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R4 RGS Proteins: Regulation of G Protein Signaling and Beyond

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Abstract

The Regulators of G protein Signaling (RGS) proteins were initially characterized as inhibitors of signal transduction cascades initiated by G-protein-coupled receptors (GPCRs) because of their ability to increase the intrinsic GTPase activity of heterotrimeric G proteins. This GTPase accelerating (GAP) activity enhances G protein deactivation and promotes desensitization. However, in addition to this signature trait, emerging data have revealed an expanding network of proteins, lipids, and ions that interact with RGS proteins and confer additional regulatory functions. This review highlights recent advances in our understanding of the physiological functions of one subfamily of RGS proteins with a high degree of homology (B/R4) gleaned from recent studies of knockout mice or cells with reduced RGS expression. We also discuss some of the newly-appreciated interactions of RGS proteins with cellular factors that suggest RGS control of several components of G-protein-mediated pathways as well as a diverse array of non-GPCR-mediated biological responses.

1. Introduction

Signal transduction mediated by heterotrimeric G proteins (Gαβγ) elicits responses in every organ system, evoking such diverse outcomes as neurotransmission, immunity, cardiovascular function, and hormone secretion (Gilman, 1987; Neer, 1995). G-protein-coupled receptors (GPCRs), which possess a heptahelical structure, catalyze guanosine triphosphate (GTP) exchange on guanosine diphosphate(GDP)-bound G protein alpha subunits (Wess, 1997). Gα-GTP and Gβγ both activate specific downstream effectors such as adenylyl cyclase, phospholipase Cβ, Rho GTPases, mitogen activated protein (MAP) kinases, and ion channels (Goldsmith and Dhanasekaran, 2007; Marinissen and Gutkind, 2001). These effectors in turn produce a number of cellular responses including proliferation, morphological changes, and gene transcription. Nearly 60% of all pharmaceutical agents in current use target GPCRs, making detailed understanding of their intracellular signaling routes critical for the treatment of human disease (Pierce, et al., 2002).

Numerous studies have suggested that cells undergo desensitization to continual GPCR stimulation due to hydrolysis of GTP by the alpha subunit, which allows Gα-GDP to re-unite with Gβγ and form an inactive heterotrimer (Tsang, et al., 1998). However, purified Gα subunits hydrolyze GTP too slowly *in vitro* to account for the rapid recovery from G-protein-mediated biological responses (Tsang, et al., 1998). Therefore, additional co-factors that could aid in desensitization to GPCR-induced activation were hypothesized.

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One candidate group of cellular proteins that probably serve this function are the Regulator of G protein Signaling (RGS) proteins, which bind to activated Gα subunits and accelerate their intrinsic GTPase activity (Blumer, 2004; He, et al., 1998). Because RGS GAP activity hastens G protein deactivation, RGS proteins would be predicted to reduce signaling output elicited by GPCR activation. The first described RGS protein, Sst2p, inhibited the pheromone-evoked mating response of the yeast *Saccharomyces cerevisiae* by binding to the yeast Gα protein, Gpa1, and acting as its principal GAP (Dohlman, et al., 1998). Shortly thereafter, RGS orthologues were discovered, which complemented the phenotype of an *SSt2*- yeast strain and inhibited GPCR-evoked signaling in mammalian cells (Druey, et al., 1996). A plethora of subsequent overexpression studies supported the conclusion that RGS proteins were negative regulators of G-protein-mediated signaling in part due to their GAP activity (Huang, et al., 1997; Willars, 2006; Yan, et al., 1997).

However, this concept has since proven to be too simplistic. RGS proteins, which number greater than 30 in mammalian cells, can be subdivided into subfamilies based on primary sequence homology and the presence of additional domains (Abramow-Newerly, et al., 2006b) (Figure 1). All RGS proteins house a ~120 amino acid RGS domain (box), which, based on biochemical and crystallographic studies, mediates direct binding to Gαi and Gαq (Tesmer, et al., 1997). Members of the R4/B subfamily, which includes RGS1-5, 8, 13, 16, 18 and 21, are the smallest RGS proteins in size, containing only short peptide sequences flanking the RGS box with one notable exception (RGS3) (Figure 1). Nonetheless, these proteins appear to bind numerous cellular signaling factors in both G-protein-mediated and non-GPCR pathways.

The biochemical and molecular determinants of RGS expression and activity have been extensively studied and are the subject of several excellent recent reviews (Neitzel and Hepler, 2006; Tinker, 2006). However, because many studies relied on RGS overexpression in transformed cell lines or analysis of purified recombinant proteins *in vitro*, the physiological function(s) of most R4 RGS proteins have not been well-defined. In this review, we focus on studies of mice containing targeted deletions of R4 *Rgs* genes as well as RNA interference analyses that have begun to clarify biological roles of individual RGS proteins in native mammalian systems. We will first describe recent findings for each R4 RGS individually that illuminate physiological function(s) before detailing novel interactions of R4 RGS proteins with proteins or other factors that either affect RGS activity and/or specificity within GPCR signaling pathways or implicate them in non-GPCR-mediated cellular responses. These studies have challenged the notion that this subfamily acts strictly as negative modulators of G-protein-coupled signaling.

2. Physiological functions of individual RGS proteins

2.1. RGS1

The major portal of RGS1 expression appears to be the hematopoietic compartment including T and B lymphocytes (Agenes, et al., 2005; Moratz, et al., 2000), natural killer (NK) cells (Kveberg, et al., 2005), dendritic cells (Shi, et al., 2004), and monocytes (Denecke, et al., 1999). In B lymphocytes, RGS1 is upregulated by B-cell receptor activation by surface immunoglobulin, and its expression is concentrated in germinal center B cells (Hong, et al., 1993). Lymphoid organ germinal centers are the site of B lymphocyte differentiation and maturation during adaptive humoral immune responses. Studies of *Rgs1* knockout mice revealed a role for Rgs1 in the control of B lymphocyte migration induced by chemokines (Moratz, et al., 2004). Rgs1-deficient B cells migrated to a greater extent after exposure to the chemokines CXCL12 and CXCL13 *in vitro*, whose receptors, CXCR4 and CXCR5, are required for germinal center formation (Allen, et al., 2004). *Rgs1*−/− B lymphocytes pretreated with CXCL12 remained responsive to subsequent challenge with either chemokine, suggesting

that Rgs1 mediates desensitization of these cells to prolonged ligand exposure. Accordingly, spleens of *Rgs1*−/− mice contained increased numbers of germinal centers even in the absence of immunization, and immune challenge induced both elevated and persistent germinal center formation. In contrast, Peyer's patches, which are lymphocyte-rich follicles in the gastrointestinal tract (GI) mucosa, were reduced in size following immunization, implying aberrant migration of lymphocytes during the immune response. Lastly, trafficking of antibody-secreting cells was abnormal in the absence of Rgs1.

Subsequent analysis of adoptively-transferred, fluorescently-labeled lymphocytes confirmed that B cell migration was substantially affected by the loss of Rgs1 (Han, et al., 2005). There were significantly more Rgs1-deficient B cells than WT cells in peripheral lymph nodes of recipient mice and decreased cell numbers in blood, suggesting enhanced homing of *Rgs1*−/− cells into lymphoid tissue. Intravital multiphoton microscopy of lymph nodes after cell injection revealed that *Rgs1*−/− cells adhered better to high endothelial venules (HEVs) of peripheral lymph nodes and moved with greater velocity within lymphoid follicles in relation to WT cells. Interestingly, B lymphocytes from *Gnai2*−/− mice, which lack Gαi2 expression, exhibited the opposite phenotype. Thus, Rgs1 regulates B cell homing to lymph nodes and motility within the lymph node microenvironment by regulating Gαi2 signaling pathways induced by chemokines.

Another recent study reported enrichment of both Rgs1 and Rgs16 in regulatory $CD4^+$ T cells and activated T cells compared with naïve T lymphocytes (Agenes, et al., 2005). This differential RGS expression correlated inversely with the ability of these subpopulations to migrate in surgical parabiosis experiments. The specific chemokines involved were not determined (Agenes, et al., 2005). The consequences of abnormal lymphocyte migration associated with Rgs1 deficiency for immune responses of whole organisms await further clarification. Preliminary analysis indicated that *Rgs1*−/− mice produced a delayed antibody response to immunization with T-cell dependent antigens, but the ultimate antibody titer and affinity profile of the immunoglobulins were normal (Moratz, et al., 2004). These studies suggest that Rgs1 may play a major role in the chemokine-mediated homing of lymphocytes to secondary lymphoid organs as well as their localization within these spaces during the immune response.

2.2. RGS2

RGS2 is expressed widely in both mouse and human tissues (Kehrl and Sinnarajah, 2002). Preliminary studies of *Rgs2*−/− mice and RGS2 knockdown in both human and mouse cells have suggested that it regulates G protein-mediated responses in the immune system, brain, heart, lung, bone, and olfactory epithelium. In an initial report, Oliveira-dos-Santos *et al.* described a variety of abnormalities in *Rgs2* knockout mice (Oliveira-dos-Santos *et al.* 2000). *Rgs2*−/− T lymphocytes proliferated less and produced less interleukin-2 (IL-2) after phorbol ester or T-cell receptor (CD3/CD28) stimulation. In whole animal studies, these mice exhibited reduced inflammation (footpad swelling) to virus (lymphocytic choriomeningitis virus) infection. Studies of central nervous system (CNS) function revealed that the brains of *Rgs2* −/− mice showed reduced density and basal electrical activity of hippocampal CA1 neurons (Oliveira-Dos-Santos, et al., 2000). These cellular abnormalities were accompanied by abnormal behavior such as increased anxiety (measured by light/dark preference) and decreased male aggression. Although no mechanistic insights were provided into how Rgs2 might control such a diverse group of biological parameters in these organ systems, the mice provided a preliminary blueprint for subsequent detailed investigation into the potential physiological functions of Rgs2. In fact, recent genetic quantitative trait analysis has confirmed that *Rgs2* is a gene that controls anxiety in mice (Yalcin, et al., 2004).

Subsequent characterization of *Rgs2*−/− mice revealed that Rgs2 controls systemic blood pressure. Both *Rgs2*+/− and *Rgs2*−/− mice were found to be profoundly hypertensive with increased systemic and renal vascular resistance and hypertrophy of the renal arterial vasculature (Heximer, et al., 2003). This parameter correlated with excessive and prolonged Ca^{++} signaling to vascoconstrictors such as ATP acting on P2Y receptors. In addition, the hypertension of Rgs2-deficient mice appeared to be especially sensitive to acute angiotensin II receptor antagonism, suggesting elevated vascular tone due to heightened signaling responses to this hormone. Despite the hypertension, there was no cardiac hypertophy change in cardiac systolic contractility or that might have contributed to the elevated blood pressure in these mice.

Several additional abnormalities may also promote hypertension in *Rgs2*−/− mice. First, the mice displayed some characteristics of increased peripheral sympathetic tone, such as elevated urinary catecholamine secretion, relative resistance to the α1-adrenergic receptor antagonist prazosin, and reduced blood pressure decrease after environmental stress (Gross, et al., 2005). These defects could lead to a re-setting of the baroreceptor reflex and hypertension. However, additional tests such as heart rate response to β-adrenergic receptor blockade were normal, arguing against a significant increase in peripheral sympathetic outflow. Further studies will be required to determine whether altered autonomic outputs contribute to the hypertensive phenotype (Stauss, 2005).

Second, the mice displayed renovascular abnormalities including increased responsiveness to vasopressin, which could result in impaired water handling and changes in plasma volume (Zuber, et al., 2007). By RT-PCR, Rgs2 expression in the mouse kidney appeared to be limited to the principal cells of the connecting tubules and collecting duct, which serve to concentrate urine in response to anti-diuretic hormone (ADH or vasopressin). Vasopressin acts on Gs-coupled V2 receptors, which generate cAMP. *Rgs2* localization mirrored expression of V2 receptors in the kidney, and vasopressin treatment lead to upregulated Rgs2 (mRNA and protein) in mouse collecting duct principal cells (Zuber, et al., 2007). In whole mice, water restriction, which induces vasopressin secretion, also upregulated Rgs2 expression. Collecting duct cells microdissected from kidneys of *Rgs2*−/− mice produced more cAMP after vasopressin treatment than WT cells, and the *Rgs2* knockout mice exhibited abnormal patterns of urine excretion after water loading. A separate study recently described inhibition of angiotensin II-mediated aldosterone secretion by adrenal cells induced by RGS2 overexpression (Romero, et al., 2006). Thus, defective signaling pathways in the kidney of *Rgs2*−/− mice could impair water and solute processing. The authors of the original study of the hypertension of *Rgs2*−/− mice hypothesized that the differential acute changes in blood pressure between WT and Rgs2-deficient mice to vasoconstrictor antagonists argued against plasma volume changes in *Rgs2*−/− mice as the primary cause of hypertension (Heximer, et al., 2003). An even more recent study has found no difference in renal sympathetic activity in WT or *Rgs2*−/− mice (Tank, et al., 2007). Although the significance of the renal abnormalities in the absence of Rgs2 for regulation of plasma volume and systemic blood pressure is far from clear, its role in renal water and salt handling merits further study. Third, RGS2 exerts control over vascular tone through an interaction with a component of the nitric oxide (NO) pathway, protein kinase G (PKG)/cGMP-dependent protein kinase, which influences vascular relaxation mediated by NO (discussed in detail below)(Sun, et al., 2005; Tang, et al., 2003). Collectively, these studies paint a complex picture of the regulation of systemic blood pressure in mice by Rgs2.

Preliminary evidence points to a role for RGS2 in the pathogenesis of human hypertension. Analysis of peripheral blood mononuclear cells (PBMCs) and skin fibroblasts from 11 normals and 12 hypertensives revealed substantially reduced RGS2 expression and increased responsiveness (Ca^{++} mobilization and Erk phosphorylation) to angiotensin II in hypertensives

compared to controls (Semplicini, et al., 2006). The reduction in RGS2 expression correlated with a polymorphism (C1114G) in the 3′ untranslated region of the *RGS2* gene. An independent analysis of RGS2 polymorphisms in hypertensives found association between two haplotypes in the 3′ non-coding region in black patients. RGS2 protein expression levels were not assessed in this study (Riddle, et al., 2006). A third report of a Japanese population identified a rare coding mutation in RGS2 (Q2L) only in hypertensives (Yang, et al., 2005), which destabilized RGS2 expression. A separate study found that this mutant was expressed at reduced levels in HEK293T cells compared to WT and failed to inhibit angiotensin II-mediated signaling (Bodenstein, et al., 2007).

Conversely, RGS2 expression may be elevated in Bartter/Gitelman (BG) syndrome, a condition that manifests as normo/hypotension due to sodium and potassium wasting and volume depletion caused by an unknown genetic defect in kidney electrolyte transporters. Decreased plasma volume leads to hyperactivation of the renin/angiotensin system and elevated serum aldosterone levels. Several compensatory changes result in decreased vascular tone and hyporeactivity to angiotensin II signaling, which may be due to decreased expression of Gαq. Calo *et al*. (Calo, et al., 2004) described significantly increased RGS2 protein levels in peripheral blood mononuclear cells of 6 BG patients compared to 6 healthy controls, which could contribute to their angiotensin II resistance. Another group recently reported upregulation of Rgs2 in vascular smooth muscle cells, which appeared to be mediated by activation of Group VIA phospholipase A2 (iPLA2β) (Xie, et al., 2007). Although the detailed molecular mechanisms remain to be determined, these studies point to a potential role for RGS2 in the control of systemic blood pressure in humans through its regulation of GPCR-evoked signals governing vascular resistance and possibly water/solute processing in the kidney.

Analysis of mouse cells with reduced Rgs2 expression also suggests that it may regulate other responses in the cardiopulmonary system. Although no changes in cardiac contractility or evidence of cardiac hypertrophy were observed in *Rgs2*−/− mice, a recent study indicated that Rgs2 could contribute to heart failure in mice. In contrast to end-stage failing human hearts with upregulated RGS4 (Mittmann, et al., 2002; Owen, et al., 2001), models of mouse cardiac hypertrophy such as transverse aortic constriction (banding) or transgenic overexpression of constitutively active Gαq were associated with significantly decreased Rgs2 mRNA and protein levels *prior* to the development of hypertrophy with no concordant change in expression of Rgs3-5 (Zhang, et al., 2006). Knockdown of endogenous Rgs2 in ventricular myocytes by selective RNAi increased inositol phosphate (IP) formation and cellular hypertrophy in response to phenylephrine and endothelin-1, which act on Gq-coupled receptors. By contrast, activation of MAP kinase pathways (Erk, JNK, and p38), which presumably would be involved in hypertrophic responses, was not affected by reduced Rgs2 expression in these cells. Thus, decreased Rgs2 levels may lead to cardiac hypertrophy by way of amplified signaling responses to catecholamines and other vasoactive mediators. Finally, in the lung knockdown of RGS2 in human ciliated airway epithelial cells by antisense oligonucleotides led to increased Ca^{++} flux and ciliary beat frequency in response to purinergic receptor stimulation with ATP (Nlend, et al., 2002). It will be of interest to determine whether these *in vitro* abnormalities translate into increased susceptibility of *Rgs2*−/− mice to hypertrophic myocardial failure or resistance to microbial pulmonary infection due to increased clearance of microorganisms.

Finally, Rgs2 may have a function in bone formation by osteoblasts. Rgs2 is upregulated by parathyroid hormone (PTH) and PTH-related peptide (PTHrP) or forskolin, which induce cAMP formation. cAMP stimulates osteoblast proliferation and differentiation, as do ATP or phorbol ester (PMA), which activate Gq effectors such as protein kinase C (PKC) (Roy, et al., 2006b). *Rgs2*−/− osteoblasts displayed no differences from WT cells in PTHrP-stimulated cAMP formation or IP generation evoked by ATP or endothelin-1. However, upregulation of Rgs2 by forskolin resulted in reduced endothelin-induced inositol phosphate formation and

ATP-evoked Ca^{++} mobilization in WT but not Rgs2-deficient osteoblasts. Similarly, pre-treatment of WT but not *Rgs2*−/− osteoblasts with ATP lead to diminished PTHrP-stimulated cAMP. The authors concluded that Rgs2 at basal levels does not regulate either Gq or Gs signaling in osteoblasts, but at higher expression levels Rgs2 may cross-desensitize both Gs and Gq signals. However, other abnormalities that could have accounted for the differences between these strains such as altered expression of GPCRs or other downstream signaling components (e.g., G proteins, phospholipase Cβ) were not examined with the exception of the sarco/endoplasmic reticulum calcium ATPase 2b, which controls calcium content in the ER. Nonetheless, these studies lend credence to the hypothesis that Rgs2 could regulate bone repair in conditions associated with elevated PTH (hyperparathyroidism) or stress, which is accompanied by increased catecholamines (e.g., infection or fractures).

2.3. RGS3

RGS3 exists as several isoforms that are splice variants of the same gene, *RGS3*. A short form, RGS3S, which contains little more than the RGS domain, was expressed in the nucleus and induced apoptosis when overexpressed (Dulin, et al., 2000). RGS3 has two longer isoforms, RGS3L and PDZ-RGS3 (Kehrl, et al., 2002), the latter of which has been linked to cell migration through interaction with Ephrin receptors and in Gβγ-evoked signaling (described below). Mice containing a targeted deletion of Rgs3 have not been reported, and few studies of its physiological function exist. Ribozyme-mediated knockdown of RGS3 in aortic smooth muscle cells lead to increased M_3 muscarinic acetylcholine (mAch) receptor-induced MAP kinase activation but no effect on angiotensin II-evoked signaling (Wang, et al., 2002). A recent study reported upregulation of RGS3 in p53-mutated tumors, and RGS3 siRNA treatment of MCF-7 breast cancer cells resulted in enhanced sensitivity to chemotherapy (docetaxel)-induced apoptosis compared to control (Ooe, et al., 2007). Further studies will be required to determine the physiological significance of this finding and the signaling pathways involved.

2.4. RGS4

In both humans and rodents, RGS4 appears to be selectively enriched in the CNS and heart (Erdely, et al., 2004; Zhang, et al., 1998). During embryonic CNS development, Rgs4 is expressed transiently in the mouse locus coeruleus, sympathetic ganglionic neurons, and cranial sensory and motor neurons, and expression is linked to the homeodomain transcription factor Phox2B (Grillet, et al., 2003). Recently, homologous recombination techniques were utilized to introduce expression of green fluorescent protein (GFP) with an internal ribosomal entry sequence (IRES) into a bacterial artificial chromosome (BAC) construct containing the *Rgs4* gene. This BAC was then expressed in mice to evaluate the expression of Rgs4 in the mouse brain (Ebert, et al., 2006). Although the extensive microanatomical localization of RGS4-GFP within brain regions cannot be detailed here, expression of the GFP reporter faithfully reproduced localization of endogenous *Rgs4* mRNA detected by *in situ* hybridization. These studies revealed widespread Rgs4 expression in most cortical neuronal layers and at all stages of development. In all cases, RGS4-GFP was detected more in grey matter than in white matter, suggesting that Rgs4 is not expressed in glial cells. Another important observation was the striking overlap between Rgs4 expression patterns and that of acetylcholinesterase, which implies a potential physiological role for Rgs4 in the regulation of mAch receptor signaling. In subcortical regions, RGS4-GFP was most abundant in the striatum and amygdala. In human brain, in situ hybridization using *RGS4* riboprobes revealed enrichment of *RGS4* in several regions of the frontal cortex with lower levels in the thalamus and striatum (Erdely, et al., 2004).

Several functional studies have linked RGS4 to regulation of opioid, cholinergic, and serotonergic signaling in the brain. After morphine treatment, RGS4 levels increase in the rat locus coeruleus (LC) and decline rapidly after opiate withdrawal. Treatment of LC neurons

with RGS4 reduced opioid-induced electrophysiological responses, suggesting a role in opioid tolerance (Gold, et al., 2003). In contrast, studies of *Rgs4* knockout mice did not support a substantial role for Rgs4 in the control of opioid signaling in sensory neurons as knockout mice displayed normal antinocioceptive responses (pain sensitivity and analgesia evoked by morphine). Nonetheless, in shock tests, the threshold for pain response (flinch test) was significantly increased in knockout mice, suggesting abnormal central processing of painful stimuli. This response presumably involves postsynaptic afferent sensory cortical neurons, which express relatively high levels of Rgs4 (Garnier, et al., 2003; Grillet, et al., 2003).

A potential role for RGS4 in Parkinson disease (PD) was revealed by a recent study of cholinergic interneurons of the striatum, which are enriched in RGS4 (Ding, et al., 2006). In PD, loss of dopaminergic neurons in the striatum is accompanied by increased acetylcholine (Ach) release, which exacerbates motor symptoms of the disease. It was previously thought that decreased dopamine levels lead to reduced D2 receptor-mediated inhibition of synaptic Ca^{++} channels (Cav2) and subsequent increased Ach release. These investigators found that while D2-evoked inhibition of Cav2 activity was unchanged after chemical dopamine depletion in mouse cholinergic interneurons, M_4 muscarinic receptor-induced suppression of channel electrical activity was markedly attenuated, which would lead to increased Ach release. It was hypothesized that the reduced M_4-mediated signaling was attributable to upregulation of Rgs4 levels by dopamine depletion (reserpine), as had been previously demonstrated (Geurts, et al., 2003) and was confirmed in this study. As proof-of-principle, dialysis of recombinant RGS4 with cholinergic interneurons inhibited M_4-evoked Ca^{++} channel activity. Interestingly, infusion of an RGS4 peptide lacking the amino-terminus (RGS4ΔN) reversed the attenuated M_4 response of reserpine-treated neurons, suggesting that RGS4ΔN acted as a dominant negative inhibitor of endogenous Rgs4. Although these results await confirmation by studies of interneurons with reduced RGS4 expression or cells from *Rgs4* knockout mice, this study implies a role for RGS4 in the control in motor symptoms of PD associated with increased striatal acetylcholine levels.

Over the past several years, considerable attention has been paid to RGS4 as a potential etiological factor for schizophrenia. Postmortem studies have identified reduced RGS4 expression in several areas of the frontal cortex of schizophrenics, such as the superior temporal gyrus (Bowden, et al., 2007). *RGS4* mapped to the schizophrenia susceptibility locus (1q23), and several single nucleotide polymorphisms have been associated with the disease (Chowdari, et al., 2007). In particular, one haplotype was most recently linked to deficit-subtype schizophrenia, in which patients have a preponderance of negative symptoms such as social withdrawal and psychomotor retardation (Bakker, et al., 2007).

Functional studies have not clearly established whether RGS4 expression levels play a role in the development of schizophrenia. A report of human subjects with an RGS4 haplotype previously associated with psychosis found significant reductions in cortical grey matter volume and connectivity that may affect cognitive responses (Buckholtz, et al., 2007). Another study employing a rat model of schizophrenia (phencyclidine treatment) demonstrated downregulated Rgs4 expression in prefrontal cortical neurons (PFCs) after phencyclidine exposure (Gu, et al., 2007). Inhibition of RGS4 function by a specific antibody potentiated serotonergic receptor ($5HT_{1A}$)-mediated regulation of NMDA receptor channels in PFCs while RGS4 overexpression inhibited the response. An RGS2-selective antibody had no effect on channel activity induced by serotonin. Single-cell studies of PFCs obtained from phencyclidine-treated rats confirmed that the heightened serotonin-evoked signaling response was limited to cells with reduced endogenous Rgs4 expression. In contrast, *Rgs4* knockout mice were found to lack any substantial behavioral abnormalities such as decreased pre-pulse inhibition, which is widely considered a standard marker for schizophrenic behavior in rodents (Grillet, et al., 2005). These studies suggest that species variability in the expression and/or

function of RGS4 in the cerebral cortex could exist. Thus, the exact role of RGS4 in the etiology of schizophrenia remains unclear.

As mentioned previously, RGS4 appears to be upregulated in failing human hearts due to dilated cardiomyopathy (Owen, et al., 2001). In murine models of cardiac hypertrophy induced by transgenic overexpression of activated Gq or in models of diabetic cardiomyopathy as a result of transgenic expression of the peroxisome proliferated-activated receptor alpha (PPARα) or treatment with streptozotocin, concomitant aortic banding markedly increased mortality in transgenic mice overexpressing RGS4, consistent with a maladaptive role of RGS4 in cardiomyopathy (Rogers, et al., 1999). However, in *Rgs4*$^{LacZ/LacZ}$ mice, which should display surrogate β-galactosidase activity in tissues expressing Rgs4, staining was detected in the large vessels of the heart including aorta and pulmonary artery trunk as well as coronary vasculature but not in cardiac muscle (Grillet, et al., 2005). It will be of interest to determine whether these mice display differences in mortality after induction of cardiac hypertrophy.

2.5. RGS5

Little is known about the physiological role of RGS5 in whole animals due to the lack of gene-targeted mice. Rgs5 is enriched in peri-endothelial cells or “pericytes” and vacular smooth muscle cells (Bondjers, et al., 2003; Cho, et al., 2003). In certain anatomical locations in the mouse, Rgs5 is enriched in pericytes of both capillaries and arterioles, and expression mirrors abundance of the tyrosine kinase receptor for platelet-derived growth factor beta (PDGFRβ) as well as its ligand PDGF. Receptor or ligand-null embryos lack Rgs5 expression and the presence of pericytes, suggesting Rgs5 as a pericyte-specific marker in microvasculature. In primate vascular smooth muscle, RGS5 was identified in larger arteries (e.g. aorta, carotid) and afferent glomerular arteioles but not coronary arteries or venous structures (Li, et al., 2004). Further, *RGS5* mRNA was downregulated in certain regions of atherosclerotic placques (Adams, et al., 2006). In contrast, RGS5 was abnormally elevated in the vasculature of renal carcinomas as well as in neovascularized pancreatic islet cell carcinomas and astrocytomas (Berger, et al., 2005; Furuya, et al., 2004). Together, these studies imply a role for RGS5 in pericyte development and vascular smooth muscle activation associated with neovascularization of tumors, as well as in arterial smooth muscle cell development or function under physiological conditions.

Another site of RGS5 expression is the heart, and beta-adrenergic receptor hyper-function has been shown to upregulate RGS5 levels (Jean-Baptiste, et al., 2005). This finding suggests a potential physiological function in cardiac conditions associated with elevated sympathetic tone. Finally, a novel splice variant of RGS5, RGS5S, which lacks 104 amino acids of the amino-terminus, was recently described. This alternate splice form appears to be differentially expressed in human tissues with high expression in the ciliary body of the eye, kidney, brain, spleen, skeletal muscle and small intestine, and undetectable transcripts in the liver, lung, and heart (Liang, et al., 2005).

2.6. RGS8

This RGS isoform garnered considerable attention soon after its discovery because of its apparent paradoxical regulation of G-protein-gated potassium channels (see below). Increased RGS8 expression was associated not only with increased “off” kinetics, which might be expected from RGS8 GAP activity, but also faster activation kinetics (Saitoh, et al., 1997). Despite this early observation, no definitive mechanism has been defined to explain the ability of RGS8 to accelerate channel kinetics nor have the physiological implications of this finding been explored in whole animals. Several theories have been proposed including “kinetic” scaffolding (Ross and Wilkie, 2000), which suggests that increased G protein deactivation induced by RGS proteins or other GAPs actually improves efficiency of the GTPase cycle by

facilitating protein-protein interactions within the GPCR-heterotrimeric G protein complex and mitigates against limiting G protein concentrations in the presence of a strong extracellular stimulus. A more recent study suggested that the RGS protein may be a component of a stable quaternary complex consisting of GPCR-G protein-RGS (Benians, et al., 2005). Fluorescence resonance energy transfer (FRET) experiments showed that RGS8-YFP protein associated with Gα regardless of the activation state or receptor-ligand interaction. In contrast, a separate set of experiments revealed that membrane association of an RGS protein (RGS2) was stable in the presence of a constitutively active, immobile G protein [Gαq(R183C)] but was only transiently recruited to the membrane by expression of a GPCR (M_3R) (Clark, et al., 2007). The localization of RGS2 after ligand stimulation of the receptor was not examined in the latter study. Thus, in some cases, depending on the receptor or G protein involved, RGS proteins could act as a physical scaffold between Gαβγ and its receptor.

In fact, the work of several laboratories has recently determined that in contrast to prevailing dogma, some G protein heterotrimers do not physically dissociate in living cells (Bunemann, et al., 2003; Digby, et al., 2006). Instead, depending on the identity of the Gα subunit, receptor stimulation induces a subunit rearrangement rather than heterotrimer dissociation (Bunemann, et al., 2003; Frank, et al., 2005). Two distinct models of G protein activation have been postulated: “collision coupling”, which postulates ligand-dependent, random interactions between GPCRs and G proteins diffusing laterally within the membrane, or “precoupling” of stably-associated receptors and G proteins. Recent FRET studies of fluorescently-tagged GPCRs and G proteins found no evidence for precoupling in living cells (Hein, et al., 2005). In a separate study, RGS2 did not affect association of fluorescently-tagged GPCRs and G proteins detected by FRET in living cells (Clark, et al., 2007). Thus, it is unclear how RGS scaffolding might affect receptor-G protein and heterotrimer interactions during the GTPase cycle as these results do not support a role for RGS proteins act as physical scaffolds, at least for this particular GPCR-G protein combination. It will be interesting to examine these interactions in cells with reduced or absent expression of one or more endogenous RGS proteins.

Expression of RGS8 appears to be concentrated in the brain (Larminie, et al., 2004). RGS8 levels can be modulated by acute and chronic electroconvulsive seizures (Gold, et al., 2002). RGS8 mRNA and protein are enriched in Purkinje cells of the granule layer of the cerebellum (Saitoh, et al., 2003; Saitoh and Odagiri, 2003). RGS8 was found to exist as a distinct splice variant containing an alternate amino-terminus (RGS8S) (Itoh, et al., 2006). RGS8S demonstrated reduced interactions with M_1 mAch receptors and impaired inhibition of M_1-evoked signaling (see below). In the hematopoietic system, analysis of leukocyte subsets revealed selective expression of Rgs8 in rat natural killer (NK) cells (Kveberg, et al., 2005).

2.7. RGS13

RGS13 exhibits relatively restricted tissue expression in T and B lymphocytes, and subsequent studies have demonstrated even higher expression in mast cells (Shi, et al., 2002); Druey Rgs13. *UCSD-Nature Molecule Pages* 2005, doi:10.1038/mp.a000020.01) RGS13, like RGS1 and RGS16, is concentrated in germinal center B cells and in activated lymphocytes (treated with anti-CD40 plus IL-4), suggesting a function in adaptive immune responses (Estes, et al., 2004; Shi, et al., 2002). In addition, RGS13 is abundant in Burkitt lymphoma, a tumor thought to represent malignant germinal center B lymphocytes, but is absent in mantle cell lymphomas (Islam, et al., 2003). In the rat brain, *Rgs13* mRNA was detected in the hippocampus and discrete thalamic nuclei (Grafstein-Dunn, et al., 2001).

Overexpression studies showed that RGS13 inhibits migratory responses and signaling induced by the chemokines CXCL12 and CXCL13 in B lymphocytes, which are required for germinal center formation in lymphoid organs (Shi, et al., 2002). These studies have recently been

corroborated by RNAi in a Burkitt lymphoma cell line, where RGS13 knockdown enhanced chemokine responsiveness (Han, et al., 2006b). Rgs13 knockout mice were recently generated by our laboratory, and preliminary studies suggest that Rgs13 has a function in IgE-mediated mast cell responses and in B cell transcriptional activity (Bansal *et al*. and Xie *et al.*, submitted). Of note, Rgs13 is also expressed in dendritic cells (Shi, et al., 2004) and is also abundant in neuroendocrine cells of the thymus, gastrointestinal, and respiratory tracts (our unpublished data).

2.8. RGS16

RGS16 was initially cloned from the retina (Chen, et al., 1996) and subsequent analysis demonstrated expression in the rat heart (Patten, et al., 2002) and brain (especially suprachiasmatic nucleus) (Grafstein-Dunn, et al., 2001), mouse liver (Kurrasch, et al., 2004), and hematopoietic cells. In blood cells, RGS16 has been found in NK cells (Kveberg, et al., 2005), platelets (Kim, et al., 2006), dendritic cells (Shi, et al., 2004), and T lymphocytes (Beadling, et al., 1999). RGS16 is upregulated in germinal center and activated T cells, suggesting a role for this RGS in adaptive immunity (Estes, et al., 2004). In the mouse liver, Rgs16 is restricted to periportal hepatocytes and demonstrates diurnally-regulated expression patterns. Rgs16 is downregulated during fasting and rapidly upregulated by refeeding (Huang, et al., 2006). These studies suggest a role for Rgs16 in circadian-regulated pathways involved in glucose and/or fat metabolism.

Rgs16 knockout mice have been generated, which are viable and fertile, but no phenotype has yet been determined (Druey, unpublished observations). In a megakaryocytic cell line, RNAi-mediated knockdown of RGS16 enhanced signaling responses to CXCL12, whereas overexpression reduced CXCL12-evoked signaling and migration (Berthebaud, et al., 2005). These preliminary results imply that RGS16 may control CXCR4-elicited migration of platelet precursors.

2.9. RGS18

Several groups recently cloned this novel RGS protein, whose expression appears to be relatively restricted to bone marrow-derived cells (Nagata, et al., 2001; Park, et al., 2001; Yowe, et al., 2001). RGS18 mRNA and protein are detectable in platelets and granulocytes, but not in lymphocytes or erythrocytes (Gagnon, et al., 2002). Few studies have addressed the transcriptional regulation of RGS expression. Interestingly, however, promoter analysis at the Rgs18 locus demonstrated highly restricted occupancy of both GATA-1 and GATA-2 transcription factors, which may partially explain its enrichment in the hematopoietic compartment (Johnson, et al., 2007). To date, most functional analysis has been limited to overexpression studies, which have shown that RGS18 is capable of inhibiting both Gi- and Gq-mediated signaling pathways. Interestingly, RGS18 overexpression had no effect on CXCR4-induced responses in a megakaryocytic cell line (Nagata, et al., 2001).

Recent work showed that RGS18 is also expressed in osteoclasts (Iwai, et al., 2007). Osteoclasts are multinucleated giant cells that promote bone resorption necessary for remodeling. Osteoclast differentiation is controlled by RANKL ligand (receptor activator of nuclear factor κB ligand), which acts on the RANK receptor to induce osteoclast differentiation. It had been previously shown that this process was mediated by upregulation of a GPCR that senses extracellular acidosis, OGR1. Proton stimulation of OGR1 activates the Gq-PLCβ pathway, which in turn increases nuclear factor of activated T cells (NFAT) activity and promotes osteoclastogenesis. RANKL reduced RGS18 expression in the osteoclast precursor cell line RAW246.7 and in primary bone marrow-derived osteoclast precursor monocytes. RGS18 knockdown in RAW246.7 cells (by RNAi) enhanced osteoclast differentiation evoked by RANKL, and this phenotype was neutralized by an anti-OGR1 blocking antibody. Further,

RGS18 siRNA increased NFAT activation induced by extracellular acidosis, while RGS18 (but not RGS2 or GAIP) overexpression inhibited OGR1-mediated responses. Taken together, the studies indicate that RGS18 levels may control osteoclastogenesis mediated by RANKL through modulation of OGR1-evoked signaling.

2.10. RGS21

The latest R4 member to be identified, RGS21, is the smallest member of the B/R4 subfamily, with 152 amino acids. It was originally described as selectively expressed in taste tissue (von Buchholtz, et al., 2004). By RT-PCR *RGS21* mRNA was detected only in sensory taste cells that express sweet taste receptors and the taste Gα subunit, gustducin. Although no functional studies of endogenous RGS21 have yet been reported, this RGS interacts with activated gustducin, suggesting a potential role in regulating taste transduction. Subsequent to the initial report, a second group cloned RGS21 from fetal brain cDNA; they reported that RGS21 was widely expressed in 16 tissues examined (Li, et al., 2005).

2. Interactions with GPCR signaling components

2.1. Role of RGS amino terminus

R4 RGS protein binding to G proteins depends strictly on the intact RGS domain (box) (Popov, et al., 1997). The RGS box is the most highly conserved region in these proteins, with 47–62% identity and 69–83% homology within the R4 subfamily. By contrast, the amino-terminal and carboxy-terminal flanking regions are less well-conserved. The amino-terminus of several R4 family members has a function in subcellular localization. Although R4 RGS proteins lack obvious membrane targeting motifs such as pleckstrin homology (PH) domains, most members contain an amphipathic α-helix at the amino-terminus that facilitates direct binding to membrane phospholipids. The amino-terminus of RGS4 (Bernstein, et al., 2000) and RGS8 (Saitoh, et al., 2001) mediates translocation to membranes and binding to phospholipids. Both the amphipathic α-helix and palmitoylation of amino-terminal cysteine residues play a role in membrane targeting of these RGS proteins (Bernstein, et al., 2000; Tu, et al., 2001).

In addition to mediating cellular localization, the amino-terminus also serves as a platform for additional protein-protein interactions unique to one or more R4 RGS proteins. For example, RGS16 but not RGS4 binds Gα13 through its amino-terminal 30 amino acids and inhibits Gα13-mediated gene transcription and cell morphological changes. (Johnson, et al., 2003). The epithelial Ca^{++} channel TRPV6, which has a function in Ca^{++} transport in placenta, pancreas, small intestine, and colon (den Dekker, et al., 2003), binds the RGS2 amino-terminus in a Ca^{++}-independent fashion (Schoeber, et al., 2006). Electrophysiological studies using whole-cell patch clamp revealed that RGS2 overexpression inhibited Na^{+} and Ca^{++} currents in HEK293 cells co-transfected with TRPV6 but not TRPV5. Deletion of the amino-terminus of RGS2 disrupted binding to TRPV6 and restored electrophysiological properties of the channel in the absence of RGS2 (Schoeber, et al., 2006).

RGS2 also interacts directly with tubulin via a short polypeptide within its amino-terminus (amino acids 41-60) (Heo, et al., 2006). This region was necessary and sufficient for RGS2 to inhibit microtubule polymerization observed microscopically in Vero cells stably expressing GFP-tagged α-tubulin. Overexpression of RGS2 enhanced nerve growth factor-induced neurite outgrowth in PC12 cells whereas RGS2 knockdown suppressed neurite outgrowth. Thus, RGS2 may have a function in neuronal cell differentiation through direct regulation of tubulin. PDZ-RGS3 has been shown to bind phosphorylated EphrinB through its PDZ domain at the amino terminus (Su et al., 2004). These are just a few examples demonstrating the importance of the RGS protein amino-terminus for diverse protein-protein interactions. More examples of RGS binding partners and the implications for various signaling pathways are discussed below.

3.2. RGS domain-dependent G protein selectivity

R4 RGS proteins bind Gαq and Gαi through their RGS domains to increase the GTPase activity of Gα. This GAP activity reduces the lifetime of activated Gα and Gβγ, attenuating GPCR signaling. As noted above, the proteins of the R4 RGS subfamily have highly homologous primary structure, and most often a given cell or tissue expresses two or more members of this subfamily. Often these proteins display roughly equivalent GAP activity for Gq or Gi family members *in vitro*. Thus, the principal question one might pose is how does an individual RGS protein achieve selectivity to regulate specific GPCR signaling pathways? Emerging evidence points to a myriad of co-factors that either confer selectivity of a given RGS protein for certain GPCRs or endow additional functions outside GPCR signaling pathways. RGS protein selectivity for certain receptors or signaling routes might be accomplished by the following: 1) measurable differences in GAP activity toward different classes of Gα subunits, which may or may not depend on additional co-factors or modification of the RGS protein or Gα; 2) direct interaction with a subset of GPCRs; 3) formation of an RGS/G protein complex that sequesters the G protein from either the receptor or downstream effectors (i.e., independent of RGS GAP activity or 4) direct binding of RGS proteins to effectors within the GPCR signaling axis.

Assessments of the relative potency of RGS proteins in cells have been mainly limited to overexpression in transformed cell lines, as noted above, with some exceptions. For example, RGS2 was found to be five-fold more potent than RGS4 in inhibiting Gαq-stimulated IP3 formation (Heximer, et al., 1999); in contrast, RGS4 was 8-fold more potent that RGS2 in complementing SSt2p in a yeast mating pheromone assay (Heximer, et al., 1999). RGS2 inhibited Gαq-activated PLCβ1 activity with an IC50 of 30 nM, while RGS4 was 10-fold less potent than RGS2 with IC50 of 300 nM (Heximer, et al., 1997b). In ventricular myocytes, RGS2, RGS3, RGS4 and RGS5 inhibited Gq-mediated hypertrophic cell growth and IP3 formation with similar potency (Hao, et al., 2006). However, RGS3, RGS4 and RGS5, but not RGS2, reversed carbachol-evoked inhibition of cAMP production induced by isoproterenol, which is thought to be mediated by Gαi (Hao, et al., 2006). Comparison of RGS activity on the Gq/G11 subfamily of G-proteins revealed that RGS1, RGS2, RGS3 and RGS4 inhibited cell morphology change in a yeast system expressing both mammalian Gαq and Gα11, whereas RGS5 and RGS16 were much less effective against Gα11 than Gαq (Ladds, et al., 2007).

The differences in efficacy of RGS proteins toward Gα subunits could be due to variable affinity of a given RGS protein for Gα family members. Assessment of relative potency of RGS proteins has proven difficult as methods used to assess GAP activity *in vitro* are somewhat crude. Using purified recombinant proteins, GAP activity is measured in either a single turnover assay or phospholipid vesicles containing both receptor and heterotrimeric G protein, which allows measurement of steady-state GTP hydrolysis. In the single turnover assay, Gα is loaded with ^{32}P-GTP in the absence of Mg^{++}, which is required for GTPase activity. Mg^{++} is then added along with excess unlabeled GTP, which allows measurement of a single catalytic cycle of GTP hydrolysis.

There are a limited number of Gαi/q family members (Gαi1-3, Gαo, Gαz, transducin, Gαz, Gαq, 11, 14/15, and 16), and most R4 RGS proteins will accelerate the GTPase activity of these Gα subunits promiscuously in single turnover assays (Berman, et al., 1996; Watson, et al., 1996). A notable exception is RGS2, which appears to be a more potent GAP for Gαq than Gαi. RGS4 binds both Gαi/o/z and Gαq, whereas RGS2 was found to bind Gαq selectively *in vitro* (Heximer, et al., 1997b). In addition, significant GAP activity of RGS2 against Gi is only detectable in the presence of a receptor in phospholipids vesicles. Whether this reflects a requirement for receptor binding or reduced affinity of RGS2 for Gi family members remains to be definitively established (Ingi, et al., 1998). In this regard, RGS2-Gαi interaction may be unfavorable due to geometry of the binding pocket of RGS2 and the switch I region of Gαi. Three RGS2 residues within this region, Cys106, Asn184 and Glu191, were critical for Gαi

binding (Heximer, et al., 1999). Mutation of amino acids in RGS2 to the analogous residues in RGS4 (C106S/N184D/E191K) rendered RGS2 able to increase GTPase activity of Gαo (Gαi subfamily) under conditions where WT RGS2 demonstrated no GAP activity (Heximer, et al., 1999). Reciprocal substitutions of these RGS2 residues into RGS4 (S85C, D163N and K170E) abolished the GAP activity of RGS4 on Gαo. Finally, RGS2, but none of the other R4 family members interacts with Gαs (see below). Although the significance of this finding has not been clarified, together these studies clearly indicate that variance in primary structure within the RGS fold determine the G-protein selectivity and specificity of some R4 RGS proteins.

In addition, recent advances in measurement of GAP activity or RGS/G protein interactions *in vitro* may illuminate more subtle differences in affinity of various RGS proteins for their substrates. Novel techniques utilizing high-throughput bead-based flow cytometry (Roman, et al., 2007) or quench-flow or spectroscopy of fluorescently-tagged GTP have been utilized to measure G protein binding and GTP hydrolysis in real time (Willard, et al., 2005). A selective small molecule inhibitor (CCG-4986) of RGS4 was discovered, which apparently does not affect activity of RGS8 (Roman, et al., 2007). By using a flow cytometry protein interaction assay, CCG-4986 inhibited RGS4 binding to Gαo in a dose-dependent manner with IC_{50} ~ 7 μM but did not affect the ability of RGS8 to bind Gαo even at 100 μM. Furthermore, incubation of permeabilized μ-opioid receptor-expressing rat C6 glioma cells with CCG-4968 reversed ~75% of the inhibitory effect of RGS4 on forskolin-induced cAMP production without affecting the activity of RGS8.

3.3. RGS-GPCR interactions

Receptor-dependent, rather than primarily G-protein-dependent, inhibition of GPCR signaling by RGS proteins has been demonstrated some time ago (Xu, et al., 1999). Recombinant RGS4 exhibited marked differences in its ability to inhibit three distinct Gq-coupled receptors mediating Ca^{++} flux and IP3 formation in pancreatic acinar cells. Recombinant RGS4 inhibited carbachol, bombesin and cholecystokinin (CCK)-induced Ca^{++} mobilization with IC50s of 35, 110 and 380 nM, respectively. In other words, cholinergic receptors were 3- and 10-fold more sensitive to RGS4 than bombesin and CCK receptors, respectively. By contrast, RGS1 and RGS16, but not RGS2, showed a similar ability to inhibit the carbachol- and CCK-stimulated Ca^{++} responses. The differential pattern of inhibition of these responses was observed in cells from mice lacking one or more individual Gαq family members. Thus, this study suggested that in certain instances R4 RGS selectivity may be determined primarily by interaction with the receptor complex rather than affinity for a specific Gq class α subunit. The amino-terminal region of R4 RGS proteins has been suggested to mediate interaction with receptors (Bernstein, et al., 2004; Itoh, et al., 2006; Zeng, et al., 1998), which is discussed below.

3.3.1. Muscarinic acetycholine (mAch) receptors—The aforementioned studies are consistent with the hypothesis that the amino terminus of certain R4 RGS proteins such as RGS4 may regulate GPCR signaling independent of GAP activity. The RGS4 amino-terminus inhibits Gq-mediated Ca^{++} signaling in pancreatic acinar cells (Zeng, et al., 1998). Recently, direct evidence of the interaction of RGS proteins with the 3rd intracellular loop (3iL) of certain GPCRs has been obtained (Bernstein, et al., 2004; Georgoussi, et al., 2006; Hague, et al., 2005; Itoh, et al., 2006), suggesting a mechanism for these findings. Recombinant GST-3iL of M_1, M_3 and M_5 mAch receptors pulled down overexpressed RGS2 from CHO cell lysates (Bernstein, et al., 2004). In contrast, RGS4 expressed in these cells interacted with GST-3iL of M_1 and M_5, but not M_3, and there was no binding of either RGS1 or RGS16 to any mAch receptors. The amino-terminal domain of RGS2 was required for the direct interaction with mAchR 3iL in this study, as well as the functional inhibition of M_1 mAch receptor-stimulated PIP2 formation (Bernstein, et al., 2004). M_1 3iL bound to recombinant RGS2 and Gαq in the

presence of AMF (aluminum magnesium fluoride), which mimics the transition state between Gα–GTP and Gα-GDP, suggesting that RGS2 forms a stable heterotrimeric complex with active Gαq and the M_1 3iL.

RGS8 was also found to interact directly with M_1 mAch at 3iL through its first 9 amino acids (Itoh, et al., 2006). Two arginine residues in that region play a key role in M_1 3iL binding, as mutation of both Arg8 and Arg9 (RGS8-R8A/R9A), reduced binding more than 60%. This double mutant displayed substantially reduced ability to inhibit acetylcholine-stimulated Ca^{++} current (M_1 mAchR-mediated) in *Xenopus laevis* oocytes. The interaction of RGS8 and M_1-mAch receptor was also observed in living 293T cells using bioluminescence energy transfer (BRET), (Itoh, et al., 2006), reaffirming the existence of the interaction *in vivo*. Collectively, these studies indicate receptor-selective interactions of individual R4 RGS proteins that may depend on less well-conserved regions outside of the RGS domain.

3.3.2. Adrenergic receptors (ARs)—Alpha-2 adrenergic receptors (α_2AR) may directly interact with RGS1 and RGS16 based on comparison of the relative efficacy of three distinct agonists to stimulate a α_2AR-Go1α fusion protein in the absence or presence of RGS proteins (Hoffmann, et al., 2001). Both the α_2AR full agonist adrenaline and partial agonist UK14304 stimulated this fusion protein expressed in COS-7 cells to induce GTPase activity from membrane preparations. RGS1 and RGS16 significantly increased high-affinity GTPase activity of the α_2AR-Go1α fusion protein induced by both adrenaline and UK14304. However, both RGS proteins reduced agonist potency for adrenaline to a much greater extent than for the partial agonist UK14304 and did not alter potency of the weak agonist oxymetazoline. This result suggests a possible interaction between RGS proteins and the α_2AR which changes the conformation of the receptor and leads to altered intrinsic agonist activity. The RGS-G-protein interaction itself would not be predicted to affect receptor-agonist interactions that determine relative agonist potency. A related study found that in some cases recovery from agonist stimulation may depend less on G-protein deactivation than agonist unbinding from the receptor, which was selectively affected by RGS8 in a receptor and agonist-dependent manner (Benians, et al., 2003). If signal termination were dependent only on RGS8 interaction with G proteins and GAP activity, increasing RGS8 expression levels should increase deactivation independent of the receptor or agonist involved. However, since a subsequent study by the same group found no direct interaction between RGS8 and the GPCR in living cells by FRET, further experiments are needed to determine if a physical association between α_2AR or other receptors and RGS proteins occurs in native mammalian cells (Benians, et al., 2005).

α_{1A}AR was shown to bind RGS2 directly in purified recombinant protein pulldown assays. Expression of α_{1A}AR in HEK293 cells recruited RGS2, but not RGS16, to plasma membrane from the nucleus, whereas co-expression of α_{1B}AR had no effect on the localization of either RGS2 or RGS16 (Hague, et al., 2005). This result suggests a selective interaction between α_{1A}AR and RGS2. Receptor selectivity of RGS2 toward α_{1A}AR was further supported by the observation that RGS2 expression suppressed norepinephrine-stimulated α_{1A}AR-, but not α_{1B}AR, -mediated IP formation by HEK293 cells. The interaction appears to be mediated through 3iL of α_{1A}AR since recombinant GST-α_{1A}AR-3iL extracted HA-RGS2 from lysates of transfected HEK293 cells. Mutational studies indicated that the amino terminus of RGS2 was required for association with α_{1A}AR and inhibition of signaling, and amino acids Lys219, Ser220, and Arg238 within the α_{1A}AR 3iL were found to be essential for this interaction (Hague, et al., 2005).

β_2ARs had been previously shown to specifically recruit RGS2, but not RGS4, to the cell plasma membrane when co-expressed in 293T cells (Roy, et al., 2003). This receptor-promoted association was not altered by agonist, implying a constitutive interaction of RGS2 with β_2AR. Further studies by the same group demonstrated a direct interaction of RGS2 with β_2AR (Roy,

et al., 2006a). RGS2, but not the closely related RGS16, interacted with the 3iL of β_2AR, and over expression of RGS2 in HEK293 cells significantly suppressed iosproterenol-β_2AR-stimulated cAMP production. Thus, receptor binding by some RGS proteins may serve a negative regulatory function independent of GAP activity since RGS2 does not exhibit GAP activity toward Gαs (Roy, et al., 2003).

3.3.3. Opioid receptors—Recombinant RGS4 directly bound the carboxy-terminal domains of both μ-opioid and δ-opioid receptors, as well as the 3iL of δ-opioid receptor (Georgoussi, et al., 2006). This interaction was agonist-dependent since it was only observed in COS-7 cells stimulated with opioid receptor ligand. The interaction also required the presence of AMF, indicating that the presence of activated Gα was essential. Overexpression of RGS4 in 293T cells significantly reduced opioid receptor-mediated inhibition of forskolin-stimulated cAMP formation. Furthermore, Gβγ appeared to compete with RGS4 for binding to the C-terminal μ-opioid receptor, as the amount of RGS4 pulled down by C-terminal μ-opioid receptor deceased in the presence of increasing amounts of Gβγ. Thus, RGS4 is recruited to the plasma membrane after receptor activation to interact with the 3iL of opioid receptor and Gαi, resulting in increased Gα GTPase activity and inhibition of GPCR signaling. These studies stand in contrast with aforementioned studies of RGS8, in which constitutive interaction of RGS8-YFP with the heterotrimeric G protein complex throughout the GTPase cycle was observed. In other words, Gα-RGS interaction was not altered by Gα binding to βγ (Benians, et al., 2005).

3.4. Gαs and Adenylyl cyclase (AC)

Gαs-GTP stimulates AC to produce cAMP in response to activation of GPCRs such as β_2AR. Although none of the R4 RGS proteins possesses GAP activity towards Gαs (Hollinger and Hepler, 2002; Roy, et al., 2003), expression of RGS1, RGS2 and RGS3, and RGS13 exerts a negative effect on Gαs/AC-stimulated cAMP production (Johnson and Druey, 2002; Roy, et al., 2003; Sinnarajah, et al., 2001). These observations suggested that RGS proteins may inhibit Gαs signaling downstream of the G protein independent of their GAP activity. Co-expression of Gαs with RGS2, but not RGS4, in HEK293 cells promoted plasma membrane translocation of RGS2 (Roy, et al., 2003) implying association of Gαs and RGS2. The interaction between RGS2 and Gαs was subsequently demonstrated by co-immunoprecipitation of proteins expressed in HEK293 cells as well as *in vitro* binding of recombinant proteins (Tseng and Zhang, 1998). BRET also revealed a significant interaction between Gαs and RGS2 in living cells (Roy, et al., 2006a).

In addition to Gαs, RGS2 interacted with several isoforms of AC. RGS2 inhibited forskolin-induced cAMP production in Sf9 insect cell membranes expressing ACIII, ACV and ACVI, but not ACI and ACII, consistent with an isoform-selective interaction (Sinnarajah, et al., 2001). RGS2 also suppressed forskolin- or Gαs-GTPγS -induced cAMP generation in olfactory membranes (Sinnarajah, et al., 2001), which express endogenous ACIII predominantly. BRET subsequently revealed an interaction between RGS2 and both ACII and ACVI in intact HEK293 cells, and this complexation was confirmed by co-immunoprecipitation (Roy, et al., 2006a). Further studies have recently shown that the first 19 amino acids of RGS2 are required for binding to ACV as deletion of the amino-terminal 19 amino acids of RGS2 abolished its ability to inhibit Gαs- and β_2AR-stimulated cAMP formation (Salim, et al., 2003). Three residues within the RGS2 amino-terminus were required for the inhibition of AC function by RGS2 (Val9, Gln10 and His11).

Thus, RGS2 may form a signaling complex with Gαs and AC to negatively regulate Gs-mediated signaling. Blockade of endogenous RGS2 by anti-RGS2 antibody significantly increased the odorant-induced inward current in whole-cell voltage clamp studies of olfactory

neurons, and pre-incubation of recombinant RGS2 blocked the antibody-induced enhancement of the current (Sinnarajah, et al., 2001). Thus, endogenous RGS2 regulates odorant-induced AC signaling in olfactory neurons. RGS2 expression in HEK293 cells reduced glucose-dependent insulinotropic receptor (GIP-R)-stimulated cAMP formation by 50%. This effect mirrored the response of cells pre-incubated with low concentration of GIP upon restimulation (Tseng and Zhang, 1998), suggesting a role for RGS2 in GIP-induced desensitization response. Expression of RGS2 in mouse insulinoma βTC3 cells attenuated GIP-induced insulin release. Collectively, these studies suggest that regulation of the Gαs-AC signaling axis by RGS2 has physiological significance.

3.5. PLCβ

PLCβ, an enzyme activated by activated Gαq and Gβγ, catalyzes the conversion of PIP2 to IP3 and diacylglycerol (DAG), which leads to the mobilization of intracellular Ca^{++} stores and activation of certain isoforms of protein kinase C (PKC). Interaction of PLCβ with RGS4 has been detected by surface plasmon resonance (Dowal, et al., 2001) and FRET techniques. Binding to RGS4 appears to require the carboxy-terminus of PLCβ1 (Dowal, et al., 2001). Further studies revealed that PLCβ1 can be regulated by RGS4 through protein kinase A (PKA) and protein kinase G (PKG) (Huang, et al., 2007). Both PKA and PKG phosphorylate RGS4, which increased its GAP activity. Phosphorylation of RGS4 by PKA or PKG also caused translocation of RGS4 from cytosol to plasma membrane. Pre-treatment of dispersed rabbit gastric smooth muscle cells expressing WT RGS4 with compounds that activate PKA or PKG suppressed PLCβ-induced IP formation in response to Ach stimulation compared to cells exposed to Ach alone (Huang, et al., 2007), indicating an increased inhibitory effect of phosphorylated RGS4. The phosphorylation site of RGS4 was identified at Ser52. Co-expression of a RGS4/S52A mutant resistant to PKA- and PKG-induced stimulation of GAP activity on Gq reversed the inhibition of Ach-stimulated IP formation induced by pre-activation of PKA and PKG.

Agonist-stimulated contraction of smooth muscle is initiated by activation of PLCβ1 via Gαq or PLCβ3 via Gαi, leading to generation of IP3 and Ca^{++} release from intracellular stores. Ca^{++} activates Ca^{++}/calmodulin-dependent myosin light chain (MLC) kinase, which phosphorylates myosin light chain kinase 20 (MLC20), a prerequisite for smooth muscle contraction. Relaxation of contracted smooth muscle is mediated by PKA or PKG through the inhibition of IP3 formation. Thus, the increased ability of phosphorylated RGS4 to inhibit PLCβ activity provided a molecular mechanism by which PKA and PKG indirectly regulate PLCβ activity, which, in turn, affects smooth muscle contraction.

RGS proteins may also suppress GPCR-evoked signaling independent of GAP activity by modulating G-protein-effector interactions. RGS2, RGS3, RGS5 and RGS16 all inhibited carbachol-induced activation of PLCβ in COS-7 cells expressing muscarinic M_3 receptors as assessed by IP formation (Anger, et al., 2004). However, RGS2 and RGS3, but not RGS5 and RGS16, inhibited IP formation stimulated by constitutively activated Gαq, which is inert to GTP hydrolysis. This finding implies a GAP-independent role for certain RGS proteins as effector antagonists of Gαq. In this regard, RGS4 was originally shown to inhibit the interaction between Gαq-GTPγS and PLCβ (Hepler, et al., 1997). RGS4 has also been suggested to reside within the Gαq/PLCβ complex acting as an effector antagonist against PLCβ with its amino-terminus bound to the receptor 3iL. This hypothesis is based on the previously noted observation that amino-terminal region of RGS4 (outside of RGS domain) inhibited carbachol-stimulated Ca^{++} signaling in rat pancreatic acinar cells, and deletion of the amino-terminus from full-length RGS4 not only reduced its inhibitory potency by 10^4-fold but also abolished the receptor selectivity of RGS4 for mAchR (Zeng, et al., 1998). In addition, as noted above,

this construct was used to compete with endogenous RGS4 activity in neurons, although the mechanism for dominant inhibition is unclear (Ding, et al., 2006).

3.6. Spinophilin/neurabin

Spinophilin (SPL) and neurabin (NRB), structurally similar scaffolding proteins with more than 30 binding partners, are involved in the regulation of membrane and cytoskeletal functions (Sarrouilhe, et al., 2006). SPL, but not NRB, binds the 3iL of some GPCRs including the dopamine D2 receptor and α_{1B}AR (Smith, et al., 1999; Wang, et al., 2007). RGS1, RGS2, RGS4, and RGS16 co-immunoprecipitated with SPL and NRB expressed in HEK293 cells. Moreover, recombinant RGS2 bound NRB/SPL *in vitro*, suggesting a direct interaction (Wang, et al., 2007). The interaction of RGS proteins with SPL was also demonstrated in *Xenopus laevis* oocytes (Wang, et al., 2007; Wang, et al., 2005) and prefrontal cortex neurons (Liu, et al., 2006). SPL binding to certain RGS proteins promotes GPCR-Gα-RGS interaction, which enhances the inhibitory effect of RGS on α_1AR-stimulated, Gq-mediated Ca^{++} signaling (Liu, et al., 2006; Wang, et al., 2007). NRB competed with SPL for the binding to RGS proteins, reversing the enhancement of RGS activity by SPL. Overexpression of NRB in *X. laevis* oocytes increased α_{1B}AR-induced Ca^{++} signaling, whereas overexpression of SPL decreased the Ca^{++} response, suggesting opposing roles for SPL and NRB (Wang, et al., 2007). Parotid duct cells from *SPL*−/− mice and *NRB*−/− mice displayed a phenotype opposite of cells overexpressing these proteins (Wang, et al., 2007). The amino-terminus of RGS proteins appears to be involved in the interaction with NRB as a mutant RGS2 lacking the amino-terminus, GST-ΔNRGS2, did not extract NRB from lysates of HEK293 cells expressing NRB exogenously. Furthermore, infusion of SPL into parotid cells from *NRB*−/− mice further enhanced its inhibition of epinephrine-stimulated Ca^{++} signaling compared to infusion of SPL into WT cells, suggesting a reciprocal effect of NRB and SPL. Thus, the following model has been proposed: SPL and NRB form a functional pair of opposing regulators that modulate the intensity of Ca^{++} signaling by GPCR-Gαq. SPL acts as a bridge between 3iL of GPCR and R4-RGS proteins. By binding to RGS proteins and 3iL of GPCRs, SPL recruits RGS proteins to the GPCR signaling complex. NRB competes with SPL for binding to RGS proteins to remove them from the complex, which would be predicted to increase the intensity of Ca^{++} signaling.

3.6. Gβγ

Although members of the R7-RGS subfamily, which contain a G-protein γ-like (GGL) domain, can bind to Gβ5 in the absence of Gγ, the R4-RGS protein RGS3, which lacks the GGL domain, was found to interact with $G\beta_1\gamma_2$ both *in vivo* and *in vitro*. RGS3 co-immunoprecipitated with Gβγ from COS-7 cells overexpressing RGS3 and Gβγ, and recombinant His-RGS3 pulled down purified Gβγ subunits (Shi, et al., 2001). RGS3 inhibited $G\beta_1\gamma_2$ -stimulated IP production and activation of MAPK and Akt. Although mutational studies mapped two regions on RGS3 for binding to Gβγ located between amino acids 313 to 390 and 391 to 458, which are located within the RGS domain, binding of RGS3 to Gβγ did not require the complete RGS domain. Since the intact RGS box is required for Gα binding, RGS3 regulation of Gβγ is therefore independent of RGS3 GAP activity (Shi, et al., 2001).

3.7. Homer-2

Homer proteins are scaffolds that interact with GPCRs, IP3 receptors, and ryanodine receptors involved in Ca^{++} signaling. Homer 2 was found to increase the GAP activity of RGS4 in M_1 mAch receptor-stimulated steady state GTP hydrolysis assays, although direct interaction of RGS4 and Homer 2 was not demonstrated (Shin, et al., 2003). Pancreatic acinar cells from *Homer 2*−/− mice showed enhanced carbachol-stimulated Gq-mediated Ca^{++} mobilization. RGS4 infusion into acinar cells from WT mice completely abolished the carbachol-induced

Ca^{++} response, but had no effect on cells from *Homer 2*−/− mice. Although the detailed mechanisms remain unclear, these data suggested that Homer 2 fine-tunes Gq-mediated Ca^{++} signaling intensity by regulating the GAP activity of RGS4.

4. Interactions with non-GPCR signaling components

4.1. 14-3-3 proteins

14-3-3 are small dimeric proteins containing seven highly conserved isoforms whose functions are largely similar. 14-3-3 acts predominantly as scaffolding regulatory proteins through their interactions with components of diverse signaling pathways. 14-3-3 exerts its effects by binding either of two conserved phosphorylated motifs: RSXpSXP or RXY/FXpSSxP (Muslin, et al., 1996), although some protein interactions are phosphorylation-independent. Most of the R4 RGS subfamily members bind 14-3-3 including RGS3, RGS4, RGS5, and RGS16 (Abramow-Newerly, et al., 2006a; Benzing, et al., 2000; Ward and Milligan, 2005).

In some cases, binding of 14-3-3 with a conserved phosphorylated Ser residue within the RGS domain inhibits RGS GAP activity. In RGS7, Ser434, which is conserved in all RGS proteins within the RGS domain, was found to be critical for the binding of 14-3-3 proteins. Furthermore, this binding diminished the GAP activity of RGS7 when measured by single turnover GTP hydrolysis assay (Benzing, et al., 2000). However, later studies contradicted some of these findings. The conserved serine in the RGS domain appeared to be dispensable for the interaction of RGS proteins with 14-3-3; mutation of the corresponding Ser residue in RGS3 (RGS3/S496A) or RGS16 (RGS16/S166A) did not prevent binding to 14-3-3 in pulldown experiments using GST-14-3-3 and lysates of 293T cells expressing the mutant RGS proteins (Ward and Milligan, 2005). However, mutants lacking the conserved Ser within the RGS domain (RGS3/S496A, RGS1/S173A and RGS16/S166A, respectively) were unable to increase adrenaline-stimulated GTPase activity by a α_{2A}AR-Cys351Ile-Go1 fusion protein expressed in membranes of HEK293 cells, suggesting impaired GAP activity of this mutant. Interestingly, two groups (Niu, et al., 2002; Ward and Milligan, 2005) have subsequently identified a Ser in the amino-terminus of RGS3 (Ser264, which lies outside the RGS domain) responsible for 14-3-3 binding. Mutation of Ser264 in RGS3 (S264D or S264A), disrupted the interaction in GST-14-3-3 and RGS proteins expressed in HEK293 cell lysates (Ward and Milligan, 2005) or in co-immunoprecipitation studies of CHO cells overexpressing myc-RGS3 (Niu, et al., 2002).

14-3-3 binding to RGS3 reduced the inhibitory effect of RGS3 on endothelin 1-induced, Gq-mediated activation of the transcription factor Elk1 (Niu, et al., 2002). These authors suggested that 14-3-3 may sequester RGS3 from Gqα as incubation of CHO cell lysates expressing myc-RGS3 with exogenous MBP-14-3-3 recombinant protein reduced the amount of Gα co-immunoprecipitated by anti-myc antibody dramatically (Niu, et al., 2002). Binding of 14-3-3 to RGS proteins appears to be direct as recombinant $His_6$14-3-3α efficiently purified GST-RGS4, GST-RGS5, and GST-RGS16 (Abramow-Newerly, et al., 2006a).

Functional consequences of the interaction of 14-3-3 with RGS proteins vary depending on the 14-3-3 isoform studied. Both 14-3-3ε and β inhibited the GAP activity of RGS4 and RGS16 but not RGS5 in steady-state GTP hydrolysis assays (Abramow-Newerly, et al., 2006a). 14-3-3τ had no effect on GAP activity of several RGS proteins including RGS3, RGS7 and RGS16 when tested on an agonist-stimulated α_{2A}AR-Cys^{351}Ile-Go1 fusion protein expressed in HEK293 cells (Benzing, et al., 2000). When GAP activities of these RGS proteins were measured by high-affinity GTPase assay in adrenaline-stimulated membrane preparations of HEK293 cells expressing α_{2A}AR-Cys^{351}Ile-Go1 fusion protein, 14-3-3τ had no effect on GAP activity (Ward and Milligan, 2005). By contrast, in a separate set of experiments, 14-3-3τ inhibited RGS7 GAP activity towards Gαi1 in a single turnover assay (Benzing, 2000),

suggesting distinct effects of 14-3-3τ on individual RGS proteins. Another recent study showed that 14-3-3ε competes with activated Gαo for RGS4 binding and that overexpression of 14-3-3ε reversed Gαi-induced translocation of RGS4 from cytosol to plasma membrane (Abramow-Newerly, et al., 2006a). Thus, in certain instances depending on the 14-3-3 isoform involved, 14-3-3 may sequester RGS proteins from Gα, resulting in mis-localization within the cell and thus indirect inhibition of GAP activity.

4.2. PKG1-α

Nitric oxide (NO) inhibits vascular contraction by activating cGMP-dependent protein kinase G (PKG), leading to vascular smooth muscle relaxation. PKG1α has been shown to attenuate thrombin receptor-stimulated Gq-mediated IP formation through activation of RGS2 (Tang, et al., 2003). PKG1α directly binds and phosphorylates RGS2 at Ser 46 and Ser 64 sites. Phosphorylation of RGS2 by PKG1α enhanced its GAP activity on Gq, therefore inhibiting thrombin-stimulated signaling. As noted, *Rgs2*−/− mice are markedly hypertensive, and their blood vessels exhibit enhanced contraction, especially in response to angiotensin II. These findings support a novel mechanism whereby NO inhibits vascular contraction and blood pressure through PKG-mediated activation of RGS2.

4.3. Receptor tyrosine kinases (RTKs)

RGS16 has been shown to interact with epidermal growth factor receptor (EGFR) by co-immunoprecipitation (Derrien and Druey, 2001). Tyrosine phosphorylation of RGS16 by EGFR enhanced RGS16 GAP activity on Gαi in single turnover assays. Whether binding of RGS16 and EGFR was direct or indirect was not determined. However, mutation of EGFR target tyrosine residues in RGS16 severely reduced the capacity of RGS16 to regulate Gi-mediated (M_2 mAch) MAP kinase activation or inhibition of adenylyl cyclase in HEK293T cells. RGS4 interacted with ErbB3 (EGFR family member) in yeast and human cells by yeast two-hybrid assay and co-immunoprecipitation, respectively (Thaminy, et al., 2003). Yeast two-hybrid analysis of RGS4 truncations mutants indicated that the residues located at the carboxy-terminus of the RGS box were important for binding to ErbB3. RGS5, which, as previously mentioned, is expressed in a pattern similar to PDGF receptors in pericytes, inhibited PDGFRβ-induced ERK-2 phosphorylation when overexpressed in NIH3T3 cells in addition to inhibiting GPCR (endothelin-1 and angiotensin II)-evoked Erk activation (Cho, et al., 2003). Although the molecular mechanism for the inhibition of PDGFβ-induced MAP kinase activation by RGS5 has not yet been determined, these three studies suggest potential cross-talk between GPCR- and RTK-induced signaling routes through RGS proteins such as RGS4, 5 and 16.

Activating mutations of RTKs cause malignant transformation of cells leading to cancers. RGS2 expression has recently been shown to be repressed by internal tandem duplications of the RTK fetal liver tyrosine kinase 3 (Flt3) gene (Flt3-ITD), a mutation which may cause acute myeloid leukemia (AML) (Schwable, et al., 2005). *RGS2* mRNA was reduced by overexpression of Flt3 with activating mutations in two myeloid cell lines. Furthermore, *RGS2* levels detected by real time PCR were decreased in the bone marrow of Flt3-ITD$^+$ AML cases compared to Flt3-ITD$^-$ AML. Overexpression of RGS2 inhibited Flt3-ITD–induced transformation of myeloid cells and inhibited Flt3-ITD-induced phosphorylation of Akt and glycogen synthase kinase 3β (GSK3β). Although the mechanism for these observations has not been elucidated, these data suggest that RGS2 has a function in oncogenic cellular transformation induced by an RTK.

4.4. Steroid hormone receptors

Steroid hormone receptors are nuclear hormone receptors that belong to the superfamily of ligand-inducible transcription factors. These receptors are structurally related and are

composed of six major functional domains. Domain A/B in the amino-terminal region is the constitutive activation domain 1 (AF-1). Domain C, the DNA-binding domain, is arranged in two zinc-stabilized DNA-binding finger motifs. Region D contains a nuclear localization signal. The ligand binding and ligand-dependent transcriptional activation function 2 (AF-2) is located in domain E/F of the carboxy-terminus of the protein. A yeast two-hybrid system using domains C and D of the rat estrogen receptor α (rERα C/D) as bait recovered a cDNA encoding a protein that was termed steroid receptor-binding (SRB) protein bearing the RGS domain at the carboxy terminus (SRB-RGS) from a rat ovary cDNA library (Ikeda, et al., 2001). The sequence of SRB-RGS was identical to *RGS3*. SRB-RGS/RGS3 interacted with ER-α by co-immunoprecipitation of [^{35}S]methionine-labeled *in vitro* translated proteins, even in the presence of ER-α–DNA binding. Gel-shift assays using nuclear extracts from rat uterine cells bound to *Xenopus* vitellogenin A2 estrogen response element (VRE) oligonucleotides demonstrated supershifting with an anti-RGS3 antibody, suggesting that SRB-RGS interacted with ER-α bound to VRE (Ikeda, et al., 2007). Expression of SRB-RGS suppressed ERα and β transcriptional activity of a VRE-chloramphenicol acetyltransferase (CAT) reporter in COS-7 cells. SRB-RGS also induced apoptosis of Hela cells in the presence or absence of the caspase inhibitor Z-VAD-FMK. These studies suggested that SRB-RGS/RGS3 regulates estrogen-induced transcription as well as caspase-independent apoptosis.

4.5. Ephrin B and Eph receptors

The ephrin-Eph signaling pathway plays important roles in developmental processes, including axon pathway selection, angiogenesis, rhombomere compartmentalization and migration (Kullander and Klein, 2002). Eph molecules resemble RTKs because they contain a cytoplasmic kinase domain. Ephrin-Eph signaling is bidirectional with a forward signal through the Eph receptor and a reverse signal through the ligand (Bruckner, et al., 1997; Holland, et al., 1996). Due to reverse signaling, cell-attached ephrins communicate cell contact signals not only to cells expressing Eph molecules, but also cells expressing the ephrins through binding of their cytoplasmic domain with intracellular effector proteins. A yeast two-hybrid screen using the intracellular domain of ephrin B as bait revealed PDZ-RGS3 as a binding partner (Lu, et al., 2001). RGS3 interacted with ephrin B in a GST pull-down assay and in co-immunoprecipitations of either transfected or endogenous proteins. Reverse signaling from soluble EphB2-Fc receptor inhibited the effect of CXCL12 on cerebellar granule cells, indicating a control over neuronal migration. These authors hypothesized that PDZ-RGS3 binding to the cytoplasmic domain of ephrin B via its PDZ domain localizes RGS3 at the membrane where it inhibits G-protein signaling through its RGS domain. This result provides evidence for direct cross-talk between ephrin-Eph and G-protein dependent pathways. Since CXCL12 and its receptor CXCR4 are required for normal granule cell migration, PDZ-RGS3 may have an essential function in neuronal development.

Recently, Src homology 2 (SH2) domain-containing Grb4 has also been shown to interact with the intracellular portion of ephrin B (Su, et al., 2004). The SH2 domain of Grb4 and PDZ domain of PDZ-RGS3 bound Ephrin B phosphorylated at Tyr304 simultaneously and independently, forming a three component molecular complex. PDZ-RGS3 also bound neuroligin, a postsynaptic adhesion molecule that associates with β-neurexin in the synaptic cleft and has a function in glutamatergic synapses (Meyer, et al., 2004). Thus, RGS3 may have several functions in neuronal signaling through its RGS domain as well as a PDZ domain that binds several receptors.

4.6. COPI

COPI, a cytosolic protein complex consisting of seven subunits (α, β, β′, γ, δ, ε and ζ-COP) plus the monomeric GTPase, ARF1 (ADP-ribosylation factor), is an integral component of intracellular transport carriers. COPI is important for maintaining Golgi integrity and normal

intracellular transport. RGS4 and RGS2 directly bound β′-COP and and the intact COPI complex in cell lysates (Sullivan, et al., 2000). These associations reduced COPI binding to Golgi membranes *in vitro* and diminished COPI-Golgi association in intact cells as determined by immunofluorescence. RGS4 overexpression inhibited secretion of alkaline phosphatase in HEK293T cells without affecting Golgi integrity. RGS4 bound COPI through two dilysine motifs in the RGS domain, but at a site not involving Gα binding. Thus, the RGS4-COPI interaction is independent of RGS4 GAP activity. Although these data need to be confirmed in native systems, additional studies showing presence of Gα subunits on intracellular organelle membranes (Denker, et al., 1996; Leyte, et al., 1992; Maier, et al., 1995; Slessareva, et al., 2006) suggest an interplay between G protein signaling, RGS proteins and COPI-mediated intracellular trafficking.

4.7. GIRK channels

G-protein regulated inward-rectifier potassium channels (GIRK or Kir3) are members of a superfamily of inward-rectifier K^+ channels that includes seven family members. Four GIRK subunits, designated GIRK1-4 (also designated Kir3.1-4), have been identified in mammals which co-assemble to form neuronal and cardiac GIRK channels (Yamada, et al., 1998). GIRK5 was identified in *Xenopus* oocytes. GIRK channels exist *in vivo* both as homotetramers and heterotetramers. In contrast to the other mammalian GIRK family members, GIRK1 cannot form functional channels by itself and must co-assemble with GIRK2, 3 or 4. GIRK channels are modulated by heterotrimeric G proteins, phosphatidylinositol 4,5-bisphosphate (PIP2), intracellular sodium, ethanol and mechanical stretch (reviewed in (Mark and Herlitze, 2000).

Kir3 channels are activated following stimulation of GPCRs that utilize the Gi/o family of G proteins. Gβγ binds to and activates Kir3 channels (Huang, et al., 1995; Reuveny, et al., 1994; Wickman, et al., 1994). Gα GTPase activity is required for terminating Kir3 activation. RGS GAP activity thus accelerates GIRK channel "off" kinetics by promoting Gα deactivation. Somewhat unexpectedly, co-expression of RGS1, 3, and 4 but not RGS2 with GIRK channels and M_2 mAch receptors in *X. laevis* oocytes or CHO cells accelerated agonist (carbachol)-evoked GIRK currents (Doupnik, et al., 1997). Several studies since then have shown that both the "on" and "off" kinetics of endogenous channels observed in tissues can be re-capitulated by expression of RGS proteins and heterologously-expressed GIRK channels.

Coexpression of RGS2, 5 and 8 accelerated mAchR-mediated GIRK kinetics in *Xenopus* oocytes (Herlitze, et al., 1999; Saitoh, et al., 1999) whereas $GABA_b$ receptor-activated GIRK currents in HEK293T cells were modulated by RGS4 (Fowler, et al., 2007; Mutneja, et al., 2005). Channels coupled to serotonin 1A and muscarinic M_2 receptors in CHO-K1 cells were differentially regulated by the alternatively spliced short RGS3 isoform (RGS3s) (Jaen and Doupnik, 2005). RGS3s dramatically reduced steady-state M_2-receptor-activated GIRK currents, but not 5-HT_{1A} receptor-coupled currents. Thus, RGS3s modulated GIRK channels in a GPCR-selective manner whereas RGS4 modulated M_2 and 5-HT_{1A}-evoked GIRK channel activity with equal potency. RGS4 co-immunoprecipitated with multiple GPCR-Kir3 channel complexes expressed in CHO-K1 cells whereas RGS3s did not (Jaen and Doupnik, 2006). Both the RGS4 amino terminus and the RGS domain were necessary for complexation with M_2-Kir3 channel complexes (Jaen and Doupnik, 2006). Precoupling of RGS4 to the GPCR-Kir3 complex lead to greater acceleration of G protein-dependent Kir3 channel gating kinetics than that was observed for RGS3s, which does not associate with the GPCR-Kir channel complex.

In the case of RGS8, GAP activity was not required for modulation of GIRK activation. Pertussis toxin (PTX)-treated rat sympathetic neurons expressing GIRK channels activated by Gβγ and a $G\alpha o_A$ mutant resistant to both PTX and RGS GAP activity displayed markedly abnormal activation and deactivation kinetics and failed to desensitize to prolonged agonist (noradrenaline) stimulation (Jeong and Ikeda, 2001). Heterologous expression of RGS8 in

neurons expressing the PTX- and RGS-insensitive $G\alpha o_A$ construct restored rapid GIRK current activation kinetics in response to noradrenaline. Expression of a mutant RGS8 protein containing only the amino-terminus affected channel activation kinetics similar to full length RGS8 whereas expression of the RGS8 RGS domain alone had no effect. These results suggested that amino-terminus of RGS8 modulated GIRK current activation kinetics independent of GAP activity.

4.8. Calcium channels

Voltage-gated calcium channels are found at presynaptic and postsynaptic terminals and play an essential role in neurosecretion. The activities of the L-, N-, P/Q- and R- type channels are modulated by G-proteins, mainly Gi/o and Gq, which in turn are inhibited by RGS proteins. R4 RGS proteins including RGS2, 3, and 4 accelerate the onset and turnoff of transmitter-mediated inhibition (Gαz, Gαq and Gαi/o-mediated) of presynaptic calcium channels (Abramow-Newerly, et al., 2006b) in recombinant as well as endogenous systems. Electrophysiological recordings of hippocampal neurons from *Rgs2*−/− mice demonstrated that endogenous RGS2 increases synaptic transmitter release and thus synaptic plasticity (Han, et al., 2006a).

The RGS3s isoform also modulated G-protein-mediated synaptic transmission in dorsal root ganglion neurons by directly binding Ca^{++} through an EF hand domain (Tosetti, et al., 2003). The direct interaction of Ca^{++} to RGS3s was confirmed by calcium-induced gel mobility-shift. Overxpression of either RGS3s or RGS3ss, two endogenous isoforms detected in these neurons, terminated G-protein-dependent inhibition of calcium channels with differing kinetics. RGS3s rapidly desensitized G-protein-dependent inhibition of calcium channels. In contrast, expression of the RGS3ss mutant, which lacks the EF hand domain, produced a slower Ca^{++}-dependent signal termination that was attenuated by a calmodulin antagonist. This result suggested distinct mechanisms of regulation of G-protein dependent inhibition of calcium channels by these RGS3 isoforms.

4.9. Calcium/calmodulin and anionic phospholipids

Levels of RGS proteins may be regulated by diverse extracellular cues depending on the cell type. Calcium-mediated signaling pathways have been implicated in regulation of RGS2 levels, and in turn RGS2 regulates Ca^{++} signaling (Popov, et al., 2000). *RGS2* mRNA expression was increased in human blood mononuclear cells treated with ionomycin (Heximer, et al., 1997a) and in cultured myometrial cells in response to oxytocin (Park, et al., 2002). In human adrenocortical cells, angiotensin II upregulated RGS2 mRNA, and this effect was reversed by both a calcium channel blocker and by calmodulin and calcium/calmodulin-dependent kinase (CaMK) antagonists (Romero, et al., 2006).

Calmodulin regulates membrane association of many intracellular signaling molecules (Popov, et al., 2000). Calmodulin binds several RGS proteins and indirectly regulates their GAP activity. RGS1, 2, 4, 10, 16 and GAIP bound calmodulin in the presence of calcium, but calcium/calmodulin did not directly affect GAP activity of RGS proteins in single turnover assays (Popov, et al., 2000). In contrast, PIP3, but not other membrane phosphatidylinositol phosphates or synthetic phospholipids, bound RGS4 and inhibited its GAP activity in a concentration-dependent manner (Popov, et al., 2000). PIP3 inhibited GAP activity of several RGS proteins including RGS1, 2, 10 and GAIP but not RGS16. Furthermore, PIP3 inhibition of GAP activity was reversed by calcium/calmodulin, suggesting calcium/calmodulin competition with PIP3 as an intracellular mechanism for feedback regulation of RGS activity and thus calcium signaling. There are two potential calmodulin binding regions in RGS4, one in the amino-terminus (amino acids 1-33) and another in the RGS fold (helices 4–5, amino acids 99-113). An independent study demonstrated that PIP3 and calcium/calmodulin

competitively bind to RGS4 within the RGS domain (Ishii, et al., 2005a). Mutation of RGS4 residues Lys99 and Lys100 impaired the interaction between the RGS box and PIP3/CaM *in vitro* and in intact HEK293 cells (Ishii, et al., 2005a; Ishii, et al., 2005b). The physiological importance of PIP3 regulation of RGS activity was demonstrated by analysis of Ach-induced GIRK channel activity in cardiac atrial myocytes by whole cell patch-clamp technique. PIP3 blocked the effect of RGS4 on GIRK channel activity, which was reversed by Ca^{++}/CaM (Ishii, et al., 2002).

Other anionic phospholipids such as phosphatidic acid (PA) also inhibit RGS4 GAP activity (Ou-Yang, et al., 2003; Ouyang, et al., 2003) in single turnover and steady-state GTP hydrolysis assays. Deletion of the RGS4 amino-terminus ablated PA binding as well as PA-mediated functional inhibition. Lys20 was essential for PA-mediated inhibition of RGS4 GAP activity, while mutation of either Arg22 or Leu23 in the amino-terminal amphipathic α-helix of RGS4 reduced PA-mediated inhibition of RGS4 GAP activity. Thus, interaction of PA and RGS4 involves basic-charged stretches within RGS4 distinct from those required for RGS-PIP3 binding. Since PA is a product of the activities of phospholipase D (PLD) and diacylglycerol kinase (DGK) downstream of G-protein dependent pathways, these results suggest feedback regulation of RGS4 activity induced by GPCR stimulation.

5. Conclusions/Future Perspectives

The importance of the RGS domain for R4 RGS protein regulation of G-protein-mediated signals is undisputed. Nearly ten years ago, crystallographic analysis of RGS4 complexed with its substrate Gαi1 demonstrated that the RGS fold mediates direct binding to the G protein through key contact residues (Tesmer, et al., 1997). Biochemical studies utilizing recombinant proteins or ectopic expression of RGS proteins in numerous mammalian cell types confirmed their ability to negatively influence GPCR effector activation through GAP activity. What this characteristic translates to in the whole organism has been slowly forthcoming. Newer studies utilizing gene-targeted mice or RNAi have now demonstrated that in addition to specifying agonist threshold responses or mediating recovery from prolonged external stimulation, RGS proteins may influence numerous components of the signaling response independent of their GAP activity.

The amino terminus of several RGS proteins, which was disordered in the crystal structure, may direct the RGS domain to specific GPCR-G protein complexes by binding the receptor itself. Thus, depending on the duration and intensity of the external cue and cellular localization of the RGS at the time of stimulation (which may be somewhat fluid), an RGS protein could actually participate in a stable complex with GPCR and heterotrimeric G protein throughout the signaling process. Depending on the effector involved, such “kinetic” (or perhaps even physical) scaffolding may enhance activation. In turn, this concept raises the possibility that RGS proteins may be required for certain cellular responses.

Small molecules that selectively inhibit the G protein interactions of an individual RGS protein are now a reality (Kimple, et al., 2007; Roman, et al., 2007). Such compounds might be predicted to profoundly affect the efficacy and potency of existing GPCR-targeted therapeutic agents. In addition, the studies summarized in this review clearly indicate that many regions of RGS proteins, particularly their amino termini, also mediate interactions with a host of factors within and outside of GPCR signaling pathways. Drugs targeting these “off” sites could represent another novel class of therapeutic agents for many processes influenced by this large and diverse family of regulatory molecules.

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Abbreviations

AC
adenylyl cyclase

AR
adrenergic receptor

AMF
aluminum magnesium fluoride

EGFR
epidermal growth factor receptor

GAP
GTPase accelerating protein

PIP3
phosphatidylinositol (1,4,5) trisphosphate

GIRK channel
G-protein regulated inward-rectifier potassium channels

GPCR G protein-coupled receptor

3iL 3rd intracellular loop

NRB neurabin

mAch muscarinic acetycholine

PKA protein kinase A

PKG protein kinase G

PLC phospholipase C

RTK receptor tyrosine kinases

RGS regulators of G protein signaling

SPL spinophilin

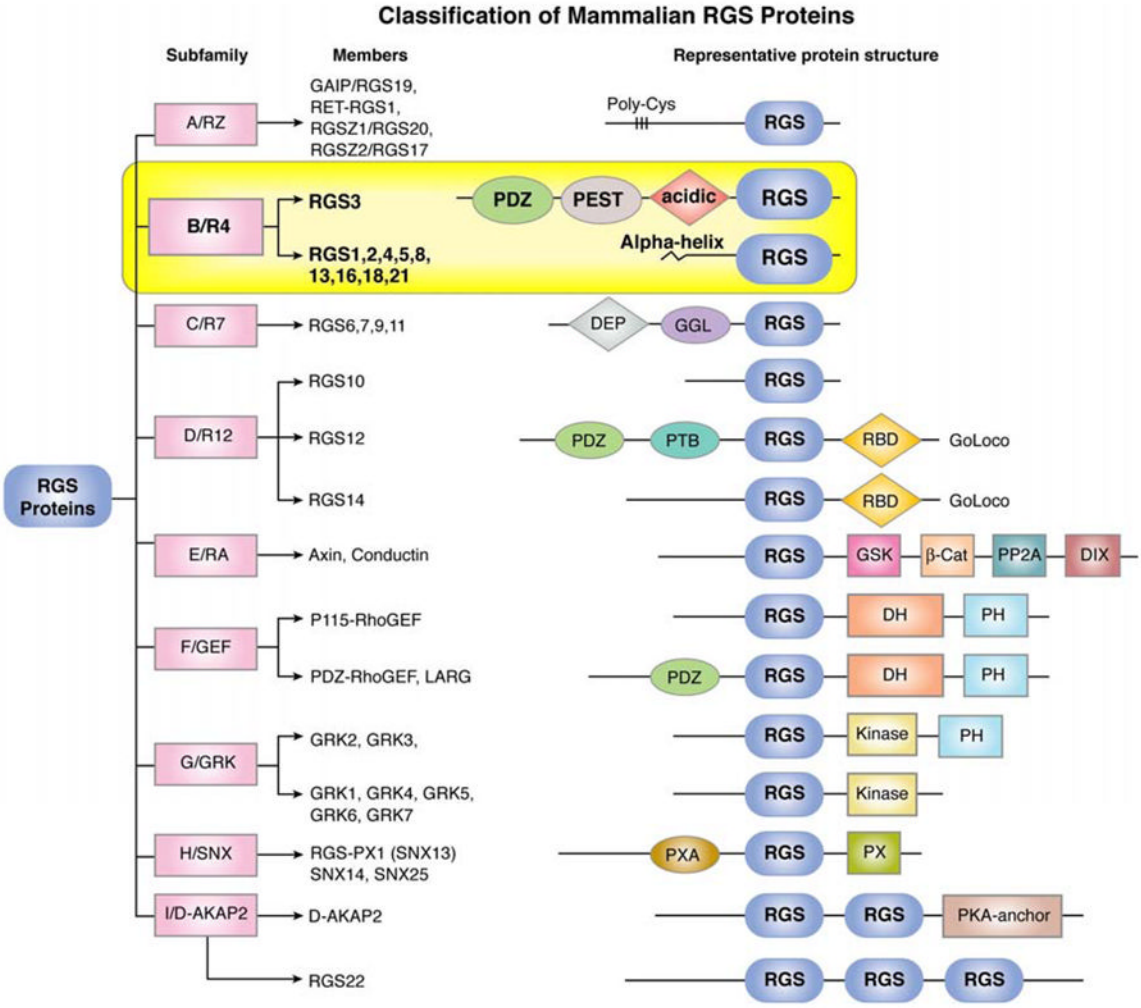

Figure 1.

Classification of mammalian RGS proteins into different subfamilies and their representative protein structure showing identified motifs or domains. Abbreviations: β-Cat, β-catenin-binding; D, dimerization domain; D-AKAP, dual-specificity A-kinase anchoring protein; DEP, dishevelled/EGL-10/pleckstrin; DH, double homology; DIX, dishevelled homology domain; GAIP, G alpha interacting protein; GEF, guanine nucleotide exchange factor; GGL, Gγ-like; GoLoco, Gαi/o-Loco; GRK, G protein-coupled receptor kinase; GSK, glycogen synthase kinase 3β-binding; PDZ, PSD95/Dlg/Z0-1/2; PEST, proline, glutamine, serine, threonine-rich; PH, pleckstrin homology; PP2A, protein phosphatase 2A; PTB, phosphotyrosine-binding; PX, phosphatidylinositol-binding; PXA, PX-associated; RBD, Ras-binding domain; RGS, Regulator of G protein Signaling domain; SNX, sorting nexin.

Table I

Major Sites of R4 RGS Expression and Associated Loss-of-Function Phenotype(s)

RGS	Major Sites of Expression	Phenotypes	References
RGS1	B lymphocytes	↑ B cell chemotaxis to CXCL12, 13* ↑ B cell LN homing and motility w/in LN	(Moratz, et al., 2004) (Han, et al., 2005)
RGS2	Widespread	↓ T cell proliferation, cytokine production, antiviral immunity; ↑ basal hippocampal neuron activity; ↓ male aggression; ↑ anxiety ↑ Hippocampal neuron NT release Hypertension Abnormal renal solute handling ↑ Gq-mediated IP formation in cardiac myocytes (phenylephrine, ET-1)* ↑ ATP-induced ciliary beat frequency in lung epithelium	(Oliveira-Dos-Santos, et al., 2000) (Han, et al., 2006a) (Gross, et al., 2005; Heximer, et al., 2003) (Zuber, et al., 2007) (Zhang, et al., 2006) (Nlend, et al., 2002)
RGS3	Widespread	↑ M_3 mAchR-evoked MAPK activation in aortic smooth muscle cells** ↑ Docetaxel-induced apoptosis of breast cancer cells*	(Wang, et al., 2002) (Ooe, et al., 2007)
RGS4	Brain	↑ pain threshold ↑ $5HT1_A$-evoked inhibition of NMDA channels in prefrontal cortical neurons***	(Grillet, et al., 2005) (Gu, et al., 2007)
RGS5	Vascular smooth muscle Pericytes	↑ Angiotensin II-induced MAPK activation in aortic smooth muscle cells**	(Wang, et al., 2002) (Cho, et al., 2003)
RGS8	Cerebellum	N/A	(Saitoh, et al., 2003)
RGS13	B lymphocytes Mast cells Neuroendocrine cells T lymphocytes Dendritic cells	↑ Chemotaxis to CXCL12,13 in B cell line*	(Han, et al., 2006b) Druey, et al., unpublished observations (Estes, et al., 2004) (Shi, et al., 2004)
RGS16	Platelets Liver T lymphocytes	↑ Ca^{++} response to CXCL12 in megakaryocyte cell line*	(Berthebaud, et al., 2005) (Huang, et al., 2006) (Beadling, et al., 1999; Johnson, et al., 2003)
RGS18	Osteoclasts Hematopoietic stem cells Platelets	↑ RANKL-induced osteoclastogenesis*	(Iwai, et al., 2007) (Park, et al., 2001; Yowe, et al., 2001) (Gagnon, et al., 2002)
RGS21	Taste bud cells	N/A	(von Buchholtz, et al., 2004)

Loss-of-function phenotypes in RGS-deficient cells. Studies of knockout mice or cells there from unless otherwise noted (

* SiRNA;

** Ribozyme;

*** Blocking antibody). N/A: not assessed; LN: lymph node

Table II

Interacting Partners of R4-RGS Proteins within GPCR pathways

RGS Proteins	Interaction Partners	References
RGS1	Gαi/αo, Gαq	(Moratz, et al., 2000; Watson, et al., 1996)
	SPL, NRB	(Wang, et al., 2007)
	α_2AR	(Hoffmann, et al., 2001)
RGS2	Gαq	(Heximer, et al., 1997b)
	SPL, NRB	(Wang, et al., 2007)
	Gαs	(Roy, et al., 2006a; Roy, et al., 2003; Tseng and Zhang, 1998)
	ACI, II, V, VI	(Roy, et al., 2006a; Salim, et al., 2003)
	β_2AR	(Roy, et al., 2006a)
	α_{1A}AR	(Hague, et al., 2005)
	M_1, M_3, M_5 mAchR	(Bernstein, et al., 2004)
RGS3	Gαi, Gαq	(Shi, et al., 2002)
	$G_{\beta 1\gamma 2}$	(Shi, et al., 2001)
RGS4	Gαi/αo	(Lan, et al., 1998; Watson, et al., 1996)
	Gαq	(Heximer, et al., 1997b)
	PLCβ	(Dowal, et al., 2001; Zeng, et al., 1998)
	SPL, NRB	(Wang, et al., 2007)
	μ-, δ-opioid R	(Georgoussi, et al., 2006)
	M_1, M_5 mAchR	(Bernstein, et al., 2004)
RGS5	Gαi/αo, Gαq	(Chen, et al., 1997; Zhou, et al., 2001)
RGS8	Gαi, Gαq	(Saitoh, et al., 2002)
	M_1 mAchR	(Itoh, et al., 2006)
RGS13	Gαi, Gαq	(Johnson and Druey, 2002; Shi, et al., 2002)
RGS16	Gαi/αo, Gαq	(Beadling, et al., 1999; Chen, et al., 1997)
	Gα13	(Johnson, et al., 2003)
	α_2AR	(Hoffmann, et al., 2001)
	SPL, NRB	(Wang, et al., 2007)
RGS18	Gαi, Gαq	(Nagata, et al., 2001; Park, et al., 2001)
RGS21	α-Gusducin (Gαi), Gαq	(von Buchholtz, et al., 2004)

Table III

Interacting Partners of R4-RGS Proteins outside of GPCR pathways

RGS Proteins	Interaction Partners	References
RGS1	Calmodulin	(Popov, et al., 2000)
	14-3-3	(Ward and Milligan, 2005)
RGS2	Calmodulin	(Popov, et al., 2000)
	β′-COP	(Sullivan, et al., 2000)
	TRPV6	(Schoeber, et al., 2006)
	Tubulin	(Heo, et al., 2006)
	PKG1-α	(Tang, et al., 2003)
PDZ-RGS3	EphrinB	(Lu, et al., 2001; Su, et al., 2004)
	Neuroligin	(Meyer, et al., 2004)
SRB-RGS3/RGS3	Estrogen receptor α	(Ikeda, et al., 2007)
	14-3-3	(Niu, et al., 2002; Ward and Milligan, 2005)
RGS4	Calmodulin	(Popov, et al., 2000)
	β′-COP	(Sullivan, et al., 2000)
	Kir3 channel	(Jaen and Doupnik, 2006)
	PIP3	(Ishii, et al., 2005a; Ishii, et al., 2005b; Popov, et al., 2000)
	Calcium/CaM	(Ishii, et al., 2005a; Ishii, et al., 2005b)
	Phosphatidic acid	(Ou-Yang, et al., 2003; Ouyang, et al., 2003)
	ErbB3	(Thaminy, et al., 2003)
	14-3-3	(Abramow-Newerly, et al., 2006a)
RGS5	14-3-3	(Abramow-Newerly, et al., 2006a)
RGS8	N/A	
RGS13	PIP3	Druey, et al., unpublished observations
RGS16	Calmodulin, PIP3	(Popov, et al., 2000)
	EGFR	(Derrien and Druey, 2001)
	14-3-3	(Abramow-Newerly, et al., 2006a; Ward and Milligan, 2005)