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# THE REGULATOR OF G PROTEIN SIGNALING FAMILY

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■ **Abstract** Regulator of G protein signaling (RGS) proteins are responsible for the rapid turnoff of G protein–coupled receptor signaling pathways. The major mechanism whereby RGS proteins negatively regulate G proteins is via the GTPase activating protein activity of their RGS domain. Structural and mutational analyses have characterized the RGS/Gα interaction in detail, explaining the molecular mechanisms of the GTPase activating protein activity of RGS proteins. More than 20 RGS proteins have been isolated, and there are indications that specific RGS proteins regulate specific G protein–coupled receptor pathways. This specificity is probably created by a combination of cell type–specific expression, tissue distribution, intracellular localization, posttranslational modifications, and domains other than the RGS domain that link them to other signaling pathways. In this review we discuss what has been learned so far about the role of RGS proteins in regulating G protein–coupled receptor signaling and point out areas that may be fruitful for future research.



## RGS PROTEINS: INTRODUCTION TO A FAMILY OF GAPS FOR Gα SUBUNITS

### Regulation of G Protein–Coupled Receptor Signaling

G protein–coupled receptor (GPCR) signaling pathways are involved in cellular responses to extracellular stimuli and need to be tightly regulated, both short-term and long-term. Most of the short-term regulation takes place at the level of the receptor and the heterotrimeric G protein. The best-described receptor regulatory mechanism is the agonist-dependent ''disconnection'' of the β-adrenergic receptor from the transducing G protein via phosphorylation of the receptor by G protein–coupled receptor kinases (GRKs), arrestin binding, followed by receptor dephosphorylation in endosomes and recycling to the plasma membrane (PM) (1). At the G protein level, phosphorylation of $G_z$ and acetylation of $G_s$ have been

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shown to play a short-term regulatory role (2, 3), and myristoylation and reversible palmitoylation changes the affinity of G proteins for membranes (4, 5). Long-term modulation of certain G protein mRNA levels by transcriptional regulation has been documented, but at the protein level G proteins are generally very stable molecules with long half-lives (6). None of the above-described regulatory mechanisms can explain the rapid turnoff of G protein signaling with satisfaction. Rapid turnoff of G proteins can be accomplished by accelerating their return to the inactivated state, and this is exactly one of the mechanisms of regulator of G protein signaling (RGS) protein function.

The ability of cells to negatively regulate signaling on both the receptor and G protein level might appear redundant, but RGS proteins and GRKs play distinct roles in turning off signaling. GRKs prevent additional ligands from acting on receptors, whereas RGS proteins shorten signals that have already been generated by inactivating G proteins.

The activation-inactivation cycle of G proteins and the factors that influence the kinetics of this cycle are represented in Figure 1. Factors that regulate the speed of the different steps in the G protein cycle clearly have a major impact on the kinetics of the GPCR signaling system. These factors are GTPase activating proteins (GAPs) and guanine exchange factors (GEFs) isolated more than 10 years ago for the small G proteins (7). In the classical G protein paradigm at the PM, the receptor activates the G protein, but a fast turnoff mechanism generally appli-

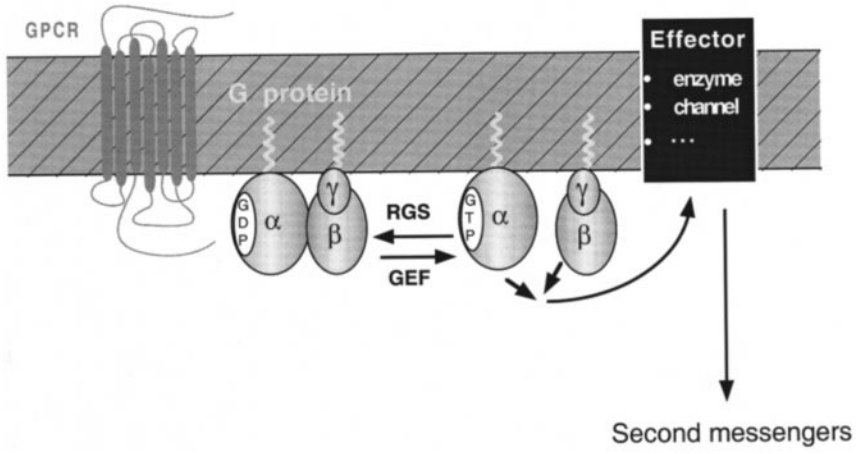


**Figure 1** Effect of regulator of G protein signaling (RGS) proteins on the classical G protein cycle at the plasma membrane. A G protein–coupled receptor (GPCR) serves as a guanine nucleotide exchange factor (GEF) that activates the G protein by enhancing GDP dissociation from the Gα subunit. Gα and Gβγ dissociate and stimulate their respective effectors. RGS proteins serve as GTPase activating proteins that accelerate GTP hydrolysis and thereby return the Gα subunit to its inactivated GDP-bound form, followed by reassembly of the heterotrimer.

cable to (almost) all G proteins emerged only 4 years ago with the discovery of the mammalian RGS protein family. Soon thereafter the GAP activity of RGS proteins was shown to be the major mechanism of negative regulation (8). Since then, a number of reviews on RGS proteins have been published (9–17).

Although all of the above-described regulatory events take place at the PM, it should be noted that G proteins have also been found on intracellular membranes such as Golgi membranes, and the regulation of G proteins on intracellular membranes might be different from the classical G protein paradigm at the PM (18).

## Discovery of the RGS Family

RGS proteins evolutionarily stem from the Sst2 gene in *Saccharomyces cerevisiae,* involved in desensitization of the yeast pheromone response (19, 20).

The recognition of a distinct mammalian family took place when a 130-residue homologous domain—defined as the RGS domain—was described in several proteins at approximately the same time (21–23), and the RGS domain was demonstrated to be responsible for binding of the RGS protein to the Gα subunit (21). Genetic studies in yeast also suggested a direct interaction between Sst2, the yeast RGS, and Gpa1, the pheromone receptor–linked Gα subunit (24–26). That RGS proteins can regulate G protein–mediated signaling events in vivo was demonstrated by the findings that RGS1, RGS2, RGS3, and RGS4 could attenuate mitogen-activated protein kinase (MAPK) activation by interleukin 8 (IL-8) and RGS2 and RGS4 can complement the defective pheromone response in yeast *sst2* mutants, which suggests that they play a role in the negative regulation of G protein signaling (23, 27). In the 4-year period since the family was originally recognized, many more mammalian RGS homologs have been isolated (17). To date, approximately 25 different RGS proteins have been identified in mammals (Figure 2). They all contain the diagnostic RGS domain, and many contain additional domains with different functions that link RGS proteins to other signaling pathways (17).

In this review we summarize our present knowledge of the function of RGS proteins in GPCR signaling and what has been learned so far about their regulation, expression, localization, and molecular structure, including the importance of non-RGS domains in certain RGS proteins. It is also our goal to point out what we do not know and by doing so to stimulate research in these directions.

# THE FUNCTION OF RGS PROTEINS IN GPCR SIGNALING PATHWAYS

## Molecular Mechanisms of RGS Protein Function

There are three known mechanisms by which most RGS proteins act in vivo to turn off signaling pathways. First and foremost, RGS proteins are GAPs, initially demonstrated by in vitro assays using recombinant proteins (8, 28–32) or receptor/

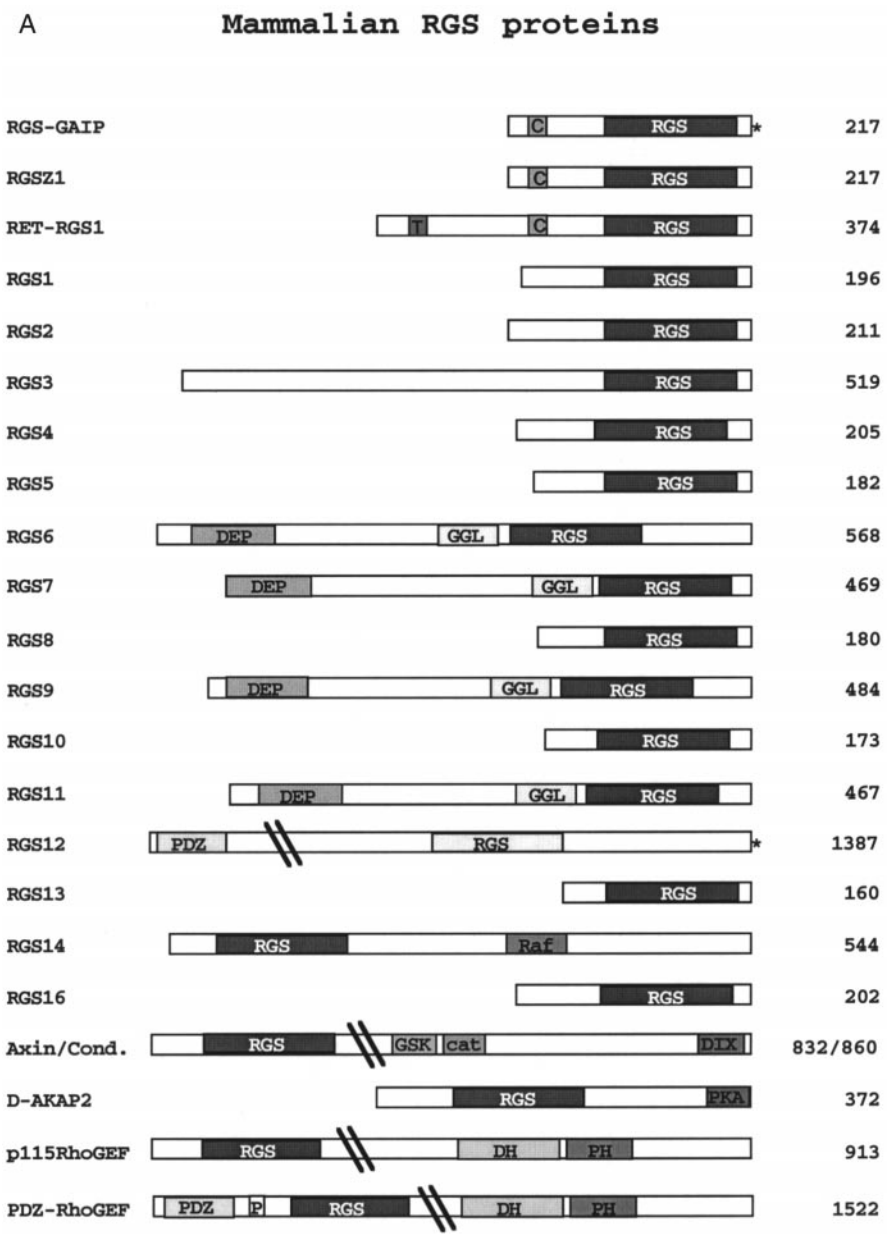


**Figure 2** For legend and Figure 2b see next page.

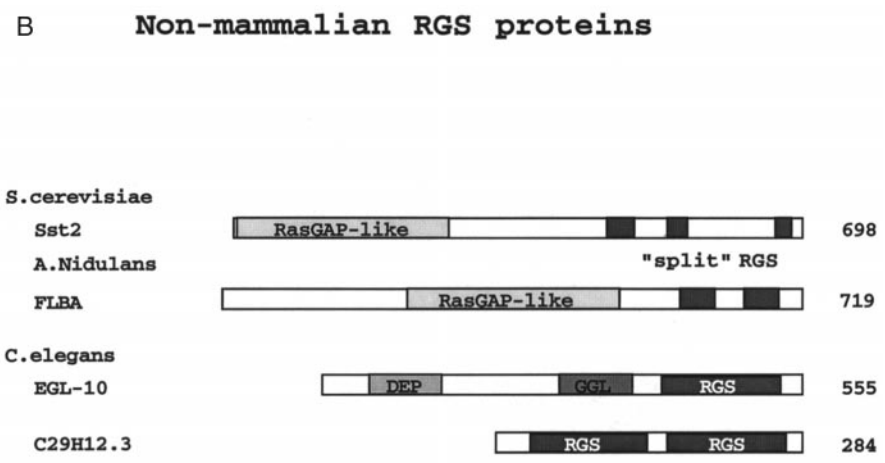


**Figure 2** Schematic representation of mammalian (*A*) and nonmammalian (*B*) regulator of G protein signaling (RGS) proteins. The total number of amino acids for each family member are indicated to the right. RGS, RGS domain. Because of space limitations, most *Caenorhabditis elegans* and none of the *Drosophila* RGS proteins are included. RasGAP, RasGAP-like domain; C, cysteine string domain; Cat, β-catenin binding domain; DEP, DEP domain (Dishevelled/EGL-10/pleckstrin homology); DH, double homology domain; DIX, dishevelled homology domain; GGL, GGL (G protein gamma-like) domain (homology to Gγ); GSK, glycogen synthase kinase 3b binding domain; *, PDZ-binding motif; PDZ, PDZ domain (PSD95/Dlg/ZO1 homology); PH, pleckstrin homology domain; PID, PID domain (phosphotyrosine interacting domain); PKA, PKA-anchoring domain; Raf, B-raf homology domain; T, transmembrane domain.

G protein/effector complexes reconstituted in phospholipid vesicles (33). Their GAP function was later confirmed in vivo in a number of systems. For example, RGS4 and G alpha interacting protein (GAIP) attenuated bradykinin ($G_q$-mediated) and somatostatin ($G_i$-mediated) signaling when overexpressed in HEK293 cells, and this effect was shown to be due to their GAP activity (34).

Second, RGS proteins can act as effector antagonists that prevent G proteins from binding to their effectors by physically blocking this interaction. For example, recombinant PLC-β1 can displace RGS4 bound to $G_q$-GDP-$AlF_4^-$ (which mimics the GTP → GDP transition state). Furthermore, RGS4 is able to inhibit PLC-β1–catalyzed phosphoinositol hydrolysis induced by GTPγS-activated $G_q$ (33).

Third, RGS proteins can alter the number of free βγ subunits available to interact with their effectors by enhancing the affinity of Gα subunits for βγ subunits after GTP hydrolysis, thus accelerating reformation of the heterotrimer. Evidence for this mechanism came from the findings that (*a*) overexpression of RGS4 attenuated MAPK activation by IL-8 ($G_i$) and bombesin ($G_q$), a process that involves the βγ subunit of the G protein (23, 35), and (*b*) overexpression of RGS1,

RGS2, RGS4, and RGS8 accelerated the turning off of G protein–coupled inwardly rectifying $K^+$ channels (GIRKs) (30, 36), known to be activated (opened) by direct binding of βγ subunits to the channel (37, 38). Unexpectedly, overexpression of the same RGS proteins also results in increased activation of GIRKs (30, 36, 39–41). Transfection of a chimeric protein that can act as a βγ ''sink'' reduced the ability of RGS4 to accelerate the kinetics of GIRK activation (39), and the results conflict with the idea that RGS proteins deactivate channels by reducing the number of available βγ subunits. The mechanism whereby RGS protein increases both the activation and the turnoff of GIRK channels remains a mystery.

## The Role of RGS Proteins on GPCR Signaling in Various Cell Types

Considerable work has been focused on demonstrating the importance of RGS proteins in the function of various cell types (Table 1). Every cell type seems to express several RGS proteins (17), but their functions have only begun to unfold. Studies involving in situ hybridization (32, 42–47) or reverse transcriptase–polymerase chain reaction (48) demonstrated that numerous RGS proteins are expressed in the brain, where they display a cell type–specific distribution pattern, but there is little or no information available on which RGSs are found in specific cell types and which GPCR pathways are regulated by each. The presence of many different types of serpentine receptors and G protein–regulated channels in the nervous system suggests that RGS proteins could play a role in the selective regulation of signaling pathways activated by neurotransmitters and psychogenic agents. Indeed there are already indications that this is the case (see below). To decipher the complexity of the regulation of GCPR signaling pathways, including the role of RGS proteins in different parts of the brain, represents a challenging task for the future.

Ten different RGS proteins have been found in cardiac myocytes (49), but the only RGS protein whose functions have been investigated in detail in this system is RGS4. Overexpression of RGS4 attenuated hypertrophy induced by phenylephrine ($G_i$- or $G_q$-coupled) or endothelin-1 ($G_q$-coupled) in neonatal rat cardiomyocytes (50). Also, in both a cell culture model and in living animals, RGS4 mRNA levels increased during cardiac hypertrophy (51). This implies that RGS4 is induced in the heart to keep the process of hypertrophy in check following cardiac overload and suggests the existence of a negative feedback loop for long-term regulation of cardiac hypertrophy.

Secretion of many hormones is mediated by GPCR signaling, but so far little is known about their regulation by RGS proteins in any system. The best examples to date are as follows: (*a*) In Cos cells, overexpression of RGS3 inhibited the ability of gonadotropin-releasing hormone to raise inositol trisphosphate levels (52), and (*b*) in the pancreatic beta TC3 cell line overexpression of RGS2 abolished the ability of glucose-dependent insulinotropic polypeptide (GIP), via its

**TABLE 1** Effects of overexpression of RGS proteins on second messengers and other cellular functions[a]

| RGE | Transfected cell lines | Responses | References |
|---|---|---|---|
| GAIP | HT-29, Caco-2 (colon) | Stimulates $G\alpha_{i3}$-inhibited autophagy | 82 |
| | $LLC\text{-}PK_1$ (kidney) | Retards trafficking of secretory protein | 103 |
| | Dorsal (microinjected) root ganglia | Accelerates GABA- and NE-induced desensitization of N-type $Ca^{2+}$ channel currents | 61 |
| GAIP, RGS4 | NG108 neuronal cells | Reduces leu-enkephalin inhibition of adenylyl cyclase | 33 |
| | HEK293T cells | Reduces SST-induced inhibition of adenylyl cyclase | 162 |
| | | Inhibits IP3 formation by bradykinin | |
| RGS1 | HS-Sultan (B cells) | Reduces PAF-induced MAPK activation | 23 |
| RGS1, −2, −4 | Myocytes, CHO | Accelerates ACh-induced deactivation of GIRK | 36 |
| RGS1, −2, −3, −4, −16 | HEK293 | Reduces IL-8–and carbachol-induced MAPK activation | 92, 36 |
| RGS2, −9 | βTC3 (pancreatic), L293 | Inhibits GIP-induced cAMP response | 53 |
| | Melanophores (electroporation) | Decreases pigment aggregation induced by morphine | 46 |
| RGS3 | HIT-15 (pancreatic) | Reverses inhibition of insulin secretion induced by epinephrine | 163 |
| | COS-1 | Suppresses IP3 release induced by GnRH | 52 |
| | HMC (mesangial) | Decreases ET-1–induced $Ca^{2+}$ response and MAPK activation | 164 |
| | L1/2 cells (lymphoi) | Inhibits chemoattractant (IL-8, MCP-1)-induced migration and integrin-dependent adhesion | 55 |
| RGS3, −8 | HEK293 | Releases carbachol-induced N-type calcium channels inhibition | 165 |

(*continued*)



$G_s$-coupled GPCR, to cause secretion of insulin (53). It is interesting that GIP induced an agonist-dependent interaction between RGS2 and $G_s$, shown by immunoprecipitation in RGS2-transfected L293 cells (53). The mRNAs of at least nine

**TABLE 1** (*continued*) Effects of overexpression of RGS proteins on second messengers and other cellular functions[a]

| RGE | Transfected cell lines | Responses | References |
|---|---|---|---|
| RGS4 | *Xenopus* oocytes (microinjected) CA1 hippocampal neurons | Blocks glutamate-induced activation of $I_{C1(Ca)}$ and $I_{Girk}$ as well as glutamate-induced activation of $I_{AHP}$ | 166 |
| | Cardiomyocytes | Blocks ANF and MLC-2 gene transcription induced by ET-1 and epinephrine as well as myofilament organization and cell growth | 50 |
| | COS-7 | Inhibits bombesin- and dopamine-induced activation of MAPK and IP3 formation | 35 |
| | Pancreatic acini (microinjected) | Inhibits $Ca^{2+}$ mobilization and $C1^-$ current induced by carbachol, bombesin and CCK | 62 |
| | *Xenopus* oocytes (microinjected) | Potentiates μ-opioid agonist-induced GIRK desensitization | 41 |
| | HEK293, CHO-K1 | Induces basal $I_{K(ACh)}$ currents; induces basal N-type $Ca^{2+}$ channels currents | 39 |
| RGS4, −10 | Superior cortical ganglions (intranuclear injections) | Accelerate deactivation of NE-induced N-type calcium channels currents | 167 |
| RGS7 | CHO transfected | Reduces 5-HT–induced $Ca^{2+}$ mobilization | 43 |
| RGS7, −8 | *Xenopus* oocytes | Accelerates dopamine- and ACh-induced GIRK deactivation | 30, 40 |
| RGS9 | CHO | Accelerates decay of quinpirole (dopaminergic agonist)-induced activation of GIRK | 47 |
| RGS12 | NIH3 | Blocks basal and agonist-mediated (thrombin and LPA) SRF activation | 85 |
| RGS16 | EcR-CHO | Suppresses PAF-activated p38 MAPK activation | 168 |
| | COS-7 | Suppresses carbachol-induced MAPK activation | 64 |

[a]ACh, acetylcholine; ANF, atrial natriuretic factor; BK, bradykinin; CCK, cholecystokinin; ET-1, endothelin 1; GABA, γ-amino-n-butyric acid; GIP, glucose-dependent insulinotropic polypeptide; GIRK, G protein–coupled inward rectifying $K^+$ channels; GnRH, gonadotropin-releasing hormone; 5-HT, serotonin; IL-8, interleukin-8; LPA, lysophosphatidic acid; MLC-2, myosin light chain-2; MCP-1, monocyte chemoattractant protein; NE, norepinephrine; PAF, platelet activating factor; RGS, regulator of G protein signaling; SEF, serum response factor.

RGS proteins are expressed in pituitary, but no cell type–specific expression has been documented in this gland (54).

Another system where GPCR signaling pathways are diverse is the immune system. RGS proteins have been shown to play a role in regulating the actions of cytokines/chemokines secreted by immune cells (15). Overexpression of RGS3 in L1/2 pre-B lymphoma cells stably expressing the chemokine IL-8/CXCR1 receptor (coupled to $G_i$) abolished IL-8–triggered chemotaxis (55). The ability of the same cells to bind to vascular cell adhesion molecule 1 when stimulated with formyl-methionyl-leucyl-phenylalanine, which has its own $G_i$-coupled receptor, was also attenuated by overexpression of RGS3 (55). This clearly suggests the involvement of RGS3 in regulating signaling during chemotaxis and cell adhesion signaling. The role of G protein in general and of RGS protein function in the immune system in particular is still in its infancy.

Nearly all the studies to date involve overexpression of RGS proteins. Overexpression studies might give misleading information about the actual role that RGS proteins play within a system. The finding that overexpressed RGS1, RGS2, RGS3, and RGS4 can all complement a yeast strain that is deficient in Sst2 (23) indicates not only that RGS proteins can function in settings that are not physiologically relevant, but also that their RGS domains can act promiscuously. The use of an antisense strategy or RGS knockouts in mice (56–58) will be helpful in determining the specific roles of different RGS proteins.



## How Do RGS Proteins Selectively Act on a GPCR Signaling Pathway?

The mechanisms identified so far are the localization of RGS proteins (cell type–specific and intracellular localization), the timing of their expression (transcription), and the presence of other domains outside of the RGS domain that connect to other signaling pathways (17).

For instance, the distinct regional and cell type–specific localization patterns of RGS mRNAs within the brain suggest that spatial segregation allows RGS proteins to specifically associate with neurotransmitter pathways found within particular brain areas (42). Spatial segregation might also be coupled to developmental regulation, as is the case for RGS9. The small transcript of RGS9 is expressed in total brain in embryonic and early postnatal stages (44) but is specifically expressed only in the retina of adult rats (59, 60).

Domains outside the RGS domain are also important for specificity. This was demonstrated in presynaptic N-type $Ca^{2+}$ channels of dorsal root ganglion neurons, which are inhibited by norepinephrine through $G_i$ and $G_o$ pathways. RGS4 and GAIP can selectively accelerate the turnoff rate of these $G_i$ and $G_o$ pathways, respectively (61). When the N terminus of GAIP was deleted, leaving only the RGS domain and the C terminus, selectivity toward the $G\alpha_o$-mediated pathway was lost. Similarly, the N-terminal domain of RGS4 is capable of discriminating between specific $G_q$-coupled receptors (62). These results collectively imply that

GAIP and RGS4 confer selectivity via two different domains: the RGS domain for Gα specificity and the N-terminal domain for receptor specificity as well as targeting to the PM (see below).

Elucidating the factors that control specificity in the interaction between specific RGS proteins and particular signal transduction pathways clearly requires more research.

## GENE STRUCTURE, TISSUE-SPECIFIC EXPRESSION, AND REGULATION

### Gene Structure and Chromosomal Localization

Although the RGS domain is present in the yeast Sst2 protein, it is not clear how RGS proteins diverged and/or duplicated from this ancestor to give rise to more than 20 mammalian RGS proteins. Analysis of the gene structure of RGS proteins should help clarify the situation.

The genomic structure of only five mammalian RGS proteins has been described to date—RGS2, RGS3, RGS9, RGS16 [also called RGS-r (63) and a28-RGS14p (64)], and Axin (46, 65–68). Gene sizes vary greatly, from 4 kb (RGS16) to 56 kb (Axin), generally reflecting protein size and number of exons. The open reading frames of the small RGS proteins—RGS2, RGS3, and RGS16—are encoded by five exons. The RGS domain itself in RGS2, RGS3, RGS9, and RGS16 is encoded by three exons, and the sites of the two introns in the RGS domain are conserved in RGS2, RGS3, and RGS16, which suggests a common ancestor gene (66, 67). RGSZ1 also possesses two introns in its RGS domain, but the site of the first intron is not conserved, which suggests it diverged separately from the other RGS genes (69). In contrast to the gene structure of other RGS proteins, the Axin gene has its entire RGS domain, located N terminally, encoded in exon 2 (68). The intron/exon boundaries of the RGS domain of the *Caenorhabditis elegans* gene, EGL-10, do not coincide with those of the small mammalian RGS proteins (22, 66).

Recently, based on protein sequence homologies of RGS domains, the existence of six subfamilies of RGS proteins has been proposed (70). So far, data coming from gene structure analysis fit well with these subfamily designations. Exploring the gene structure should facilitate the classification of RGS proteins into subfamilies and will determine sites of alternative splicing.

Chromosomal localization (see Table 2) should be helpful in determining the potential involvement of RGS proteins in genetic diseases. At least seven RGS genes have been mapped to chromosome 1 by radiation hybrid and fluorescent in situ hybridization analyses (65, 67, 71, 72) (L De Vries, unpublished data). The RGS9 gene has been mapped to the same site on chromosome 17 (q23–24) as the retinitis pigmentosa gene, which suggests a potential involvement of RGS9 in this degenerative retinal disease (47). RGS6 has been mapped to chromosome 14q24.3, a region known to contain an early onset Alzheimer disease gene (72).

**TABLE 2** Multiple RGS mRNAs found in tissues and cell types analyzed for their presence by in situ hybridization and Northern blotting[a]

| RGS | Chromosomal localization | Tissue expression | Alternative splicing | References |
|---|---|---|---|---|
| GAIP | 20 | Ubiquitous, low in brain | NC | 21 |
| RET-RGS1 | ND | Retina | Yes | 32 |
| RGSZ1 | ND | Brain | No | 69 |
| RGS1 | 1q31 | B-lymphocytes, lung | Yes | 71, 75 |
| RGS2 | 1q31 | Ubiquitous | No | 169 |
| RGS3 | 9q31 | Ubiquitous | Yes | 23, 66 |
| RGS4 | 1q21 | Brain, heart | No | 23 |
| RGS5 | 1q23 | Ubiquitous | Yes | 170 |
| RGS6 | 14q24.3 | Brain | Yes | 72, 141 |
| RGS7 | 1q42 | Brain, B-cells | Yes | 156 |
| RGS8 | ND | Brain | Yes | 30 |
| RGS9 | 17q23-24 | Retina, neurons | Yes | 47, 60 |
| RGS10 | 10 | Brain | No | 28 |
| RGS11 | 16p13.3 | Brain | Yes | 138 |
| RGS12 | 4p16.3 | Lung, brain, spleen, testis | Yes | 151 |
| RGS13 | 1 | Lung | ND | unpublished data |
| RGS14 | 5qter | Brain, spleen, lung | Yes | 151 |
| RGS15 | ND | ND | ND | unpublished data |
| RGS16 | 1q25-q31 | Retina, pituitary, liver, ubiquitous? | NC | 63, 67 |
| Axin | 16 | Ubiquitous, greatest in thymus, testis | NC | 68 |
| Conductin (Axil) | 17q23-q24 | Lung, thymus | No | 159, 171 |
| D-AKAP2 | ND | Ubiquitous, greatest in testis | Yes | 34 |
| p115RhoGEF | ND | Ubiquitus, leukocytes | Yes | 145 |
| PDZ-RhoGEF | ND | Ubiquitous, low in liver, lung, colon | Yes | 133 |

[a]Information compiled from published data or data submitted to GenBank. For some regulator of G protein signaling (RGS) proteins, little information is available. ND, Not determined; NC, not clear.



## RGS mRNAs Are Widely Expressed and Show Multiple Alternatively Spliced Forms

Multiple RGS mRNAs have been found in all tissues and cell types analyzed for their presence by in situ hybridization and Northern blotting (Table 2). At least nine different RGS mRNAs are expressed in pituitary (54) and in specific regions

of the brain (42). Some RGS mRNAs, such as RGS3, RGS5, and GAIP, show a broad tissue distribution, which suggests a more general function (21, 23, 73). Other RGS mRNAs, such as RET-RGS1 (retina), RGSZ1 (caudate nucleus-brain), RGS8 (brain), and RGS1 (lymphocytes), show a narrow tissue expression (30, 32, 69, 74, 75), hinting that they have more specialized roles. The expression of RET-RGS1 has recently been shown to be restricted to the plexiform layers of the retina, implicating this molecule in retina-specific synaptic transduction rather than in phototransduction (76). The detection of mRNAs of RGS proteins by Northern blot analysis or in situ hybridization has provided useful information on cell type– or tissue-specific expression of these RGS proteins, but only in combination with data coming from tissue distribution, intracellular localization of the protein, and in vivo studies can we hope to establish the function of RGS proteins in specific locations.

The existence of alternatively spliced forms of RGS proteins was suggested when two major transcripts of different size were detected for RGS3 (23). Since then, alternative transcripts have been observed for many RGS proteins, but for the most part they have remained uncharacterized (Table 2). The most interesting examples of alternatively spliced RGS mRNAs are RGS12 and RGS9. RGS12 contains an N-terminal PDZ domain (PSD-95–Dishevelled–ZO1 homology, see below) and an extreme C-terminal PDZ-binding motif (ATFV). Four alternatively spliced forms of RGS12 allow a full combination of presence or absence of both its PDZ domain and its PDZ-binding motifs. Results obtained by surface plasmon resonance suggest that in the case of the alternatively spliced form of RGS12 that contains both motifs, the PDZ domain interacts with the PDZ-binding motif (77). This intramolecular interaction would create an additional regulatory mechanism for RGS12.

The RGS9 gene produces two major alternatively spliced mRNAs that give rise to substantially different C termini. RGS9–1—which is 191 amino acids shorter than RGS9–2—is specifically expressed in the retina, where it serves as a specific GAP for transducin (59, 60), and RGS9–2 is specifically expressed in the striatum, where it is involved in desensitization of $G_{i/o}$-coupled μ-opioid receptors (46, 47). The specific functions of the different C termini of RGS9–1 and RGS9–2 are currently unknown, but this region is likely to contain information that dictates specific interactions (17).

It is clear that alternative splicing of RGS proteins significantly expands the interaction possibilities of these proteins in GPCR signaling pathways and their possible functional consequences.

## Transcriptional Regulation of RGS Proteins: Role in Desensitization

The majority of the data on desensitization or long-term (as opposed to short-term) regulation by RGS proteins fits into the paradigm of the negative feedback loop, summarized as follows: The continuous action of a ligand on a GPCR-

linked signaling pathway increases the level of a particular RGS, which then turns off signaling through one of the three mechanisms mentioned earlier. The negative feedback paradigm was first shown for the yeast Sst2 gene: Prolonged pheromone treatment induces increased transcription of Sst2 mRNA (24, 26). The first mammalian negative feedback loop was described for RGS1, which is induced by platelet activating factor via a GPCR. Transiently induced RGS1 attenuates platelet activating factor–induced MAPK activity (23).

Generally, the levels of G protein expression do not vary significantly under various physiological conditions, but as already indicated, several RGS mRNAs have been shown to do so. This suggests that transcriptional regulation of RGS proteins is an important factor in turning down G protein signaling. But higher levels of a particular RGS mRNA do not necessarily mean more GAP activity on a specific G protein signaling pathway, as regulation at the protein level is also important (see posttranslational modifications). Enhanced expression of RGS proteins would be expected to lead to long-term regulation, resulting in diminished signaling through G protein linked pathways (i.e. desensitization).

RGS mRNAs can be induced by a variety of factors. RGS1 and RGS2 were originally isolated because their mRNA is transcriptionally up-regulated in B-lymphocytes and mononuclear cell mitogens (phorbol myristate acetate) and concanavalin A, respectively (65, 71, 75, 78). It is assumed that they negatively regulate GPCR signaling leading to cell proliferation in hematopoietic cells, where mediation via Gα subunits has been implicated (15). RGS2 mRNA levels increase in response to the calcium ionophore, ionomycin, in mononuclear cells (79) and to GIP, which raises cAMP levels in pancreatic β-cells (53), which suggests that agonist-stimulated $Ca^{2+}$ and cAMP induce RGS2 mRNA. Again, these data fit the negative feedback loop scenario mentioned above.

In brain, RGS2 mRNA is rapidly induced in neurons of the hippocampus, cortex, and striatum by plasticity-evoking stimuli, and thus RGS2 may play an important role in the long-term regulation of neuro- and psycho-pharmacological signaling pathways (80). A ''survey'' study of RGS mRNA expression in the rat striatum after a single amphetamine injection showed that the level of RGS2 mRNA rapidly increases and subsequently decreases, whereas RGS3 and RGS8 mRNAs increase steadily and RGS9 mRNA decreases steadily (81). Prior exposure to amphetamine did not the reduce the induction of RGS2 and RGS3 mRNAs. This suggests that transcription of these genes is not turned off as a result of amphetamine-induced tolerance (81). These findings suggest that transcription of RGS mRNAs are differentially regulated by neuronal stimuli, which may give clues as to the function of RGS proteins.

The mRNA level for GAIP decreases during enterocyte differentiation. This decrease was mimicked in undifferentiated enterocytes by interrupting the $G_{i3}$ GTPase cycle (pertussis toxin treatment or overexpression of a GTPase-deficient mutant). Thus, the expression of GAIP is dependent on $G_{i3}$ activity and on the differentiation state of the enterocytes (82).

RGS16 mRNA is induced by serum and by the p53 tumor suppressor, and overexpression of the protein inhibits the activation of the MAPK pathway (64). These findings define RGS16 as a transcriptionally inducible component of a p53-controlled negative-feedback mechanism involved in cell proliferation and/or apoptosis.

To date, the promoters of relatively few RGS genes have been studied. In the case of RGS2, binding sites for several transcription factors (AP1, CRE, and others) have been described (65). Of particular interest is the fact that the RGS2 promoter also has putative binding sites for NFAT, a transcription factor activated by cyclosporin A. Because cyclosporins are involved in cell cycle progression, this suggests a role for RGS2 in regulating cell cycle progression of mononuclear cells (83). The RGS16 promoter probably contains serum response elements and p53 binding sites, because both serum and p53 induce RGS16 gene expression (64).

An important issue that has not been addressed is the role of RGS mRNA stability on the expression levels of RGS proteins. The exception is the study of the RGS4 mRNA, whose half-life of 3 h remained unchanged after forskolin treatment of PC12 cells (84).

There is evidence that RGS proteins can themselves indirectly regulate the transcription of other genes. A recent and very interesting example is the inhibitory role of RGS12 in the activation of the serum response factor, which is mediated by $G_q$ and $G_{12}$ (85, 86).

Promoter analysis and studies on transcriptional regulation of RGS genes should contribute significantly to our knowledge of incoming signaling pathways that induce or silence their expression.



## INTRACELLULAR LOCALIZATION OF RGS PROTEINS

### Posttranslational Modifications

Posttranslational modifications of signaling proteins generally play a role in their cellular localization and their stability and/or conformation. Palmitoylation (on cysteine residues in the N terminus) and myristoylation (on glycine residues in the N terminus) are two such modifications that have been reported to play a role in the affinity of G proteins for membranes and their translocation from the cytosol to the plasma membrane (87, 88). A parallelism can be drawn for certain RGS proteins whose posttranslational modifications may also affect their function.

GAIP, RGSZ1, and RET-RGS1 have a cysteine string motif in their N terminus (32, 69, 89). This motif is heavily palmitoylated in the cysteine string protein family and is responsible for their membrane attachment (90). Palmitoylated GAIP is detected only in membrane fractions (89), but the direct involvement of the cysteine string motif as well as of the palmitoylation itself in membrane association, although likely, remains to be demonstrated. It is intriguing that

RGS4, which lacks a cysteine string motif, can also be palmitoylated, but palmitoylation is not required for the translocation of RGS4 from the cytosol to the PM. Instead, the entire N terminus seems to be necessary for its PM localization (91). Similar results were obtained for RGS16, which contains the same conserved palmitoylation sites as RGS4 and RGS5. The level of its membrane association and its in vitro GAP activity was unchanged in palmitoyl-defective RGS16. However, its negative effect on $G_i$ (cAMP levels) and $G_q$ (MAPK activity) pathways was significantly attenuated in vivo (92). The authors suggest that palmitoylation might be important for localizing RGS and G proteins on the same membranes or membrane microdomains or it might play a role in the affinity of these proteins for one another.

Several RGS proteins contain putative myristoylation sites in their N termini, but the function of myristoylation, including its role in membrane attachment of RGS proteins has not yet been investigated.

Many of the components of the GPCR cascade undergo phosphorylation, including receptors (93), α and β subunits of G proteins [for review see Neer (94)] and effectors (95). Indications are that this may also be true for RGS proteins. Several RGS proteins, including GAIP (21), contain conserved potential phosphorylation sites for casein kinase II and for protein kinase C in their RGS domain as well as nonconserved sites outside their RGS domain. Recently it was shown that GAIP is phosphorylated and that only the membrane-associated pool is phosphorylated (95a). This suggests that the membrane association of GAIP may be regulated in part by phosphorylation. Putative phosphorylation sites are located in GAIP's RGS domain, which could regulate its GAP activity, and in its N terminus, which could modify its membrane association. Recombinant GAIP could be phosphorylated at its N terminus by purified casein kinase II, and Ser24 was identified as one of the phosphorylation sites (95a). The role of phosphorylation of RGS proteins has not yet been been linked to their function. Phosphorylation can be expected to represent an important regulatory event in controling the effect of RGS proteins on G protein signaling and localization.

## Membrane Attachment and Intracellular Localization

The specificity of an RGS protein depends on its intracellular localization: It cannot assume its role as a GAP without making direct contact with its Gα partner. G proteins are classically assumed to be localized on the cytoplasmic face of the PM in close contact with transmembrane receptors, but some Gα subunits have also been localized on intracellular membranes (96, 97), where they have been assumed to play a role in vesicular trafficking (18). G proteins have also been detected on caveolae and reported to interact directly with caveolin (98). This suggests RGS proteins may also be found on several types of membranes, including membrane microdomains defined as lipid rafts, which are rich in cholesterol and sphingolipids (99).

Most RGS proteins are present in the cell in two pools, a cytosolic and a membrane-bound pool, and the available evidence suggests that many RGS proteins can be translocated from a cytoplasmic pool onto membranes (17, 25).

Few RGS proteins have been precisely localized inside the cell. Sst2, GAIP, RGSZ1, RGS3, RGS4, RGS7, and RGS9 are all membrane bound to a certain extent (25, 52, 59, 60, 69, 89, 91, 100, 101), but only RGS4, Sst2, RGS9, and GAIP have been localized at the subcellular level. Both membrane-bound and cytosolic pools of endogenous GAIP were found whose ratio seems to depend on the cell type (89, 102–104). By immunoelectron microscopy, endogenous GAIP was localized on clathrin-coated pits and vesicles (CCVs) close to the PM in liver and on budding CCVs in the *trans* Golgi region in pituitary (102), which suggested a role for GAIP in vesicular transport. In addition, GAIP on isolated liver CCVs was shown to be an active GAP in vitro for $G_{i3}$ (105). GAIP was also reported on nonclathrin-coated vesicles close to Golgi stacks in LLC-PK1 epithelial cells (103). In this cell type, overexpression of GAIP retarded trafficking along the secretory pathway. Sst2 was found both at the PM and in the Golgi region by immunofluorescence in yeast (25). The membrane attachment of RET-RGS1 is likely because it has a putative transmembrane region and a cysteine string motif (32). RGS9, the specific GAP for transducin, is tightly associated with membranes and not detectable in a cytosolic form (59, 60). The presence of a DEP domain (from Dishevelled, EGL-10, and pleckstrin) in the N terminus of RGS9 might facilitate its tight membrane attachment (see below), but electrostatic interactions with membranes have also been proposed (59).

The membrane-bound pool of RGS4 localizes at the PM and overexpression of the activated form of $G_{i2}$ ($G_{i2}$Q205L) results in translocation of RGS4 from the cytoplasm to the PM, as detected by immunofluorescence in 293T cells (100).

Each RGS protein may have its own mechanism for binding to membranes, but a common factor seems to be that attachment of RGS proteins to membranes in the proximity of Gα subunits would enhance their performance, and translocation could be a supplementary regulatory component. One key question remains: If multiple RGS proteins are expressed and localized on membranes in the same cell, are they associated with the same or with different membranes?

In summary, the subcellular distribution of RGS proteins is still poorly documented, yet in order to elucidate the G-protein signaling pathways they regulate, their precise intracellular localization is essential.



## MOLECULAR STRUCTURE OF RGS PROTEINS

### Primary Structure of RGS Proteins

An alignment of RGS domains from different mammalian RGS proteins is depicted in Figure 3. From the alignment it can be seen that most of the RGS domains (RGS1–16, GAIP, RGSZ1, RET-RGS) are closely related, displaying

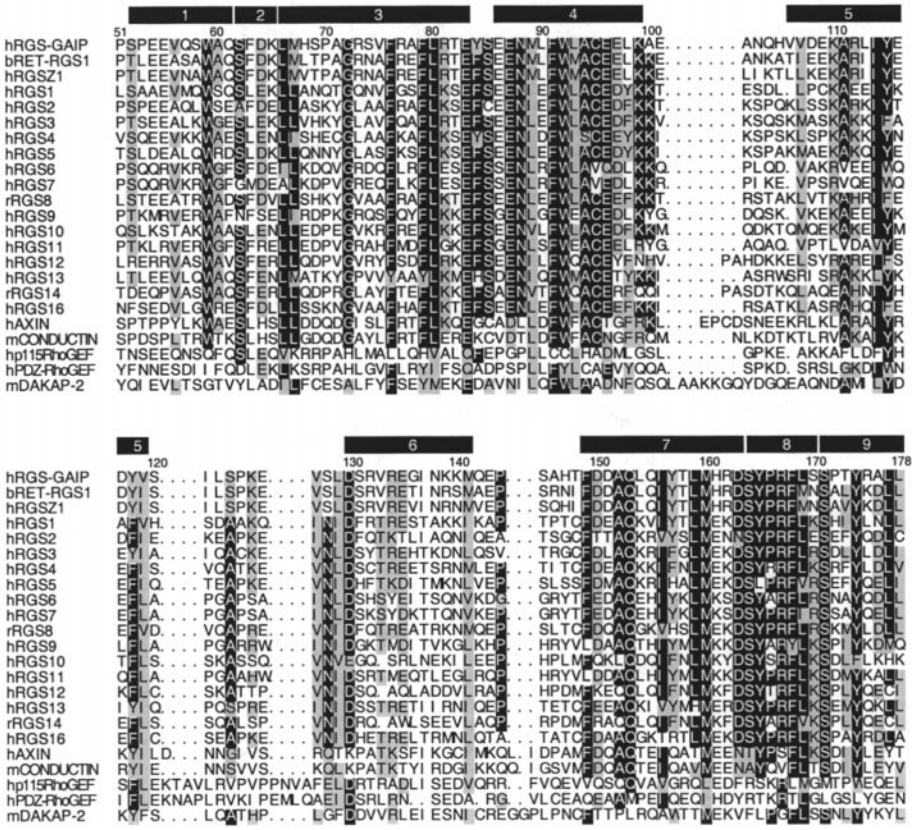


**Figure 3** Alignment of RGS domains for mammalian regulator of G protein signaling (RGS) proteins. Amino acid sequences of RGS domains from 23 mammalian RGS proteins were aligned using Clustal W 1.7 and manual adjustments. (*Black, gray*) Conserved residues and similar residues, respectively, shaded by MacBoxshade2.15. The regions of RGS domains are defined based on work by Tesmer et al (106). Regions of the nine alpha helixes found in RGS4 are indicated (*black frames above the alignment*). h, *Homo Sapiens;* m, *Mus musculus;* r, *Rattus norvegicus;* b, *Bos taurus.* The accession numbers for the sequences used are as follows: hRGS-GAIP, P49795; bRET-RGS1, P79348; hRGSZ1, NP_003693; hRGS1, NP_002913; hRGS2, NP_002914; hRGS3, P49796; hRGS4, P49798; hRGS5, NP_003608; hRGS6, NP_004287; hRGS7, AAD34290; rRGS8, BAA23680; hRGS9, AAC64040; hRGS10, NP_002916; hRGS11, AAC69175; hRGS12, O14924; hRGS13, NP_002918; rRGS14, O08773; hRGS16, O15492; hAxin, AAC51624; mConductin, AAC26047; hp115-RhoGEF, NP_004697; hPDZ-RhoGEF, BAA20834; mD-AKAP2, AAC61898.

approximately 35–55% identity, whereas the RGS domains in Axin, D-AKAP2, conductin, p115 RhoGEF, and PDZ-Rho GEF are more distantly related to the others, sharing only ~10–30% identity.

By contrast, the regions outside the RGS domain are diverse (Figure 2). Some RGS proteins are relatively small, approximately 20–25 kDa in size, and contain very short regions around the RGS domain. Others contain large N-terminal and/or C-terminal regions. The regions outside the RGS domain usually have other structural and functional features, such as coiled-coil, DEP, DH, GGL, PDZ, PH, and PTB domains (discussed below).

RGS proteins have also been found in other eukaryotic organisms. In the *Saccharomyces* Sst2 protein and the *Aspergillus* FlbA protein, the RGS domains are interrupted and divided into segments (Figure 2*B*), which reportedly would not cause dramatic changes in their three-dimensional structure (106). Analysis of the nearly complete *C. elegans* genome revealed that it has 12 RGS domain–containing proteins (107). One contains two RGS domains (Figure 2*B*). In *Drosophila*, four RGS domain–containing proteins—dRGS7 (108), Loco (109), d-Axin (110), and dRhoGEF2 (111)—have been identified. The functional roles of most of the invertebrate RGS proteins remain to be investigated.

An intriguing finding is that the N-terminal region of most GRKs share significant sequence similarity (up to 30%) with the RGS domain (27). The function of this region in GRKs is currently under active investigation.

## Three-Dimensional Structure of RGS Proteins

***RGS4-Gi1 Complex*** The crystal structure of the RGS domain of RGS4 in complex with $G_{i1}$ in its transition state (RGS4-$G_{i1}$-$Mg^{2+}$-GDP-$AlF_4^-$) was determined by Tesmer and colleagues (106). In this complex, the RGS domain is made up of nine alpha helices that form two subdomains—a terminal subdomain (α1–α3, α8–α9) and a four-helix bundle subdomain (α4–α7) (Figure 4). The base of the four-helix bundle, including the α3–α4, α5–α6, and α7–α8 loops, constitutes the $G_{i1}$-contacting surface. In $G_{i1}$, the RGS4 binding sites are located in the three switch regions, where there are dramatic conformational changes during the GTP cycle and GTPase hydrolysis (112).

***Mechanism of GAP Activity: Comparison with GAPs for Small GTPases*** Besides $G_{i1}$-RGS4, the crystal structures of several small GTPases in complex with their corresponding GAPs were also resolved recently, including Ras-RasGAP (113), Rho-RhoGAP (114), Cdc42-Cdc42GAP (115), and ARF1-ARFGAP (116). The structures of these complexes revealed two common features that characterize the molecular mechanism of GTPase activation: (*a*) A catalytic ''arginine finger'' is present at the active site of the GTPase in *trans* (from the GAP) or in *cis* (from the GTPase), and (*b*) switch regions of the GTPase, particularly a catalytic glutamine residue, are stabilized on the binding of the GAP to the GTPase (117–119).

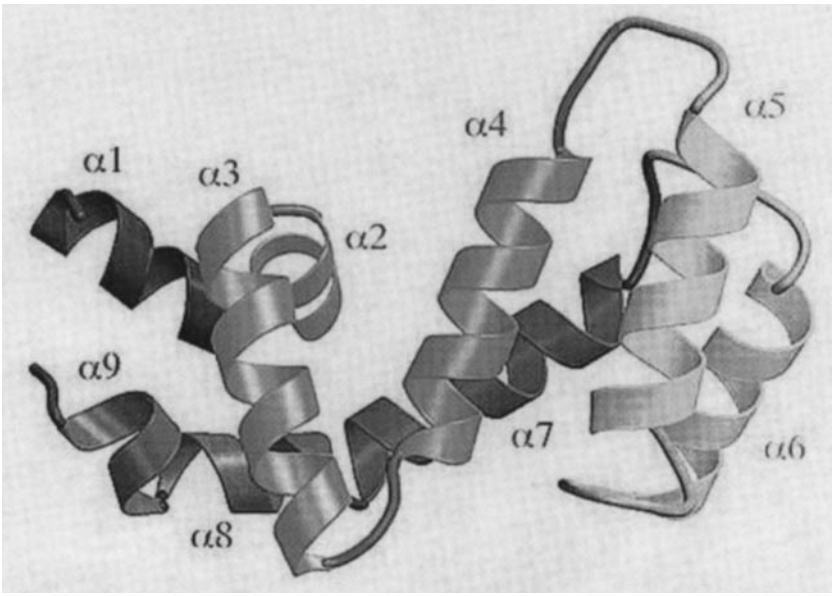


**Figure 4** Ribbon diagram depicting the tertiary structure of RGS4. The RGS4 box consists of nine helices that form two subdomains. The terminal subdomain is formed by α1, α2, α3, α8, and α9, and the bundle subdomain is formed by α4, α5, α6, and α7. The majority of residues that contact $G_{i1}$ are found along the bottom of the bundle subdomain, as shown here. Insertions found in lower eukaryotes occur at the top of the bundle subdomain, opposite the $G_{i1}$-binding surface, and between α1 and α2 (106).

It was shown that both RasGAP (113) and RhoGAP (114) contribute a highly conserved arginine finger to the active site of Ras and Rho, respectively. At the same time, they can also stabilize the switch regions of the GTPase through protein-protein interaction. Unlike RasGAP and RhoGAP, it was deduced that RGS4 does not directly contribute an arginine residue or any other catalytic residue to the active site of $G_{i1}$ for GTP hydrolysis (106). Subsequent mutational studies (discussed below) confirmed this assumption. In fact, the Gα subunit has a ''built-in'' arginine residue in the extra helical domain that projects into the catalytic active site in the opposite direction compared with Ras and Rho (117). It is inferred that RGS4 exerts its GAP activity mainly through binding to the switch regions, reducing the flexibility and stabilizing the transition state of $G_{i1}$ (106). In the case of ARF1-ARFGAP complex, the arginine finger is speculated to be supplied by the ARF effector coatomer instead of ARFGAP or ARF1 itself (116). Similar to RGS4, ARFGAP functions by stabilizing the switch regions.

## Mutational Analysis of RGS Proteins

Mutagenesis has been used to study the mechanisms of GAP function and to search for dominant negative mutants of RGS proteins that would be useful for in vivo studies.

Early experiments demonstrated that the intact RGS domain itself is sufficient and necessary for the GAP activity in vitro but not for functionin vivo (31, 32, 54). Before the crystal structure of the RGS4-$G_{i1}$ complex was known, Chen et al (54) performed mutagenesis analysis on the RGS domain of RGS16 based on the conserved residues in several RGS proteins. For example, the RGS16 (G74R) mutant that retained binding ability to the transition state of $G_i$ fails to attenuate pheromone signaling in yeast (54). This showed for the first time that the physical interaction between the RGS domain and the Gα subunit could be separated from the GAP activity of the RGS protein on G protein signaling pathways. These results, however, need to be confirmed in a mammalian signaling system.

***Implications for the Mechanism of GAP Activity*** With the solving of the crystal structure of RGS4-$G_{i1}$, several mutagenesis studies have been conducted to further investigate the mechanisms of GAP activity. From the crystal structure of the RGS4-$G_{i1}$ complex, the Asn128 residue of RGS4 appears to be important for GAP activity by making contact with the catalytic glutamine residue of $G_{i1}$ (106). The RGS4 (N128A) mutant essentially lost all its GAP activity on $G_i$ and its affinity for $G_{i2}$ in vitro, which confirms that this residue is critical for RGS4 activity (120). When different Asn128 mutants of RGS4 (Ala, Gly, Phe, Ser, Val) were tested on four Gα targets ($G_o$, $G_{i1}$, $G_q$, and $G_z$) by two distinct GAP assays (solution-based single-turnover assay and vesicle-based steady state assay), several mutations significantly decreased the apparent affinity for Gα subunits but showed only modest effects on GTP hydrolysis (121). Based on these findings, it was suggested that Asn128 of RGS4 is predominantly involved in Gα substrate binding. Similarly, when the equivalent Asn131 residue in RGS16 was mutated (into Ala, Asp, Gln, His, Leu, Ser) or deleted (122), the mutants showed substantial decreases in their ability to bind $G_t$ to different degrees. The GAP activity of four of these mutants was essentially abolished, but two, N131S and N131Q, retained partial GAP activity ($V_{max}$ ~80% and 60%, respectively).

It is intriguing to note that the critical N128 residue in RGS4 is not strictly conserved in all RGS proteins; it can be a Ser (GAIP, RET-RGS1, and RGSZ1), Gln (Axin), or Gly (D-AKAP2) (Figure 3). GAIP with a Ser in the equivalent position is a GAP, but no GAP activity has yet been demonstrated for Axin. A recent nuclear magnetic resonance study on the RGS domain of GAIP revealed it has a nine-alpha helical structure similar to that of RGS4 (123). The major differences between the RGS domains of RGS4 and GAIP are a displacement in the packing of some of the helices and the relative orientation of the loop connecting helices α5 and α6, where the critical residue for GAP activity is located (RGS4-Asn128, GAIP-Ser156). Biochemical data have shown that GAIP has comparable binding affinities for the transition state (GDP-$AlF_4^-$) and the GTP-

bound state of $G_{i3}$ (89), whereas RGS4 has a much higher affinity for the transition state than for the GDP- or GTP-bound states of $G_i$ (124). The connection between the structural variations and the functional differences between these two proteins is still unknown. Further understanding is likely to come from structural studies of other RGS domains.

An extensive mutational analysis of the RGS domain of RGS4 using GAP activity and Gα binding properties of these mutants supports the idea that RGS4 exerts its GAP activity primarily through stabilizing the transition state conformation of the switch regions of the Gα subunit (120). All the GAP-defective RGS4 mutants tested were unable to bind Gα in vitro. Besides residues directly involved in the RGS4-Gα binding, other conserved residues distal to the interface are also critical for GAP activity, which suggests these residues may contribute to the overall conformational stability of RGS4.



***Dominant Negative Mutants*** Dominant negative mutants, i.e. those that uncouple the interaction between Gα subunits and RGS proteins, represent valuable tools to determine the importance of a particular RGS protein in a specific signaling pathway. Mutational analysis and genetic screening have been employed to search for dominant negative mutants of RGS proteins and Gα subunits, respectively.

Evidence was obtained that R167M/A and F168A mutants of RGS4 are incapable of binding to the transition state of $G_{i1}$. Instead, in contrast to the wild-type protein, they bind preferentially to the activated (GTPγS bound) form of $G_{i1}$ (124a). More intriguing, it was shown that these mutants serve as RGS antagonists in a $G_{i1}$ GAP assay and in an IL-8–induced $G_i$-mediated MAPK activation assay. However, the dominant negative effects of these mutants were not substantial. For example, the GAP activity of wild-type RGS4 was inhibited only 25% by a 10-fold excess of R167A mutant. It would be interesting to test similar mutants of other RGS proteins to examine their potential dominant negative roles.

Two recent reports demonstrated the usefulness of Gα mutants for uncoupling RGS-Gα interaction. A yeast Gα mutant ($Gp\alpha 1^{sst}$) in which a conserved Gly residue in the switch I region was mutated into Ser was obtained that shows reduced binding to Sst2p (125). A similar mutation in $G_o$, $G_{i1}$, and $G_q$ was shown to be insensitive to the GAP activity of RGS4 and RGS7, presumably because of the low affinity between the RGS proteins and the Gα mutants (125, 126). The Gly-to-Ser mutations in Gα did not significantly change effector coupling, intrinsic GTPase hydrolysis, or GDP release behavior. These Gα mutants represent useful tools to study the relationship of an endogenous RGS protein and a particular Gα subunit.

## Relationship Between Gα Effectors and RGS Proteins

***Structural Basis for RGS as an Effector Antagonist*** As indicated earlier, biochemical data have suggested that besides GAP function, some RGS proteins serve as effector antagonists. It was shown that RGS4 and GAIP are able to

compete with phospholipase Cβ (PLCβ) for binding to GTPγS-$G_q$, and to inhibit the activation of PLCβ by GTPγS-$G_q$ (33). Similar partial attenuation effects were also observed for RGS16 (33), RGS4 (127), and GAIP (127) with $G_t$ and the cGMP phosphodiesterase γ subunits (PDEγ), the inhibitory subunit of PDE. These results are in agreement with the initial prediction based on the crystal structure of RGS4-$G_{i1}$ that Gα-effector binding sites may partially overlap with $G_i$-RGS binding sites at the switch regions (106). However, subsequent analysis of the crystal structure of $G_s$ associated with the catalytic domain of adenylate cyclase indicated that $G_i$ may be able to accommodate RGS4 and adenylate cyclase simultaneously (128). Mapping studies of PDEγ-binding sites on $G_t$ also suggested that there is no significant overlap between PDEγ and RGS16 binding surfaces on $G_t$ (129), but it should be noted that RGS16 is not the physiological partner for $G_t$.

Thus, the structural basis for the competition between RGS and effector is not yet well established. It is to be hoped that the structural analysis of other Gα-effector complexes will provide insight into the mechanisms underlying the effector-antagonist behavior of some RGS proteins.

***Cooperative Action Between Effector and RGS*** It has become clear that an effector-antagonist function does not apply to every RGS protein. Instead, unlike the RGS proteins mentioned above, the GAP activity of RGS9 on $G_t$ is enhanced by the PDEγ subunit (60). Similar to RGS9 and PDEγ, ARFGAP and the ARF effector, coatomer, were shown to have a synergistic effect on the GAP activity of the small GTPase ARF (116). The kinetic analysis of RGS9 binding to the $G_t$-PDEγ complex suggested that there was no steric hindrance to assembly of the ternary $G_t$-RGS9-PDEγ complex, which may provide the structural basis for this cooperative action (130). A more recent study (131) suggested that the α3-α5 region in the RGS domain of RGS9 is responsible for the effect of PDEγ on the GAP activity of RGS9 for $G_t$. It would be interesting to study whether this cooperative action is a unique feature for RGS9 or whether it is also found for other RGS proteins, at least for the closely related RGS6, RGS7, and RGS11 proteins.

## RGS-Gα Partner: Specificity or Promiscuity?

One major question in the RGS field is whether there is specificity in the RGS-Gα interaction. Most of the RGS proteins tested so far are GAPs for $G_i$ and/or $G_q$, except that p115RhoGEF is a specific GAP for $G_{12}$ and $G_{13}$ (132) and probably also PDZ-RhoGEF (133). No RGS protein has yet been found to serve as a GAP for $G_s$. This has been explained by the finding that Asp229 in the switch 2 region of $G_s$ is a major structural barrier to its interaction with known RGS proteins (134, 135).

Regarding specificity toward different $G_i$ and $G_q$ subunits, in vitro data accumulated from GTPase assays show that some RGS proteins (e.g. RGS4) are relatively promiscuous, displaying little preference for different $G_i$ and $G_q$ subunits,

whereas others (e.g. RGSZ1) have selectivity for certain Gα subunits. RGS2 was shown to bind selectively to $G_q$ and had no GAP activity on $G_i$ in a solution-based single-turnover GTPase assay (136). However, in a reconstituted lipid vesicle–based GAP assay, RGS2 displayed GAP activities to both $G_{i1}$ and $G_q$ (80). These results are an indication of the limits of in vitro assays, and assessments of the specificity of RGS proteins for their Gα partner(s) should include in vivo approaches whenever possible.

GAIP interacts more strongly with $G_{i3}$, $G_{i1}$, and $G_o$ than with $G_{i2}$ (89). A single residue at the far N terminus of switch 3 in $G_i$ (Asp 229 of $G_{i1}$ and the equivalent Ala 230 of $G_{i2}$) was shown to be the determinant for the selectivity for $G_{i1}$ over $G_{i2}$ (137). RGS9 was shown to have a high specificity for $G_t$ over other $G_i$ subunits in a GAP assay using urea-washed rod-outer-segment (ROS) membranes, and the molecular determinant for this specificity appears to be located in the helical domain of $G_t$ (130). Finally, by an unknown mechanism, RGSZ1 displayed great selectivity of binding affinity and GAP activity for $G_z$ over other Gα subunits (69).

Although only a few examples of selective in vitro interaction between RGS and Gα partners have been shown so far, greater specificity of RGS proteins for different Gα subunits may arise in vivo because of different tissue expression patterns and different subcellular localizations of RGS proteins from their Gα partner.



## NON-RGS DOMAINS IMPLY LINKS TO OTHER SIGNALING PATHWAYS

### GGL (Giggle) and DEP Domains

RGS6, RGS7, RGS9, RGS11, and EGL-10 (*C. elegans*) contain a domain with homology to Gγ subunits, termed G protein gamma-like (GGL or Giggle) (138) and a DEP domain (from Dishevelled, EGL-10, and Pleckstrin) (144). The fact that the 100–amino acid-long DEP domains are found exclusively in RGS proteins that have GGL domains suggests some functional link between the two. A first step toward understanding the role of the GGL domain was made when RGS7 was identified in a complex with the G protein β5 subunit in the retina (139) and when RGS11 was shown to specifically interact with Gβ5 (138). The photoreceptor-specific RGS9 protein also forms a complex with the long splice variant of Gβ5 (140). In the case of RGS6, the specificity of interaction was narrowed to a specific tryptophan residue in the GGL domain (141). More detailed studies revealed that Gβ5-RGS/GGL complexes may play a role in the affinity or selectivity for Gα subunits (138, 142). The functional implication of Gβ5 binding to the GGL domain in RGS proteins is still not clear and should be studied in its physiologically relevant context.

The presence of GGL domains in some RGS proteins significantly extends their interaction possibilities and suggests that these RGS proteins are part of a macromolecular complex that includes the receptor, G protein, and effector. This would certainly enhance the overall efficiency of the signaling pathway.

The DEP domain present in all RGS proteins that have a GGL domain may dictate membrane localization, as the DEP domain of the Dishevelled protein from *Drosophila* is responsible for localization of the protein to membranes (143). In keeping with this assumption is the finding that EGL-10 fused to GFP localizes to sarcoplasmic reticulum–like structures in *C. elegans* (22), and RGS9 is tightly attached to ROS membranes (59, 60), but this remains to be confirmed for other members of this RGS subfamily (70).

## DH/PH Domains and Links to Small GTPases

An exciting direct link between heterotrimeric G proteins and small GTPases was recently unveiled, and the linking molecule p115 RhoGEF belongs to the RGS family. p115RhoGEF was discovered as a GEF of Rho GTPase, whose GEF activity is mediated through its DH (double homology) domain (145). Activated $G_{12}$ and $G_{13}$ were shown to induce cytoskeletal and mitogenic changes typically transmitted by the Rho family of small GTPases, but the nature of the connection to Rho remained obscure (146, 147). This link became obvious when it was demonstrated that activated $G_{12}$ and $G_{13}$ stimulate the GEF activity of p115RhoGEF, which has an N-terminal RGS domain that shows GAP activity on $G_{12}$ and $G_{13}$ (132, 148). This connection solved the longtime outstanding enigma of how GPCR ligands such as thrombin and lyso phosphatidic acid can induce cytoskeletal changes in the cell (149). Thus, p115RhoGEF serves as an effector molecule for $G_{13}$, which activates the GEF function of the DH domain by $G_{13}$/RGS domain interaction. The RGS protein p115RhoGEF occupies a crucial position in the signaling pathway that links an extracellular ligand via a heterotrimeric G protein to a small G protein that promotes downstream morphological changes in the cell. It is also the first example of an RGS protein that acts as an effector.

The Rho GEF family has recently been expanded to include p115RhoGEF, PDZ-RhoGEF, and the lsc oncogene. All have PH domains (pleckstrin homology) located immediately C-terminal of their DH domains and are required for the in vivo activity of these GEFs (150). PH domains may also facilitate translocation to membranes (150), but this remains to be verified.

Although p115RhoGEF is a GAP for both $G_{13}$ and $G_{12}$ in vitro, its GAP activity was 10-fold higher on $G_{13}$ than on $G_{12}$. Furthermore, $G_{12}$ does not stimulate the GEF activity of p115RhoGEF and does not activate the downstream serum response factor (85, 86, 132, 148). Clearly, the relationship between $G_{12}$ and p115RhoGEF still needs to be worked out, but some of the possibilities include the following: (*a*) $G_{12}$ activates the GEF of another family member of

p115RhoGEF, or (*b*) $G_{13}$ but not $G_{12}$ interacts with an additional site in p115RhoGEF.

A new member of the RhoGEF family, PDZ-RhoGEF, that also interacts with $G_{12/13}$, was shown to have a PDZ domain N-terminal of its RGS domain (133). The role of the PDZ domain in this molecule remains unknown, but it was suggested that it may interact with the C terminus of the lyso phosphatidic acid receptor, which is coupled to $G_{12/13}$ and has a putative PDZ binding motif.

Another example of a link between heterotrimeric and small G proteins is provided by RGS14. It was also reported (151) to be a Rap1/Rap2 interacting protein (GenBank# U85055), which suggests that RGS14 might also link to small GTPases. Although the Rap1/Rap2 binding site in RGS14 has not been established, a 70–amino acid region with homology to B-raf kinase, an effector of Ras, is a potential candidate. In Raf kinase, this region homologous to B-raf kinase is part of the Ras-binding site, and the Raf-binding domain of Ras (amino acids 32–40, the effector domain) is very conserved in Rap1A (115). It is important to determine the in vivo Gα specificity of RGS14 to establish which signal transduction pathways RGS14 might connect.

## PDZ and PDZ Binding, PID/PTB and PKA Anchor Domains

Two RGS proteins contain PDZ domains, which bind to consensus C-terminal motifs in target proteins and play an important role in organizing protein networks on membranes (152). Generally, a PDZ domain in one protein binds to a PDZ-binding motif in another. RGS12, the largest RGS protein described so far, has an N-terminal PDZ domain that binds in vitro to the CXCR2 interleukin-8 receptor, but its in vivo target remains to be identified (77, 151). The C-terminal PDZ binding motif of RGS12 is also in search of a partner, but theoretically it could form an intramolecular link with its own PDZ domain. GAIP also has a PDZ binding motif in its short (extra-RGS) C terminus. A novel protein—GIPC (for GAIP interacting protein C terminus)—was recently isolated that binds, via a central PDZ domain, to the C terminus of GAIP (153). GIPC's function is unknown but, like GAIP, it is found on vesicles close to the cell membrane, which suggests a role in intracellular transport. Recently, GIPC was also isolated through interaction of its PDZ domain with the C terminus of several transmembrane proteins, including the glucose 1 transporter (154) and semaphorin F (154a). This suggests that GIPC, like other PDZ domain proteins, may serve to cluster signaling molecules and membrane proteins (152).

RGS12 contains a PID/PTB or phosphotyrosine interacting domain, that directly binds to a phosphotyrosine residue on the alpha subunit of the N-type $Ca^{2+}$ channel (M Diversé, personal communication).

The protein kinase A (PKA) anchoring protein, D-AKAP2, has an N-terminal RGS-like domain and a C-terminal region that binds the regulatory subunits of PKA (PKA anchor), but no GAP activity or Gα binding was yet shown for this

protein (34). D-AKAP2 could form a novel link between G protein signaling and cAMP signaling, in addition to the established $G_s/G_i$–adenylate cyclase pathway, but the functional implications of this connection remain to be established.

## PEST Domains

PEST (proline, glutamine, serine, threonine-rich) domains reportedly confer protein instability and are often found in signal transduction molecules with rapid turnover (155). RGS3 has four PEST domains in its N terminus (23), and GAIP also has one (L De Vries, unpublished results). RGS7 has two PEST sequences within its GGL domain and is rapidly degraded via the ubiquitin-proteosome pathway unless it interacts with the C-terminal tail of polycystin, an integral membrane protein (156). Rapid degradation and/or stabilization through interaction with membrane proteins could become a more general mechanism of regulation of RGS proteins.

## Wnt Signaling

Axin and its homolog conductin (also named axil or axin-like) are important scaffold proteins in the Wnt signaling pathway, which involves the seven transmembrane Frizzled receptors (with no G protein connection demonstrated yet) and plays a role in cell-cell signaling and tissue development (157). Both axin and conductin negatively regulate Wnt signaling and have an N-terminal RGS domain for which an interacting Gα partner remains to be found (68, 158–160). However, the RGS domain of both proteins was shown to interact directly with the tumor suppressor, adenomatous polyposis coli (APC), a non–Gα-like molecule, which suggests that APC and a Gα might compete for the RGS domain. The region in APC that interacts with conductin is limited to three previously unidentified repeated motifs with the SAMP (Ser-Ala-Met-Pro) signature; mutation of the serine to alanine in the signature abolished binding to the RGS domain (158, 161). Deletion of the RGS domain down-regulates signaling through β-catenin or its *Drosophila* homolog armadillo (68, 110), which normally activates specific transcription factors inducing genes involved in cell adhesion, morphology, and motility. These results imply that Axin and conductin are involved in switching the cell from a proliferative to a more differentiated state. Future research should establish whether the Frizzled receptors are true GPCRs and which Gα subunit binds to the RGS domain of axin/conductin. This would also strengthen the association of heterotrimeric G proteins and RGS proteins with developmental processes.

## SUMMARY

In this review we have summarized what is known to date on RGS proteins. The RGS protein family is only 4 years old, and already it has had a major impact on the way we think about the mechanisms of G protein signaling. The data available

today reflect major progress in our understanding of the molecular mechanisms of the RGS-Gα interaction, but we have only begun to recognize the importance of factors that regulate RGS function, such as tissue-specific expression, transcriptional and posttranslational modifications, and intracellular localization. In order to establish the specificity of RGS proteins on the vast number of G protein signal transduction pathways implicated in processes such as cell proliferation, differentiation, motility, and vesicular trafficking, many more in vivo studies will be necessary.

## PERSPECTIVES

The fact that levels of RGS proteins are tightly regulated suggests their importance in influencing G protein–coupled signaling pathways. Dysregulation might lead to a pathological state. For example, a defect in the transcriptional machinery that leads to overexpression of RGS proteins could affect the turnoff of GPCR signaling. Likewise, if there is a defect in the transcriptional machinery so that RGS proteins could not be expressed, or if RGS proteins are mistargeted and thus become nonfunctional, G protein signaling would be prolonged. The observation of pathological phenotypes in either transgenic or targeted knockout mice as well as chromosomal mapping will be helpful in determining the role of RGS proteins in normal cell processes as well as in disease. It should be noted, however, that several Gα knockout mice did not show evident phenotypes. The same may hold for RGS knockout mice.

Given that overexpression of RGS proteins alters signaling cascades, RGS proteins represent potential targets for therapeutic agents. Administering a compound that results in up-regulation of an RGS protein might be as effective as administering an antagonist to prevent initiation of the signaling pathway. Understanding the particular signaling pathways with which specific RGS proteins associate will be critical in designing such therapeutic agents. The use of RGS proteins in the context of therapeutics is one of the most difficult but arguably the most important challenges currently facing investigators studying RGS proteins.



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## NOTE ADDED IN PROOF

After this paper was submitted, a new member of the RGS family, RGS17, was reported (171). It appears to be a member of the subfamily A and shares the characteristic cysteine string and subfamily-specific Ser residue (70).

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