against the amount of RGS4. B, varying concentrations of GTP-G$_{o}$ were incubated with 2.1 nm RGS4 and 15 mM MgSO$_{4}$ at 4 °C, and the linear release of 32P$_{i}$ was monitored over 1 min (7 time points). These initial rates are plotted against the concentration of GTP-G$_{o}$ (determined by the maximal amount of P$_{i}$ released). Inset, Lineweaver-Burk analysis of these data. These data represent one of two similar experiments.

RGS4 and the maximal rate of turnover of GTP-G$_{o}$ by RGS4 (Fig. 1B). These values are 2.5 μM and 14/s, respectively, but should be considered estimates, since the substrate concentration was varied over a relatively limited range. This turnover rate could be limited by the maximal intrinsic rate of GTP hydrolysis by G$_{o}$.

RGS4 Stabilizes the Transition State for GTP Hydrolysis—The crystal structures of the GDP-AlF$_{4}^{2-}$G$_{o}$ complexes of G$_{ia}$ (27) and G$_{ia}$ (28) revealed that AlF$_{4}^{2-}$ occupies the position normally taken by the γ-phosphate of GTP but, remarkably, that the fluorine atoms assume a square planar configuration about the central aluminum atom, in contrast to the tetrahedral geometry of a phosphate group. The AlF$_{4}^{2-}$ complex is octahedrally coordinated to a β-phosphate oxygen and to the putative hydrolytic water molecule. Furthermore, an Arg residue and a Gln residue that are known to be critical for catalysis are dramatically reoriented in the GDP-AlF$_{4}^{2-}$ structure (compared to their positions in the GTP-γS-bound protein), contacting the fluorine atoms and the hydrolytic water. These facts (and oth-
RGS4 Stabilizes the Transition State of $G_{\alpha}$

**Fig. 3.** Specificity of the inhibition of RGS4 by various GDP-AlF$_4$-protein complexes. The substrate was 140 nM [$\gamma$-32P]GTP-G$\alpha_1$, RGS4 (29 nM) was incubated with GDP-AlF$_4$ complexes of G$_\alpha_1$ (¥), G$_\alpha_2$ (●), G$_\alpha_3$ (●), or G$_\alpha_6$ (□) for 5 min on ice prior to incubation with the substrate. The time course of GTP hydrolysis was determined under the indicated conditions, and each point of the graph represents data obtained from similar experiments with each competitor protein.

The data presented above strongly imply formation of a high affinity complex with the GAP. Free guanine nucleotide or the combination of AlCl$_3$, NaF, and MgSO$_4$ had no effect on nucleotide hydrolysis in these assays.

Competition assays (at 150 nM GTP-G$\alpha_1$) with different concentrations of GTP-$S$-G$_\alpha$ or GDP-AlF$_4$-G$_\alpha$ highlight the preferential affinity of RGS4 for the transition state conformation of the G protein (Fig. 2B). Stimulated GTP hydrolysis is nearly completely inhibited at concentrations of GDP-AlF$_4$-G$_\alpha$ that approximate those of RGS4. The apparent $K_d$ for RGS4 for GDP-AlF$_4$-G$_\alpha$ is thus below 100 nM. Again, GTP-$S$-G$_\alpha$ is a poor competitor (Fig. 2B), consistent with the $K_m$ for GTP-G$_\alpha_6$ estimated in Fig. 1B, as were the GTP-$S$-bound forms of G$_\alpha_1$, G$_\alpha_2$, and G$_\alpha_3$ (not shown).

**Fig. 4.** RGS4 stimulates the GTPase activity of G$\alpha_3$ but not G$\alpha_1$. [$\gamma$-32P]GTP G$\alpha_1$ and G$\alpha_3$, substrates were prepared as described under “Experimental Procedures.” A, [$\gamma$-32P]GTP-G$\alpha_1$ (2.4 nM) was incubated with (●) or without (□) 12 nM RGS4, and the rate of GTP hydrolysis was measured at 15°C. B, [$\gamma$-32P]GTP-G$\alpha_3$ (3 nM) was incubated with (●) or without (□) 0.6 µM RGS4, and the initial rate of GTP hydrolysis was measured at 15°C. In both panels, data shown are averages of duplicate determinations from a single experiment, which is representative of three such experiments.

RGS4 interacts with G$_\alpha_1$, Subfamily Members and with G$_\alpha_3$, but Not with G$_\alpha_6$, or G$_\alpha_2$.—The competition assay just described was utilized to examine the interactions of RGS4 with other G protein $\alpha$ subunit family members. We demonstrated previously that RGS4 stimulates GTP hydrolysis by G$_\alpha_1$, G$_\alpha_2$, G$_\alpha_3$, and G$_\alpha_6$. G$_\alpha_1$, G$_\alpha_2$, and G$_\alpha_3$ were similarly effective in the competitive assay as the GDP-AlF$_4$-bound species (Fig. 3). Two additional G$_\alpha$ subfamily members were also tested for interactions with RGS4. GDP-AlF$_4$-G$_\alpha_6$ was an effective competitor (Fig. 3), indistinguishable from G$_\alpha_1$ and G$_\alpha_3$. The $k_{cat}$ for GTP hydrolysis by G$_\alpha_6$ was measured directly and was increased from its normal low value of 0.02/min to 0.12/min by 12 nM RGS4 (Fig. 4A). It thus seems likely that the GTPase activity of all G$_\alpha$ subfamily members is stimulated by RGS4. We have attempted direct estimation of affinities between GDP-AlF$_4$-bound $\alpha$ subunits and RGS4 by observation of surface plasmon resonance (Pharmacia Biosensor). Appropriate protein-protein interactions were detected, but the rate of dissociation of hexahistidine-tagged RGS4 from the Ni-NTA-derivatized chips of the Biosensor instrument was faster than the rate of dissociation of GDP-AlF$_4$-G$_\alpha$ from RGS4. Thus, we can only estimate an upper limit for the $K_d$ from these experiments, roughly 100 nM.

We also noted previously the inability of RGS4 (or GAIP) to 2 stimlate GTP hydrolysis by G$_\alpha_1$; accordingly, the GDP-AlF$_4$-bound form of G$_\alpha_1$ did not compete with G$_\alpha_1$ (Fig. 3). Of interest, GDP-AlF$_4$-G$_\alpha_1$, did interact with RGS4, although its apparent affinity for the protein is 10- to 100-fold or more lower than those of the G$_\alpha_1$ subfamily members. However, the assumption that GDP-AlF$_4$-bound complexes of all G protein $\alpha$ subunits are transition-state mimics may be unwarranted. G$_\alpha_4$ must be reconstituted with an appropriate receptor to examine the effect of RGS4 on nucleotide hydrolysis; the rate of nucleotide exchange is too low to permit preparation of GTP-G$_\alpha_4$ substrate by the present methods. A high concentration of RGS4 did not stimulate GTP hydrolysis by G$_\alpha_1$, which proceeded with a $k_{cat}$ of 0.06/min at 15°C (Fig. 4B).

**RGS4 Forms a High-affinity Complex with GDP-AlF$_4$-G$_\alpha_1$.**—The data presented above strongly imply formation of a high affinity complex between RGS4 and the GDP-AlF$_4$-bound forms of various G$_\alpha$ proteins. To demonstrate this directly and to prepare material for crystallographic analysis, we have performed gel filtration chromatography on mixtures of RGS4 and
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**FIG. 5.** RGS4 and GDP-AlF\(_4\) \(-G_{\alpha}\) form a high-affinity complex. A, RGS4 (2.2 mg) or GDP \( G_{\alpha}\) (2.2 mg; activated with 20 \( \mu \)M AlCl\(_3\), 10 mM NaF, and 5 mM MgSO\(_4\) for 10 min at 4°C) was applied to a 16/200 Superdex gel filtration column equilibrated and eluted with 50 mM NaHepes (pH 8), 1 mM EDTA, 10 mM NaF, 20 \( \mu \)M AlCl\(_3\), 5 mM MgSO\(_4\), 2 mM dithiothreitol, and 50 mM NaCl. Fractions (1.5 ml) were collected, and \( A_{280} \) was monitored. Arrows indicate the position of molecular weight standards. B, after incubation of GDP-AlF\(_4\) \(-G_{\alpha}\) with AlCl\(_3\), NaF, and MgSO\(_4\) (Fig. 5B) was applied to a 16/60 Superdex gel filtration column equilibrated and eluted with 50 mM NaHepes (pH 8), 1 mM EDTA, 10 mM NaF, 20 \( \mu \)M AlCl\(_3\), 5 mM MgSO\(_4\), 2 mM dithiothreitol, and 50 mM NaCl. Fractions (1.5 ml) were collected, and \( A_{280} \) was monitored. Arrows indicate the position of molecular weight standards. C, after incubation of GDP-AlF\(_4\) \(-G_{\alpha}\) with GDP-AlF\(_4\) \(-G_{\alpha}\) and GDP-AlF\(_4\) \(-G_{\alpha}\) eluted from a Superdex 200 column at positions 1 and 2, respectively, as expected (Fig. 5A).

We conclude that RGS4 has a relatively low affinity (greater than 1 \( \mu \)M) for its substrates, GTP-G\(_{\alpha}\) family members, but interacts directly and with high affinity with the transition-state conformations of these \( \alpha \) subunits. Stabilization of the transition state lowers the activation energy barrier for hydrolysis of GTP, accounting for the large rate enhancements that are seen. We surmise that RGS4 is not an effector for \( G_{\alpha} \) proteins, based on the relatively poor affinity of RGS4 for their GTPyS-bound forms compared to those of other known effectors of G protein \( \alpha \) subunits. We note, however, that some other RGS proteins are considerably larger than are RGS4 or GAIP, and generalization of this point may be unwarranted. Finally, we call attention to the recent observation by Mittal et al. (29) that wild-type p21ras protein interacts with AlF\(_4\) only in the presence of its GAPs. Thus, the ground state of p21ras must be too distant, conformationally, from the transition state to recognize AlF\(_4\) (unlike heterotrimeric G proteins), but GAPs for low molecular weight GTPases and RGS proteins both appear to act predominantly by stabilizing the transition states for nucleotide hydrolysis and not by elevating the energy level of the enzyme-substrate complex.

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