Mechanism of RGS4, a GTPase-activating Protein for G Protein α Subunits*

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GTP hydrolysis by guanine nucleotide-binding proteins, an essential step in many biological processes, is stimulated by GTPase-activating proteins (GAPs). The mechanisms whereby GAPs stimulate GTP hydrolysis are unknown. We have used mutational, biochemical, and structural data to investigate how RGS4, a GAP for heterotrimeric G protein α subunits, stimulates GTP hydrolysis. Many of the residues of RGS4 that interact with Gαi1b are important for GAP activity. Furthermore, optimal GAP activity appears to require the additive effects of interactions along the RGS4-Gi interface. GAP-defective RGS4 mutants invariably were defective in binding Gi subunits in their transition state; furthermore, the apparent strengths of GAP and binding defects were correlated. Thus, none of these residues of RGS4, including asparagine 128, the only residue positioned at the active site of Gαi1b, is required exclusively for catalyzing GTP hydrolysis. These results and structural data (Tesmer, J. J. G., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 89, 251–261) indicate that RGS4 stimulates GTP hydrolysis primarily by stabilizing the transition state conformation of the switch regions of the G protein, favoring the transition state of the reactants. Therefore, although monomeric and heterotrimeric G proteins are related, their GAPs have evolved distinct mechanisms of action.

By cycling between active GTP-bound and inactive GDP-bound states, guanine nucleotide-binding proteins control many biological processes, including translation, vesicular trafficking, cytoskeletal organization, and signal transduction (1–3). GTP hydrolysis is required to deactivate guanine nucleotide-binding proteins, as shown by the effects of hydrolysis-resistant GTP analogs, or mutations and toxins that block this reaction. Because guanine nucleotide-binding proteins have slow intrinsic rates of GTP hydrolysis, they are acted upon by GTPase-activating proteins (GAPs),1 achieving rates of GTP hydrolysis in the physiological range (4).

RGS (regulators of G protein signaling) proteins are GAPs for α subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins), principally Gq and members of the Gi family (5, 6). RGS proteins, which are unrelated in primary sequence to GAPs that act on monomeric guanine nucleotide-binding proteins such as Ras, have been identified in the yeast Saccharomyces cerevisiae (Set2p) (7–9), Aspergillus nidulans (FlbA) (10), Caenorhabditis elegans (Egl-10) (11) and in mammalian cells (at least 16 family members) (reviewed in Refs. 5 and 6). In addition to acting as GAPs, certain RGS family members are capable of inhibiting signaling by binding activated (GTP-bound) Gα subunits, antagonizing effector binding (12). Therefore, RGS family members are thought to govern the strength and duration of physiological responses triggered by an array of G protein-dependent signaling pathways.

The mechanisms whereby RGS proteins, or other GAPs, stimulate GTP hydrolysis are unknown. They could introduce residues into the active sites of guanine nucleotide-binding proteins, participating directly in catalyzing GTP hydrolysis as has been suggested by mutational studies of p120GAP (13–15); however, proof of this mechanism requires determining the structure of Ras-p120GAP complexes. Alternatively, RGS proteins or other GAPs could act allosterically by stabilizing the transition state structures of the “switch” regions of guanine nucleotide-binding proteins that change conformation during the GTPase cycle, stimulating intrinsic GTPase activity. Consistent with this mechanism, p120GAP and certain RGS family members bind preferentially to the transition state conformations of Ras and Gα subunits, respectively (16–18).

The structure of RGS4-Gαi1 complexes in the transition state has recently been solved to a resolution of 2.8 Å (19). The structure suggests that RGS4 could act allosterically and/or catalytically to stimulate GTP hydrolysis. An allosteric mechanism is suggested because binding of RGS4 stabilizes the switch regions of Gαi1. However, a catalytic role is possible because an asparagine residue (Asn-128) of RGS4 interacts with an active-site glutamine residue (Gln-204) of Gαi1 that is thought to bind or polarize the attacking water molecule in the GTPase reaction. Furthermore, modeling studies suggest that asparagine 128 of RGS4 potentially binds a water molecule when it associates with GTP-bound Gαi1, earlier in the GTPase reaction mechanism (19). Therefore, determining whether RGS4 acts allosterically or catalytically requires mutational and biochemical data that reveal which residues of RGS4 are required specifically to bind Gα subunits and/or catalyze GTP hydrolysis.

EXPERIMENTAL PROCEDURES

Mutagenesis of RGS4—RGS4 was expressed in yeast from the ADH promoter using the polymerase chain reaction to clone the coding region of an RGS4 cDNA (rat (20); rat RGS4 was used for structural studies (19)) into pVT102U cut with BamHI and XhoI, creating pADH-RGS4. A C-terminally Myc-tagged form of RGS4 (which did not affect RGS4 function in yeast; data not shown) was generated by ligating an XhoI fragment encoding three in-frame copies of the c-Myc epitope (in pUC119; gift of D. Pellman, Whitehead Institute) with XhoI-cut pADH-RGS4, creating pADH-RGS4–3xMyc. N-terminally His-tagged RGS4

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1 The abbreviations used are: GAPs, GTPase-activating proteins; GTP, guanosine 5'-O-triphosphate.
was expressed in Escherichia coli using plasmids described previously (17). Point mutations in the RGS4 coding regions of yeast and E. coli expression plasmids were generated using the QuickChange mutagenesis kit (Stratagene). N- and C-terminal truncation mutations of RGS4 were generated by the polymerase chain reaction and cloned into pET15B (Novagen). The RGS4 coding regions of all constructs were sequenced to verify that only the desired mutations had been introduced. Phoromone response of yeast cells (BC180, an sst2 mutant) expressing wild-type and mutant forms of RGS4 was determined by performing quantitative growth arrest (halo) assays (20). Expression of Myc-tagged RGS4 in yeast cells was examined by performing immunoblot experiments using 9E10 monoclonal antibodies and enhanced chemiluminescence detection (Amersham Corp.).

**Purification of Proteins, GTPase Measurements, and G Protein Binding Assays—**N-terminally His-tagged forms of RGS4 (rat) and Gαs were expressed in and purified from E. coli (BL21(DE3)) by Ni2+-nitrilotriacetic acid chromatography as described previously (17). Recombinant myristoylated Gαs (provided by M. Linder) was purified from E. coli as described previously (21). The activities of G proteins were assessed by determining the stoichiometry of [32P]GTP-γS binding, which was >70%.

GTP hydrolysis by Gαs subunits during a single catalytic turnover was determined as described previously (17). Briefly, His-tagged Gαs (100 nM in 400 μl) was incubated with [γ-32P]GTP (0.1 μM, 20,000–30,000 cpm/μmol) in the absence of Mg2+; stoichiometries of nucleotide binding were determined by scintillation counting. Aliquots (50 μl) were removed 15 s before and 45 s after the addition of GTPase assay mixture (200 μM GTP, 0.1 mM MgCl₂, 10 mM NaF, 10 mM MgSO₄, 100 mM Tris, pH 7.4). GTPase activity was determined as described previously (17). Briefly, purified myristoylated Gαs (5 μg) was incubated with GDP, GTP-γS, or GDP and AlF₄⁻. Purified His-tagged wild-type or mutant forms of Gαs (10 μg) were added. After a 30-min incubation on ice, Ni2+-nitrilotriacetic acid beads were added, incubated for 30 min with agitation at 4 °C, washed three times, and boiled in Laemmli sample buffer to elute bound proteins. Eluted proteins resolved by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose; Gαs subunits were detected by immunoblotting with antiserum P960 (Gαs-common antiserum) and peroxidase-labeled secondary antibodies and by enhanced chemiluminescence.

**Graphics—**Images were produced using RasMol and the coordinates of the transition state structure of RGS4-GTP-γS (85–205) (13–21). Alternatively, if RGS4 catalyzes GTP hydrolysis strictly by binding and stabilizing the transition state of G protein subunits during a single catalytic turnover was determined as described previously (22). Truncation extending into the RGS homology domain resulted in the production of RGS4 in an insoluble form (Table I), indicating a defect in protein folding or stability. The RGS homology domain of RGS4 therefore appears to be a single functional domain.

To identify amino acids in RGS4 that are functionally important, we constructed a set of 43 point mutations (most of which were alanine substitutions) that affect many of the charged or conserved residues of its RGS homology domain (Fig. 1). As a means of screening rapidly for loss-of-function mutations, C-terminally Myc-tagged forms of the RGS4 mutants were tested for their ability to inhibit G protein-dependent signaling (mating pheromone response) when expressed in yeast (20) (see “Experimental Procedures”; results are summarized in Table I). The results indicated that many of the mutants were as functional as wild-type RGS4, whereas other mutants were nonfunctional. Mutants showing an intermediate activity in this in vitro assay were not obtained, even though subsequent experiments (see below) revealed that many of the nonfunctional mutants were partially defective in GAP activity in vitro. This was expected because the in vitro assay is much more sensitive. None of the loss-of-function mutations significantly decreased expression of RGS4 in yeast, as indicated by immunoblotting (data not shown).

We subsequently determined whether these loss-of-function mutations affected the stability or GAP activity of recombinant RGS4 expressed in and purified from E. coli (Table I); as controls, several mutations that did not affect RGS4 function in yeast were also analyzed. Equivalent results were obtained using either Gαs or Gαz as a substrate (data not shown). The results are interpreted below in light of the structure of RGS4-Gαz complexes (19).

### TABLE I

| Protein | GAP activity (P, released relative to wild type (%)) |
|---------|-----------------------------------------------|
| None    | 100 ± 4                                       |
| 1–205   | 72 ± 2                                       |
| 13–205  | 75 ± 5                                       |
| 58–177  | 74 ± 6                                       |
| 85–205  | ND                                            |
| 1–166   | ND                                            |
| E87A,N88A | 3 ± 1 (1)                                 |
|         | 4 ± 0.5 (1)                                  |
| I114D   | 8 ± 2 (1)                                    |
| 114A    | 4 ± 1 (1)                                    |
| N128A   | 4 ± 2 (1)                                    |
| L159A   | 12 ± 2 (1)                                   |
| R167A   | 3 ± 2 (1)                                    |
| R167H   | 2 ± 1 (1)                                    |
| R167K   | 5 ± 3 (1)                                    |
| E83A    | 29 ± 1 (1)                                   |
| Y94A    | 38 ± 1 (1)                                   |
| E87A    | 32 ± 1 (1)                                   |
| N88A    | 45 ± 3 (1)                                   |
| G272R   | 79 ± 7 (1)                                   |
| F91A    | 66 ± 2 (1)                                   |
| W92A    | 70 ± 9 (1)                                   |
| I114A   | 70 ± 3 (1)                                   |
| M180A   | 85 ± 2 (1)                                   |
| D165A   | 96 ± 1 (1)                                   |
| E64A    | 104 ± 2 (1)                                  |
| E68A    | 96 ± 1 (1)                                   |
| E96A    | 96 ± 2 (1)                                   |
| E97A    | 101 ± 2 (1)                                  |
| K99A    | 93 ± 1 (1)                                   |
| K110A   | 101 ± 3 (1)                                  |
| K130A   | 87 ± 1 (1)                                   |
| F79A    | ND                                            |
| F149A   | ND                                            |
| F168A   | ND                                            |

* ND, not determined; protein was insoluble.

**RESULTS AND DISCUSSION**

**Identification of Mutations That Disrupt the GAP Activity of RGS4—**To determine the mechanism whereby RGS4 stimulates GTP hydrolysis by G protein α subunits, we analyzed the effects of mutations affecting RGS4. If certain residues of RGS4 are required exclusively for catalysis, they should be dispensable for binding Gαs subunits, similar to what has been shown for p120GAP (13–15). Alternatively, if RGS4 catalyzes GTP hydrolysis strictly by binding and stabilizing the transition state of Gαs subunits, then mutations that disrupt GAP activity should invariably cause a corresponding defect in Gαs binding. Because these experiments were initiated before the structure of RGS4-Gαs complexes was reported, our first objective was to define the minimal region of RGS4 (a 205-residue polypeptide) that possesses normal GAP activity in vitro. This domain could then be subjected to extensive point mutagenesis.

Analysis of purified forms of several truncation mutants indicated that the domain characteristic of RGS family members (RGS homology domain, residues 58–177 of RGS4) had normal GAP activity toward Gαs (Table I), similar to results obtained using the RGS-δ (22). Truncation extending into the RGS homology domain resulted in the production of RGS4 in an insoluble form (Table I), indicating a defect in protein folding or stability. The RGS homology domain of RGS4 therefore appears to be a single functional domain.

To identify amino acids in RGS4 that are functionally important, we constructed a set of 43 point mutations (most of which were alanine substitutions) that affect many of the charged or conserved residues of its RGS homology domain (Fig. 1). As a means of screening rapidly for loss-of-function mutations, C-terminally Myc-tagged forms of the RGS4 mutants were tested for their ability to inhibit G protein-dependent signaling (mating pheromone response) when expressed in yeast (20) (see “Experimental Procedures”; results are summarized in Fig. 1).
Mutations That Apparently Affect the Stability of the RGS Domain—The RGS homology domain of RGS4 forms a bundle of nine α-helices. The binding site for G_{i,1} is a cleft consisting of conserved amino acids at the ends of helices 4, 7, and 8 and loops between helices 3 and 4 and helices 5 and 6. However, many other conserved amino acids in RGS4 are located distal to the G_{i,1}-binding site. Some of these residues are important for the folding and/or stability of the RGS domain. These include a pair of phenylalanine residues (Phe-79 and Phe-168) apposed at the interface of helices 3 and 8; substituting either with alanine resulted in an insoluble protein when expressed in E. coli (data not shown). Other interacting pairs of residues may also maintain the stability or rigidity of the RGS fold because mutations affecting them resulted in soluble proteins with reduced GAP activity (Table I). These interacting residues include an isoleucine-phenylalanine pair (Ile-114 and Phe-149) apposed between helices 4 and 5, and an isoleucine-tryptophan pair (Ile-67 and Trp-92) between helices 2 and 4. In contrast, two highly conserved serine residues (Ser-164 and Ser-171), which were predicted to stabilize the RGS fold by acting as helix breakers between helices 7, 8, and 9 (19), were not required for RGS4 function in vivo (Fig. 1).

Mutations Affecting Residues on the Surface of RGS4 That Interact with G_{i,1}—In remarkable agreement with structural data, the remaining loss-of-function mutations affect residues on the surface of RGS4 that interact with G_{i,1} (Fig. 2A and Table I). As discussed below, this indicated that many of the interactions between RGS4 and G_{i,1} are important for GAP activity (Fig. 2A and Table I). Furthermore, this allowed us to investigate the relative functional importance of specific protein-protein contacts and the mechanism whereby RGS4 stimulates GTP hydrolysis. In describing these results, we have used the convention of Tesmer et al. (19), in which residues of RGS4 are preceded with “a-” and those of G_{i,1} with “b-”.

Interactions occurring at the edge of the RGS4-G_{i,1} interface are functionally important (Fig. 2B), as indicated by the effects of various alanine substitutions in RGS4. These include hydrophobic interactions between the side chains of r-Tyr-84 and a-His-121, and a salt bridge between r-Glu-87 and a-Lys-210. r-Glu-83 is also functionally important, probably because it interacts with the side chain of r-Arg-167 (Fig. 2A). Potentially, this interaction pulls r-Glu-83 and the adjacent residue, r-Tyr-84, into place such that r-Tyr-84 interacts effectively with a-His-213. Alternatively, the side chain of r-Glu-83 may be important for GAP activity because it contacts the side chain of a-Val-185 (Fig. 2B).

In the center of the RGS4-G_{i,1} interaction footprint, a-Thr-182 in switch I of the G protein binds a pocket in RGS4 consisting of several conserved residues, including r-Ser-85, r-Asn-88, r-Leu-159, r-Asp-163, r-Ser-164, and r-Arg-167 (Fig. 2B) (19). With the exception of r-Ser-85 and r-Ser-164, the side chains of residues forming the pocket are functionally important because an alanine substitution at each site diminished the GAP activity of RGS4 (Table I).

Based on these results and the structural data of Tesmer et al. (19), we propose the following roles for residues forming the pocket that binds a-Thr-182. Three residues of the pocket are important because they interact directly with a-Thr-182. The bulky side chain of r-Leu-159 may determine the size or shape of the pocket because it interacts extensively with the side chain of a-Thr-182 (Fig. 2, B and C) and because the r-L159A substitution causes a severe defect in GAP activity. Furthermore, the side chain of r-Leu-159 may be of critical importance because it also participates in a hydrophobic interaction with the side chain of a-Lys-180 (Fig. 2, B and C). The side chains of r-Asn-88 and r-Asp-163 appear to be important because they form hydrogen bonds with the side chain hydroxyl and backbone nitrogen of a-Thr-182, respectively. Although r-Ser-85 and r-Ser-164 form part of the pocket that binds a-Thr-182, their side chain hydroxyl groups are unimportant because alanine substitutions at these sites did not affect RGS4 function in vivo (Fig. 1). However, this does not exclude the possibility that the β-carbon atoms of r-Ser-85 and/or r-Ser-164 that do interact with a-Thr-182 are functionally important.

Residues forming the pocket, but which do not interact extensively with a-Thr-182, are also functionally important, but probably for different reasons. r-Met-160, which lies at the floor of the pocket but does not contact a-Thr-182, may be important because its side chain interacts with that of r-Asn-88 (Fig. 2D), potentially orienting it toward a-Thr-182. Similarly, because r-Arg-167 does not contact a-Thr-182 extensively (their side chains are a minimum of 3.9 Å apart), its principal function may be to stabilize two parts of the RGS4 surface such that each can interact with G_{i,1}. This is suggested because 1) the side chain of r-Arg-167 interacts with side chain carboxyl groups of r-Glu-83 and r-Asp-163 (Fig. 2B), both of which are important for GAP activity; 2) an alanine substitution of r-Arg-167 leads to a stronger defect than an alanine substitution affecting either r-Glu-83 or r-Asp-163 (Table I); and 3) changing r-Arg-167 to alanine, lysine, or histidine disrupted GAP activity (Table I).

There also was evidence that normal function of RGS4 requires the additive effects of two sites that bind different regions of G_{i,1}, r-Glu-87 and r-Asn-88, which are adjacent to one another, are likely to function cooperatively because they in-
Asparagine 128 of RGS4 Is Critical for GAP Activity—A cluster of residues in Gαi1 (including a-Lys-180 of switch I and a-Gln-204, a-Ser-206, and a-Glu-207 of switch II) cradles r-Asn-128, which interacts with the active-site residues of Gαi1. All the residues of Gαi1 that interact with ASR4 are colored, each according to the magnitude of the defect in GAP activity (see Table I). The backbones of RGS4 (white ribbon) and Gαi1 (cyan ribbon) are indicated. Nearly all the residues of Gαi1 that interact with RGS4 are conserved in the Gα subunits we have used for in vivo (Gpa1) and in vitro (Gαo1 and Gαol subunits assays of RGS4 function; an exception is that in Gpa1, a threonine residue occurs at the position equivalent to valine 185 of Gαo1.

Effects of Mutations on Binding of RGS4 and Gα Subunits—To investigate the mechanism of RGS4-stimulated GTPase activity, we determined whether the panel of GAP-defective RGS4 mutants can bind Gαi1 subunits in their active (GTP-bound) or transition state (GDP + AlF4-). The results of the binding assays, although somewhat qualitative, suggested that the severity of the defects in Gαi1 binding and GAP activity were correlated. Mutations that severely reduced or eliminated GAP activity (E87A,N88A double mutant, I114D, N128A, L159A, and R167A) appeared to cause severe defects in binding (binding was not detected) (Fig. 3, third row), whereas those that partially disrupted GAP activity caused less severe binding defects (binding was less efficient than with wild-type RGS4) (Fig. 3B, first and second rows; 10% of the bound material was analyzed for each mutant in the first row and 20% for those in the second and third rows). Because all of these mutations disrupted or impaired the binding of RGS4 to Gα subunits, none of the residues they affect, including r-Asn-128, is required exclusively to catalyze GTP hydrolysis.

Proposed Mechanism of RGS4-stimulated GTP Hydrolysis—

![Functional map of the surface of RGS4 that binds Gαi1-A](image)
regarding the mechanism whereby RGS4 stimulates GTP hydrolysis is thought to stabilize the developing negative charge of Gα subunits. Measurements of GAP activity (pmol of 32Pi, released after 45 s of incubation at 5 °C, relative to that obtained with wild-type RGS4 (0.2 μM)) were used to indicate whether mutant forms of RGS4 can bind GTP-bound Gα subunits, thereby inhibiting the ability of wild-type RGS4 to stimulate GTP hydrolysis. Assays were performed using the indicated concentrations (μM) of purified wild-type (WT) and mutant RGS4 and Gα subunits (0.1 μM). The results shown are the average of three assays; standard errors are indicated (bars). B, effects of mutations on the ability of RGS4 to bind (GDP + AlF4−)-activated Gα(GTPαS) (incubated with the indicated nucleotides) was incubated with polyhistidine-tagged wild-type RGS4 and the indicated mutant forms of RGS4. RGS4-Gα(GTPαS) complexes bound to Ni2+-nitrotetracycline beads were detected by SDS-polyacrylamide gel electrophoresis and immunoblotting. The portion of input (In) and bound fractions analyzed was as follows: 10% input, 10% bound (first row; wild-type and mutants having weaker GAP defects); and 5% input, 20% bound (second and third rows; mutants having moderate and strong GAP defects, respectively). The experiment was performed three times, with equivalent results.

Our functional analysis of RGS4 mutants and the structural data of Tesmer et al. (19) lead to the following conclusions regarding the mechanism whereby RGS4 stimulates GTP hydrolysis by Gα subunits. First, a principal function of asparagine 128 of RGS4 is to bind and stabilize switches I and II of Gα subunits, contributing to the overall stability of the RGS4-Gα transition state complex. High affinity binding of RGS4 and Gα1 may not require a hydrogen bond formed by the amide nitrogen of r-Asn-128 and the carbonyl oxygen of the side chain of a-Glu-204 because in several other RGS proteins, including GAIP, r-Asn-128 is replaced by a serine residue. Therefore, interactions between r-Asn-128 and a-Lys-180, a-Ser-206, or a-Glu-207 may be important for stabilizing RGS4-Gα complexes. Indeed, a-Glu-207 of Gα1, which is highly conserved in Gα subunits, is critical for RGS binding and RGS4-stimulated GTP hydrolysis (18, 23), but it is dispensable for intrinsic GTPase activity and effector recognition.

Second, our finding that GAP-defective RGS4 mutants are defective in binding Gα subunits provides direct evidence in support of the hypothesis of Tesmer et al. (19) that RGS4 stimulates GTP hydrolysis primarily, if not exclusively, by binding and stabilizing the transition state conformation of Gα subunits. Apparently, the rigidity or stability of RGS4 is important for the mechanism because we found that pairs of interacting conserved residues distal to the Gα1-binding site of RGS4 are important for GAP activity. Furthermore, the mechanism appears to involve the additive effects of several binding interactions along the RGS4-Gα interface, as indicated by the analysis of double mutants. Thus, structural and functional data indicate that RGS4 stimulates GTP hydrolysis by binding the switch regions of Gα subunits, creating a rigid environment that favors the transition state of the reactants.

Finally, the mechanism used by RGS4 to stimulate GTP hydrolysis is different in certain respects from that used by p120GAP. Binding of p120GAP is believed to introduce one or two invariant arginine residues into the active site of Ras (13–15). One of these conserved arginine residues, which are required for GAP activity but are dispensable for binding of p120GAP to Ras, is thought to stabilize the developing negative charge of the γ-phosphate during GTP hydrolysis, a role subserved by a-Arg-178 in Gα1 (24). Direct support of this conclusion has been provided recently from the crystal structure of p120GAP bound to Ras (25). However, a common feature of p120GAP and RGS4 is that they both stabilize the structures of the switch regions of their cognate GTP-binding proteins in the transition state (19, 25, 26). Therefore, although monomeric and heterotrimeric GTP-binding proteins are evolutionarily related, their attendant GAPs are distinct in terms of their sequences, structures, and mechanisms of stimulating GTP hydrolysis.

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