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Review

Regulatory mechanisms involved in modulating RGS function

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Abstract. Regulator of G-Protein Signaling (RGS) refers to a conserved 120–125 amino acid motif that was first identified by its ability to negatively regulate G-Protein-Coupled Receptor (GPCR) signalling. Mechanistically, RGSs were found to regulate GPCR responses by binding to and stimulating the GTPase activity of the receptor-activated GTP-bound Gα subunits. There are now over 25 mammalian RGSs containing proteins that are reported to carry out a variety of functions, many of which are unrelated to GPCR signalling. RGS proteins range in size from small proteins that contain little more than an RGS box to very large proteins that contain a variety of domains. The selectivity of function of the RGS proteins is attributable to the divergence of the RGS sequences as well as the presence of a variety of functional motifs, which allow them to interact with other proteins. Here we focus on the RGSs that are involved in modulating GPCR signalling by reviewing the diversity of the mechanisms involved in regulating these RGSs.

Keywords. RGS, G-protein coupled receptor (GPCR), interacting proteins, yeast, endogenous expression.

Introduction

G-Protein-Coupled Receptors (GPCRs) are seven transmembrane domain (7 TMD) protein receptors that serve to transduce the intracellular effects of a large variety of extracellular factors [1]. Stimuli that serve as agonists for different GPCRs include light, ions such as calcium, small molecules such as cAMP (cyclic AMP) and catecholamines, peptides such as somatostatin and large glycoprotein hormones such as follicle-stimulating hormone. In addition, most tissues and cell types express multiple GPCRs. As such, GPCRs serve key regulatory functions for a large number of biological processes as diverse as vision, smell, blood clotting, physiological responses to numerous hormones and regulation of blood pressure. GPCRs are associated with a heterotrimeric G-protein that consists of α, β and γ subunits, with the G_α subunit bound to GDP [2]. Upon occupancy by the agonist, the receptor conformation is shifted to the active state, which results in the displacement of the GDP by GTP on the G_α subunit (Fig. 1). The GTP-bound G_α subunit then dissociates from the $G_{\beta\gamma}$ subunit. Both the G_α and $G_{\beta\gamma}$ subunits are thus activated and can stimulate or inhibit effector proteins such as adenylyl cyclase, phospholipases and a variety of ion channels [3, 4]. The observed diversity in the physiological effects of GPCR agonists is in large part dictated by the large repertoire of available G-proteins and effector systems [2–4]. Although the basic tenets of this model have stood up to vigorous testing, it is increasingly apparent that the model is still oversimplified [5, 6]. For example, GPCRs exist and function as homo- and heterodimers or oligomers, 7 TMD receptor signalling can occur in a G-protein-independent manner and other non-7 TMD proteins such as Activator of G-protein Signalling 1 (AGS1) and Cysteine String Protein (CSP) function by activating hetero-

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trimeric G-proteins [5, 7–9]. There is also evidence that the activation of some heterotrimeric G-proteins may not require that the G_α subunit physically dissociate from $G_{\beta\gamma}$ [10]. More recently, GPCRs have been demonstrated to be part of multimeric complexes of different proteins, including G-proteins, effectors and regulatory proteins that are involved in mediating the specificity, efficiency and regulation of GPCR responses [11, 12].

It is well known that continuous stimulation of GPCRs (minutes to hours) leads to a gradual decrease in the ability of the receptor to respond to further agonist [13, 14]. This tackyphylatic response is called homologous desensitization and has been observed for most GPCRs. Molecular analysis suggests that this process is initiated by the phosphorylation of the ligand-bound receptor by a family of G-Protein Receptor Kinases (GRKs). The phosphorylated receptor shows a decrease in its affinity for ligand, a decrease in its ability to activate heterotrimeric G-proteins and hence is desensitized. Further ligand stimulation of the phosphorylated receptor leads to its internalization within the cell, and as a consequence there is a loss of ligand binding sites on the cell surface (Fig. 1). The receptor-activated G-proteins must also be inactivated in order for signalling to terminate. Hydrolysis of GTP to GDP causes the inactivation of the G_α subunit and the reassociation and inactivation of the heterotrimeric G-protein complex. Thus, temporal differences in the rate of GTP hydrolysis can account for some of the observed heterogeneity in GPCR signalling [15]. Although a great deal of knowledge has been gained regarding the molecular mechanisms involved in turning off the ligand-bound receptor, very little is currently known regarding the mechanisms regulating the lifetime of the activated GTP-bound G_α proteins. In fact, prior to 1996, despite a few reports to the contrary, it was generally believed that the intrinsic GTPase activity of the G_α subunits was sufficient to regulate the G-protein activation/deactivation cycle [16, 17]. It should be noted that in addition to the hydrolysis of GTP, a number of other mechanisms exist that serve to regulate signalling from heterotrimeric G-proteins [4, 10]. These include the post-translational modification of G_α subunits that serve to regulate its subcellular localization and the levels of G_α protein [18]. For example, chronically activated G_α

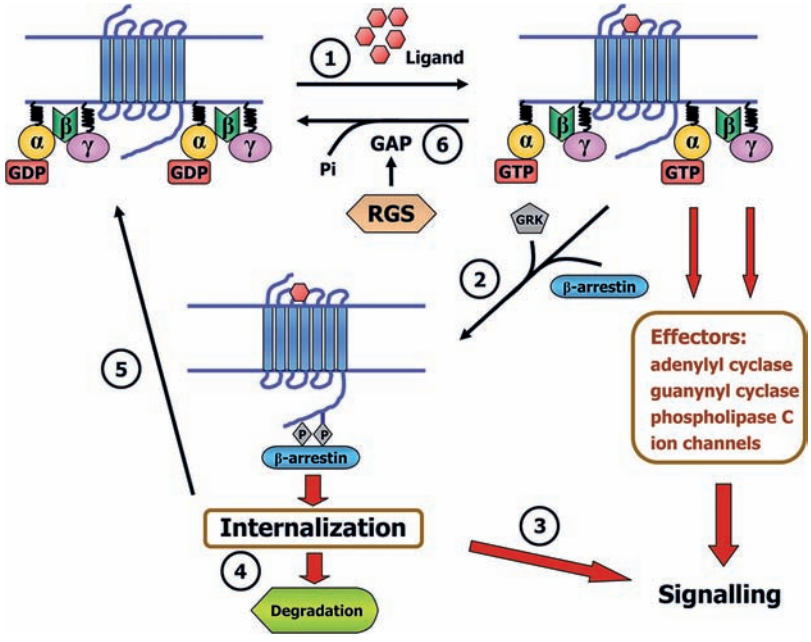

Figure 1. G-Protein Coupled Receptor (GPCR) structure, activation and deactivation of heterotrimeric G-proteins. GPCRs consist of seven transmembrane domains (TM1-7), three extracellular loops, three cytoplasmic loops, and N-terminal extracellular (N) and C-terminal (C) cytoplasmic domains. Inactive GPCRs are loosely associated with inactive heterotrimeric G-proteins consisting of G_α, G_β and G_γ subunits. Activation and inactivation of GPCRs and GPCR-mediated signalling occur in the following steps: 1, ligand binding produces a conformational change in the receptor, which is then transduced to the inactive heterotrimeric G-protein. This interaction induces the exchange of GDP for GTP on the G_α-subunit and the dissociation of the G-protein into constituent G_α and $G_{\beta\gamma}$ subunits. The G-proteins are thus activated and are now able to activate or inhibit downstream effectors leading to receptor, G-protein and cell-type characteristic signalling responses. 2, the activated receptor is recognized and phosphorylated by a G-protein Receptor Kinase (GRK). This leads to a decrease in receptor-mediated activation of heterotrimeric G-proteins. The phosphorylated receptor then recruits *β*-arrestin, leading to the internalization of the receptor, and the internalized receptor has three possible fates: 4, it can recruit and activate other proteins, such as Src, leading to the activation of different signalling responses; 5, it can be degraded likely via the lysosome; or 6, it can be recycled back to the cell surface where it can bind ligand once again. 6, deactivation of G-protein-mediated signalling occurs via the inherent GTPase activity of the G_α-subunit, which causes the exchange of GTP for GDP, and subsequent reassociation of the G-protein heterotrimer. The RGS protein family largely regulates G_α-protein deactivation, essentially increasing the rate of GTP hydrolysis and favoring heterotrimer reassociation.

proteins can likely be degraded by a mechanism involving ubiquitin-mediated proteolysis [19].

RGSs: historical perspective and current overview

An early indication that the G-protein cycle might be regulated by extrinsic factors came from the observation that the product of the yeast *SST2* gene could negatively regulate heterotrimeric G-protein signalling [20, 21]. The FlbA-encoded protein in *Aspergillus nidulans* having similar structure and function to Sst2p served to indicate that regulation of G-proteins by extrinsic factors was not confined to *Saccharomyces cerevisiae* [22]. By 1996, a large number of proteins in *Caenorhabditis elegans* and mammalian cells with similar structures were identified and called Regulators of G-protein Signalling (RGSs) because of their ability to attenuate GPCR signalling [23–25]. These proteins are identified by the presence of a conserved ~125-amino acid motif, which is referred to as the RGS box. The remaining portions of these proteins are mostly dissimilar in size and amino acid composition [26, 27]. With the use of recombinant proteins in *in vitro* biochemical assays, a number of RGS-containing proteins were shown to stimulate the intrinsic GTPase activity of GTP-bound G_α subunits with a minimum of 40-fold over basal levels [28–30]. In humans, there are over 20 G_α proteins encoded by 16 genes, which are subdivided into four subfamilies based largely on sequence identity and shared effector systems: $G_{s\alpha}$ ($G_{s\alpha}$ and $G_{olf\alpha}$), $G_{i\alpha}$ ($G_{i\alpha1}$, $G_{i\alpha2}$,$G_{i\alpha3}$,$G_{o\alpha}$, $G_{z\alpha}$, $G_{t\text{-}cone\alpha}$, $G_{t\text{-}rod\alpha}$ and $G_{gust\alpha}$), $G_{q\alpha}$ ($G_{q\alpha}$, $G_{11\alpha}$, $G_{14\alpha}$, and $G_{16\alpha}$), and $G_{12\alpha}$ ($G_{12\alpha}$ and $G_{13\alpha}$) [2–4]. Early *in vitro* biochemical evidence as well as *in vivo* experiments showed that RGSs could function as GAPs for GTP-bound $G_{q\alpha}$ and $G_{i\alpha}$, but some RGSs, such as RGS2, nevertheless were shown to have a strong preference for $G_{q\alpha}$ [27, 31–40]. Only a few individual RGSs have been shown to serve as GAPs for the $G_{12/13\alpha}$ proteins [36, 41, 42]. Thus RGSs were originally thought to function largely as GTPase-Activating Proteins (GAPs) for heterotrimeric G-proteins in much the same way that GAPs regulate small G-proteins such as ras [28, 29]. RGSs are nevertheless functionally different from GAPs. Biochemical and crystallography experiments revealed that they preferentially bind a transition state of G_α proteins that occurs as an intermediate during the hydrolysis of GTP [43–45]. Crystallography of RGS4 bound to the GTP-Gα hydrolysis intermediate mimicked by $G_{i\alpha1}$-Mg^{+2}·GDP-ALF_4 suggests that RGS4 does not contribute catalytic function; instead, it promotes the hydrolysis of GTP by stabilizing the GTP hydrolysis intermediate form [43].

There are now over 25 proteins that contain RGS or RGS Homology (RH) domains. Although GAP activity towards G_α proteins is a common functional feature of many RGSs, a number of RGSs have very weak or no GAP activity when assayed *in vitro* [4, 46]. Many or all members of the E (Axin), H (SNXs) or atypical (D-AKAP2) subfamilies show significant sequence divergence from other RGS subfamilies and appear to have no GAP activity. Alternatively, the RGS box of members of the F subfamily, such as p115-Rho GEF, appear to require some adjacent non-RGS sequences in order to demonstrate GAP activity [47]. Although RGSs are best known for regulating GPCR signalling, not all RGS domains appear to subserve this function. Even RGSs that have strong G_α GAP activity, such as the members of the R4 or R7 subfamily, can also serve to inhibit GPCR signalling in a manner that is independent of GAP activity [48, 49]. For example, RGS2 is well known for its ability to prevent GPCR signalling by serving to inhibit effector activation [50]. This so-called effector antagonism appears to require the N-terminal non-RGS portion of RGS2 [51]. In addition, RGS GAP activity may be used to recycle Gα and promote an increase in the local concentration of inactive Gα that can be reactivated by GPCRs [52]. Alternative mechanisms of RGS function such as these may account for some atypical GPCR signalling events [52, 53].

Yeast as a model system to understand RGS function

Understanding the complete repertoire of how RGSs function to regulate GPCR responses in mammalian cells will be a complex task considering that there are multiple RGSs containing proteins that serve to regulate multiple Gα proteins that are differentially coupled to a large number of different GPCRs [1]. The situation is rendered more difficult by the fact that most cells express multiple RGSs and GPCRs [41, 54–56]. Yeast has proven to be an excellent model system to delineate the basic framework for a number of different aspects of cell biology, including cell cycle regulation, ubiquitin-mediated protein degradation and G-Protein Coupled Receptor (GPCR) signalling [57–59]. For example, yeast cells lacking Sst2p were originally instrumental in identifying and cloning mammalian RGSs [24, 25]. In fact, they continue to be extensively used as a model system to study the structure and function of heterologous RGSs [60–64]. To get insight into RGS function in mammalian cells, it is therefore instructive to look at what is known about the role of RGSs regulating GPCRs in yeast. Compared with mammalian cells, *S. cerevisiae* is a relatively simple cell since it possesses only two different GPCR signalling pathways. The pheromone response pathway is an extensively characterized GPCR-mediated signalling cascade that consists of a single GPCR (Ste2p for **MATa** and Ste3p for **MAT**α cells), G_α-protein (Gpa1p), G_β-protein (Ste4p), G_γ-protein (Ste18p) and RGS (Sst2p) [65]. As a consequence of this simplicity and the fact that yeast

is highly amenable to genetic analysis, RGS function is probably best understood in this organism. Yeast mutants devoid of their RGS-containing gene, *SST2*, show hyperresponsive GPCR-mediated responses and a greatly reduced ability to desensitize in the presence of continued agonist stimulation [21]. This suggests that the basal GPCR responses are limited by the presence of basally expressed RGSs. In contrast, yeast cells overexpressing *SST2* show a dramatic reduction or hyporesponsiveness in GPCR signalling. This suggests that the basal levels of RGSs exist in a rate-limiting amount. The *SST2* gene is regulated by transcriptional and post-translational mechanisms. *SST2* mRNA (messenger RNA) is increased by chronic GPCR stimulation. Prolonged GPCR stimulation also increases Sst2 protein phosphorylation, leading to an increase in the stability and levels of the Sst2 protein. Both mechanisms presumably serve as a feedback regulatory mechanism to limit pheromome responsiveness. Although it is less well characterized, yeast also contains a second GPCR signalling cascade that consists of a single GPCR (Gpr1p), G_{α}-protein (Gpa2p) and RGS (Rgs2p). Interestingly this receptor appears to have a dual role in regulating both vegetative growth and pseudohyphal formation. The specificity of the function of individual RGSs is demonstrated by the fact that *RGS2* cannot functionally replace the *SST2*-encoded RGS [66]. Thus, two RGSs can be expressed in the same cell all the while regulating two different GPCR signalling pathways. Finally, both yeast RGSs are structurally distinct, RGS2 is a 309 protein that has characteristics of small mammalian RGS proteins [65, 66]. In contrast, Sst2p is a 699-residue protein that contains a DEP domain in addition to its RGS box. In addition, there is evidence that the N-terminal non-RGS domain of the yeast Sst2p carries out functions that are unrelated to the termination of GPCR signalling [67].

Although the number of RGS-encoding genes that are involved in regulating GPCR responses is larger, the repertoire of RGS function and regulation shows a number of parallels in mammalian cells. For example, mice models of RGS gene knockouts show hyperresponsive GPCR responses, while cells or mice overexpressing RGSs show reduced GPCR responses (see below). The results obtained with mice serve to illustrate two important points regarding the role of RGSs that is also observed in yeast. The knockout studies suggest that the basal level of RGS serves to limit the magnitude of GPCR-mediated responses, while the overexpression studies suggest that the basal level of RGS is in fact rate limiting for the ability of these proteins to inhibit GPCR responses. Other parallels include the diversity in RGS structure, the regulation by GPCR agonist and the specificity of RGS function. Here we will highlight advances in RGS biology that focus on the different aspects of RGS function and regulation that confer specificity of function on mammalian RGSs involved in regulating GPCR responses. A number of excellent reviews on general as well as specific aspects of mammalian RGSs have recently been presented, and the reader is invited to consult these in order to obtain more information on different aspects of RGS biology [12, 27, 41, 42, 68–71].

RGS-containing proteins are divided into distinct subfamilies

The sizes of the different RGS-containing proteins show significant divergence ranging from small RGS proteins that contain little more than an RGS box to the large RGS-containing proteins that have identifiable functional sequence motifs, such as PDZ, GGL and DEP (Fig. 2). These non-RGS regions or domains are one of the more obvious characteristics that confer specific functions on the different RGSs. On the basis of sequence identity and the presence of shared domains, RGS proteins have been classified into a number of different subgroups or families [46, 72]. The family of RGS proteins includes the A/RZ, B/R4, C/R7, D/R12, E/RA, F/GEF, G/GRK and H/SNX subfamilies (Fig. 2). Here is a brief description of the structural characteristics and major functions of the different subfamilies of RGS-containing proteins. A number of recent reviews provide a more detailed analysis of the structure and function of RGSs [42, 45, 46].

A/RZ subfamily

Subfamily A or RZ regroups RGS17, also called RGSZ2; RGS19, also called GAIP (G_{α}-interacting protein); RGS20, also called RGSZ1; and the retinal-specific RGS protein, RET-RGS1 [46] (Fig. 2). Aside from the conserved RGS domain, all these proteins share an N-terminal cysteine-rich region, the cysteine string motif. This region is palmitoylated and is likely involved in membrane targeting and attachment and protein-protein interactions [73]. RGSZ1 is a highly selective GAP for the $G_{z\alpha}$ proteins [74, 75], RGSZ2 has been shown to regulate $G_{i/o\alpha}$, $G_{z\alpha}$ and $G_{q\alpha}$ signalling [40], while RET-RGS1 and GAIP also act almost exclusively as GAPs for the $G_{i\alpha}$ subfamily [76]. RET-RGS1 also contains a putative transmembrane domain at its N-terminus, which raises the possibility that it may be an integral membrane protein [33]. On the other hand, the presence of a PDZ binding motif in the C-terminal of RGS-GAIP allows the specific interaction with the PDZ domain-containing protein GIPC, which confers additional roles to this RGS protein [77]. For example, in kidney proximal tubule epithelium, the function of the endocytic receptor megalin is partly regulated by a G-protein-dependent event involving $G_{\alpha i3}$, GAIP and GIPC, suggesting that the binding of GAIP to both $G_{i3\alpha}$ and GIPC may link G-protein signalling and megalin-mediated endocytosis or trafficking [78].

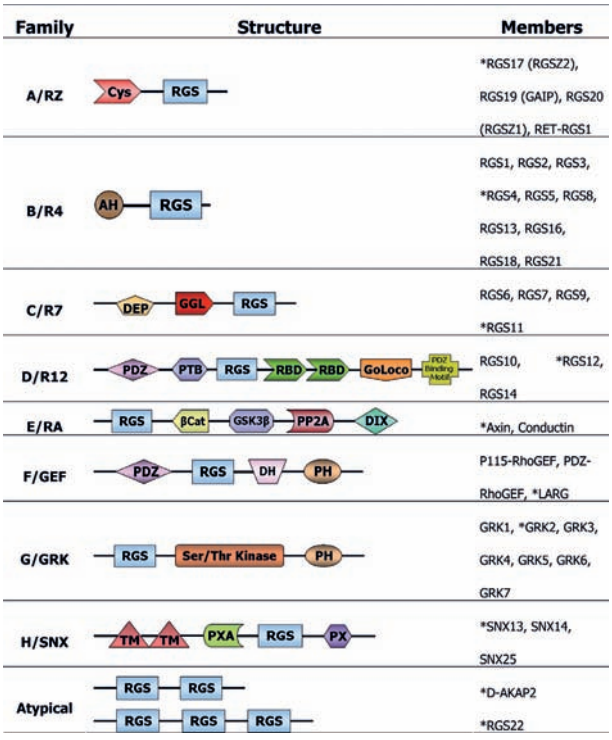

Figure 2. The RGS families of proteins. Sequence identities within the RGS domain establish nine mammalian subfamilies: RZ (A), R4 (B), R7 (C), R12 (D), RA (E), GEF (F), GRK (G), SNX (H) and atypical. A schematic diagram depicting the structural motifs present in a representative member (shown by a '*') of each RGS family is shown and defined in the boxed inserts. The names of all the members of each family are listed on the right. Individual members of the different subfamilies do not contain all the motifs of the represented member of the subfamily. For example, the R12 RGS10 is a small protein that contains only the RGS domain. Abbreviations used to describe the different domains are as follows: AH, Amphipathic Helix; βCat, β-Catenin interacting domain; CYS, polycysteine region or cysteine string; DEP, Dishevelled domain; DH, dbl homology domain; DIX, Dishevelled-Interacting domain; GGL, Gγ-like domain; GoLoco, Gαi/o-Loco-interacting domain; GSK3β, Glycogen Synthase Kinase 3β-interacting domain; PDZ, PSD-95/Dlg/ZO-1 domain; PDZ Binding Motif, PSD-95/Dlg/ZO-1 Binding Motif; PP2A, Phosphatase 2A-interacting domain; PTB, Phospho-Tyrosine Binding domain; PX, phosphatidylinositol binding domain; PXA, phosphatidylinositol-associated domain; RBD, Rap½- or Ras Binding Domain; PH, Pleckstrin Homology domain; RGS, Regulator of G-protein-Signalling domain; Ser/Thr Kinase, Serine/Threonine Kinase; TM, Trans-Membrane domain.

B/R4 subfamily

Subfamily B or R4 contain a number of small proteins, namely RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16, RGS18 and RGS21, and is considered the largest subfamily of RGS proteins [24, 27, 46, 70, 79] (Fig. 2). With the exception of RGS3, which possess a relatively long N-terminal stretch of ~300 amino acids [24, 80], their structure consists of a short N-terminal sequence and a C-terminal RGS domain. RGS4, -5 and -16 also contain a highly conserved cluster of basic residues within their N-terminus, known as the amphipathic helix, and conserved cysteine amino acids, thought to be palmitoylated and involved in membrane anchoring [81, 82]. RGS21 is the smallest RGS protein identified to date, with very short N- and C-terminal tails flanking the RGS domain, and represents the most basic RGS protein. Most RGSs of the R4 subfamily have been shown to act as GAPs for $G_{q\alpha}$ and $G_{i/o\alpha}$ proteins [45, 46, 70, 83]. Nevertheless, individual R4 RGSs have *in vivo* preferences. A good deal of evidence now exists which demonstrates that the GPCR itself is important in determining which R4 RGS can functionally interact with different G-proteins [12, 84]. For example, RGS4 is more potent at inhibiting Ca^{2+} release mediated from M3 muscarinic receptor than that mediated by the bombesin and cholecystokinin receptors. Since all three GPCRs activate $G_{q\alpha}$ proteins, this suggests that the receptors serve to modulate RGS function [85]. The existence of a trimeric complex involving the receptor, the G_{α}-protein and the RGS could explain the observed selectivity of the small RGSs [12, 84]. For example, RGS2, but not closely related RGS16, was found to bind simultaneously to the third intracellular loop (i3) of the M1 muscarinic cholinergic receptor and the coupled $G_{q\alpha}$ protein [86]. Deletion of the N-terminal region of RGS2 abolished both the RGS2-M1mAChR interaction, as well as the RGS2-mediated inhibition of PLCβ activity, suggesting that the N-terminal portion of RGS2 is responsible for mediating this interaction [86]. The C-tail of certain GPCRs has also been implicated in mediating this interaction [87]. A more recent report suggests that linker proteins such as spinophilin serve to bind to and link both specific GPCRs and RGSs. [88]. R4 RGSs also interact with effectors, act as effector antagonists and inhibit G-protein signalling independent of their GAP activity [49, 50, 89–91]. Indeed, the inhibitory effect of RGS2 and RGS3 on G_q-coupled muscarinic M_3 receptor is observed through diminished activation of PLCβ in the absence of accelerated GTPase activity [49]. The non-RGS sequences appear to directly interact and inhibit the effector enzymes. For example, the N-terminal non-RGS portion of RGS2 appears to be required for its ability to inhibit adenylyl cyclase [50]. RGSs from other subfamilies such as the R7 RGSs also can serve as effector antagonists [48].

C/R7 subfamily

RGS6 [92], RGS7 [93, 94], RGS9 [95] and RGS11 [93] constitute the C or R7 subfamily of RGS proteins (Fig. 2). They all share a N-terminal Disheveled/EGL-10/Pleckstrin (DEP) domain [96], a Gγ protein-Like (GGL) domain [93] and a C-terminal RGS domain. All of the R7 RGS proteins have the capacity to act as GAPs for $G_{i\alpha}$ proteins [97] as well as effector antagonists. The latter is well documented in the case of desensitization of μ-opioid receptors in the central nervous system [48]. The 64-

residue GGL domain region shares similarity with the Gγ subunit of heterotrimeric G proteins [98]. The GGL interacts with Gβ_5 proteins to create an RGS-Gβ_5 heterodimer analogous to that of Gβ with Gγ [46, 93]. The exact function of the RGS/Gβ5 complex is still under investigation, although one report by Chen et al. suggests that the RGS/Gβ5 interaction is required for the stability of R7 RGS proteins [99]. Conventional G$\beta\gamma$ heterodimers bind inactive Gα-GDP and are capable of activating several signalling cascades. Because of the structural properties shared by both dimers, one might expect that the RGS/Gβ5 complex would share a similar function. Indeed, Ajit et al. have shown that RGS9/Gβ5 positively regulates pheromone responses when heterologously expressed in yeast [100]. However, several groups have reported the inability of the RGS/Gβ5 complex to either activate adenylyl cyclase or PLCβ, or bind GDP-Gα subunit in mammalian systems [93, 101]. Only recently did Witherow et al. [102] report a direct interaction between the RGS7/Gβ5 complex and $G_{q\alpha}$ protein, with subsequent inhibition of $G_{q\alpha}$-mediated signalling in mammalian cells. At least two independent studies have implicated the DEP domain of RGS9-1 in membrane anchoring and subcellular targeting [103, 104]. One study reported that membrane association of the RGS9-1-Gβ5L-$G_{t\alpha}$ complex required the integral membrane protein R9AP, which serve to anchor RGS9-1 to rod outer segment membranes, and the DEP domain-containing N-terminal region of RGS9-1 [103]. Selective targeting of RGS proteins to specific GPCRs have also been proposed as a possible role of the DEP domain [26]. For example, deletion of the DEP domain of the RGS9 splicing product, RGS9-2 (DEPless RGS9-2), failed to specifically co-localize with D2-like dopamine receptors (D2DR) but not m2-mAChRs and lost the ability to accelerate the deactivation of D2DR-mediated GIRK responses. The R7 RGSs have also been shown to functionally interact with other proteins. The interaction of RGS9, via its DEP domain, with the integral membrane protein R9AP may serve to anchor the protein to outer rod segment membranes [103]. A more recent report describes the interaction of all four R7 RGS proteins with R7BP (for R7-binding protein), a SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor)-related neuronal protein involved in vesicular trafficking and exocytosis [105]. Interestingly, R7BP is closely related to R9AP and binds R7 RGS proteins via their DEP domain.

D/R12 subfamily

Subfamily D or R12 is made of only three members, namely RGS10, RGS12 and RGS14 [46] (Fig. 2). RGS10 is a small protein of 173 amino acids, characterized by a very short N-terminal tail and a unique functional RGS domain. RGS10 structure resembles that of the R4 subfamily of RGS proteins and exhibits both GAP activity and effector antagonist properties [106]. In contrast, RGS12 and RGS14 are much larger proteins [107] that contain, in addition to the N-terminal RGS box, many other functional domains, including Ras-binding (RBD), $G_{i/o\alpha}$-Loco (GoLoco), Phosphotyrosine binding (PTB) and PSD-95/Disk-Large/ZO-1 (PDZ) domains. The tandemly repeated 80-amino acid RBD domains, similar to the RBDs of B-Raf serine/threonine kinases, have been shown to bind Ras-like molecules [108]. The 19-amino acid C-terminal GoLoco motif is a $G_{i/o\alpha}$ binding motif that exhibits Guanine nucleotide Dissociation Inhibitor (GDI) activity [109, 110]. GDIs bind to GDP-bound G-proteins and serve to prevent GDP release and thus serve to oppose G-protein activation. Thus, both the RGS and GoLoco domains of RGS14 are able to contribute, in an additive manner, to downregulate Gi-dependent signalling in HEK293 cells [111]. GoLoco-motif containing proteins are involved in *Drosophila* neuroblast mitosis, a well-studied example of asymmetric cell division (ACD), where they direct cell polarity, mitotic spindle organization and chromosomal segregation [109, 110]. Similarly a role for R12 RGSs in ACD has been proposed [112]. For example, $RGS14^{-/-}$ embryos are defective in zygotic cell division [113]. PSD-95/Disk-Large/ZO-1 (PDZ) and Phosphotyrosine binding (PTB) domains are also present within the N-terminus of RGS12, but not the two other members of this subfamily. Like other bona fide PDZ domains, PDZ-containing RGS12 binds a C-terminal A/S-T-X-L/V-COOH motif found in proteins such as the interleukin-8 receptor (CXCR2), as well as a PDZ binding motif present in its own C-terminus [114].

E/RA subfamily

The E or RA subfamily of RGS proteins is made up of only two members, Axin and Conductin [46] (Fig. 2). Axin2 and axil are the respective human and rat homologues of conductin. Although both proteins contain an N-terminal RGS domain, they have not been shown to act as GAPs or negative regulators of G-protein responses [46]. Nevertheless, there is a single report demonstrating that there is a direct interaction of the RGS domain of axin with a $G_{s\alpha}$ subunit [115]. RA RGSs function as key regulators of the Wnt-β-Catenin signalling pathway [116]. Wnt proteins constitute a class of extracellular molecules that bind heptahelical frizzled receptors to induce a wnt signalling cascade that serves to regulate diverse biological processes, including embryonic development and tumorigenesis [117]. Both axin and conductin contain binding sites for three components of the Wnt cascade, namely β-catenin, Glycogen Synthase Kinase 3β (GSK3β) and the tumor suppressor Adenomatous Polyposis coli (APC). Binding consensus for the APC lies

within the RGS domain region [116]. Both axin and conductin have been found to exist in a complex (axin/conductin complex) with all three proteins. Murine axin and conductin contain a C-terminal DIX domain that interacts with Dsh. Binding of Wnt ligands to their corresponding receptors induces the activation of Dishevelled (Dsh) protein, which perturbs the axin/conductin, APC, GSK3β, β-catenin complex. The mechanism through which Dsh disrupts the axin/conductin complex is not fully understood. A Protein phosphatase (PP2A) binding domain is also present within the C-terminal region of RA RGS proteins; the exact outcome of PP2A binding to axin is controversial [118, 119].

F/GEF subfamily

The F or GEF subfamily of RGS proteins consist of a family of three proteins – p115-RhoGEF, PDZ-RhoGEF, LARG – known as Ras homology (Rho)-specific Guanine nucleotide Exchange Factors (GEF) that serve to activate Rho [46] (Fig. 2). Rho proteins belong to the Ras superfamily of small GTPases and regulate several aspects of actin and microtubule cytoskeleton organization, including cell morphology and migration. p115RhoGEF was the first Rho-specific GEF identified that contains an NH2-terminal RGS domain [120], followed by Dbl-homology (DH) and pleckstrin-homology (PH) domains [36]. The DH domain regulates the GEF activity of these proteins, while the PH domain is involved in subcellular localization [36, 121–123]. The dual nature of p115RhoGEF is well studied. Its RGS domain binds to activated $G_{12/13\alpha}$ proteins, and this leads to the activation of its GEF domain and subsequent activation of Rho. By doing so, p115RhoGEF has emerged as an intermediate that links heterotrimeric G-protein activation to Rho-dependent signalling pathways. PDZ-RhoGEF [123] and LARG [122] contain an additional N-terminal PDZ domain capable of interacting with intracellular C-terminal PDZ binding motifs not only of GPCRs [124] but also other receptors such as the Insulin-like Growth factor (IGF)-1 [125] and the semaphorin plexin B1 receptor [126]. In all cases, this interaction is required for the receptor in question to activate Rho-dependent signalling. The GEF subfamily of RGS proteins thus constitutes a class of Gα effectors that transduce positive signals from activated receptors and/or G-proteins to the Rho machinery. Finally, a novel sequence in PDZ-RhoGEF that mediates interaction with the actin cytoskeleton was recently identified [127].

H/GRK subfamily

The G or GRK subfamily of RGS proteins consist of serine/threonine kinases that share an N-terminal RGS domain, a central catalytic kinase domain and a carboxyl-terminus bearing either membrane translocation/anchoring signals or various protein-protein interaction sites (Fig. 2). To date, seven mammalian cDNAs (complementary DNAs) encoding GRK proteins have been identified: GRK1 or Rhodopsin kinase, GRK2 and GRK3, also known as the β-Adrenergic Receptor Kinase (βARK) 1 and 2 respectively; GRK4 or IT-11, GRK5, GRK6 and GRK7 [46, 128]. These kinases are related to the Protein Kinase C (PKC) and cAMP-dependent protein kinase (PKA) families and are mainly involved in homologous desensitization of GPCRs, a process that serves to terminate signalling at the level of the receptor [13, 14]. Indeed, GPCR signalling is tightly regulated by GRKs, which phosphorylate intracellular loops and C-terminal tails of agonist-occupied receptors on serine and threonine residues. Phosphorylated residues on activated receptors provide docking sites for the versatile arrestin proteins, which subsequently bind phosphorylated receptors to induce signal termination or desensitization, GPCR internalization/recycling or degradation (Fig. 1). Studies of knockout animals demonstrate that GRKs are crucial in GPCR-mediated desensitization [128]. Finally, the GAP function of the RGS sequences present in the GRKs has yet to be fully characterized. One report by Carman et al. [129] showed that GRK2 possesses weak GAP activity towards $G_{q/11\alpha}$ and is capable of inhibiting $G_{\alpha q/11}$-mediated phospholipase C activation. This suggests that the receptor kinase domain of GRKs may be more potent at negatively regulating GPCR signalling than its RGS domain.

I/SNX subfamily

The sorting nexins constitute a family of 29 proteins believed to be involved in intracellular trafficking [130]. They are characterized by a SNX phox homology (PX) domain, which acts as a phosphatidylinositol 3-phosphate-binding domain that is required for the targeting of PX domain-containing proteins to phosphoinositide-enriched membranes such as endosomes. Three of the sorting nexins, SNX13 (RGS-PX1), SNX14 and SNX25, contain an RGS domain and comprise the H or SNX subfamily of RGS proteins [46] (Fig. 2). RGS-PX1 (SNX13) was found to act as a functional nexin by impairing the agonist-induced lysosomal targeting/degradation of the EGF receptor (EGFR) [131]. RGS-PX1-overexpressing cells also displayed prolonged EGF signalling, further suggesting a delay in EGFR degradation. A single report has identified RGS-PX1 as the first and only RGS to exhibit GAP activity towards $G_{s\alpha}$ proteins [131]. Given that this report was published in 2001, the importance of this observation remains to be determined. The GAP activity of SNX14 and 25 has not been examined.

D-AKAP2 and RGS22: the not so typical RGS proteins

There are two other RGS-containing proteins, D-AKAP2 and RGS22 [46] (Fig. 2). Sequence analysis suggests that they do not belong to any of the eight subfamilies of RGS proteins. D-AKAP2 (dual A kinase anchoring protein 2) belongs to a family of proteins that serve to regulate the subcellular localization of cAMP-dependent protein kinase A (PKA). Full-length human D-AKAP2 contains two putative RGS domains, but like many other RGSs, no physical interactions with G-protein subunits or GAP activity have been demonstrated. RGS22 is a recently identified protein that has not yet been characterized [46].

Tissue distribution of RGSs

Differential tissue-specific gene expression is an important factor that serves to limit the role of the different RGSs [41, 54]. For example, RGS5 expression is limited to the heart, skeletal muscle, smooth muscle, pericyte and a variety of subregions within the brain [55, 70, 132–136]. Therefore, RGS5 will be limited to regulating GPCR signalling within these cell types. Although a good deal of information regarding the tissue or cell type distribution of RGSs has been determined, the systematic detailed analysis of the expression of all RGSs in any given cell or tissue has not been carried out. Nevertheless the analysis of the expression of most RGSs, at least by RT-PCR (reverse transcriptase-polymerase chain reaction), has been carried out. Some R4 RGSs, such as Rgs2, Rgs3, Rgs10 and Rgs19, are widely distributed, while many of the others show a more narrow distribution, such as RGS8, which seems to be expressed only in the brain [41, 54]. Thus, the restricted tissue distribution is one of the most obvious characteristics that will limit the *in vivo* function or selectivity of the different RGSs.

Alternative splicing appears to be a common mechanism that serves to increase the repertoire of different RGS proteins. Many of these alternatively splice variants also show distinct tissue distributions and functions. For example, one of the Rgs9 splice variants, RGS9-1, is expressed predominantly if not exclusively in the retina, where it regulates rhodopsin receptor signalling [137]. In contrast, the alternatively spliced variant RGS9-2 is widely expressed in the brain where it has been shown to play an important role in regulating opioid receptor signalling. The alternative splicing of Rgs19 leads to an N-terminally truncated form of the protein that may have different receptor specificity than the full-length RGS19 protein, but their tissue distribution appears to be the same [138]. Thus, alternative splicing may not only serve to increase the diversity of RGS expression. It may also serve to increase the diversity of RGS function within the same cell.

Of interest, a number of RGS genes are tightly linked to other genes involved in GPCR signalling [71]. Many of these genes appear to share the same promoter. For example, the opioid receptor gene OPR1 and the RGS-encoding gene for Rgs19 are linked head to head and appear to share a bidirectional promoter. This suggests that co-expression may play an important role in selectively regulating the distribution of the expression of Rgs genes with genes that encode functionally-dependant proteins.

Induction of RGSs by GPCR stimulation: feedback inhibition of signalling

In yeast, the levels of the Sst2p RGS are increased by pheromone (GPCR agonist) [21]. The analogous situation exists in mammalian cells, as the levels of a number of different RGSs, especially the R4 RGSs, are increased by a variety of different GPCR agonists [139–144]. This regulation is both tissue- and receptor-specific. For example, RGS1 is induced by platelet activating factor (PAF), RGS16 is induced by carbachol, while RGS2 is induced in response to a number of different agonists in different tissues, including Angiotensin II (Ang II), atropine and parathyroid hormone. The simplest interpretation suggests that an increase in RGS levels in response to the stimulation of a given GPCR will serve as a negative feedback loop to limit signalling responses to the GPCR itself [140]. This implies that upregulated RGSs serve to attenuate GPCR responses in an agonist-dependent manner that is analogous to the process of homologous desensitization. A single agonist may also upregulate more than one RGS in the same tissue. For example, Ang II upregulates both the R4-RGS RGS2 and the GEF-RGS GEF leukemia-associated Rho-GEF (LARG) in vascular smooth muscle cells [145, 146]. The increase in RGS2 may be responsible for the ability of AngII to decrease Gi-mediated inhibition of adenylyl cyclase. In contrast, an increase in LARG appears to be responsible for the ability of AngII to activate RhoA signalling. Upregulation of multiple RGSs may therefore be involved in regulating different aspects of a given GPCR response. Although a single agonist may upregulate more than one RGS, the GPCR-mediated increase in RGS gene expression is nevertheless selective. For example, the expression of RGS2 but not that of RGS3, 5, 6, 9 10, 12, 14 and 16 was found to be upregulated by the thyroid-stimulating hormone (TSH) in primary thyroid cultures [147]. This suggests that individual RGSs are upregulated to carry out specific functions. For example, RGS16 and RGS18 are both upregulated in differentiating megakaryocytes [148]. Overexpression of RGS16 but not RGS18 was found to inhibit stromal-cell-derived factor 1 (SDF-1)/chemokine receptor 4 (CXCR4) signalling. In agreement with this specificity, it was found that RNAi (RNA interference)

knock down of endogenous RGS16 resulted in the potentiation of CXCR-4 responses. Thus, upregulation of RGS16 may serve to prevent developed megakaryocytes from further activating specific CXCR4-mediated responses. Conversely, an increase in RGS levels may lead to decreased responsiveness for other GPCRs, thereby serving to contribute to the phenomena of heterologous desensitization. For example, nerve growth factor (NGF) was found to decrease RGS4 mRNA and protein levels in PC12M cells [149]. This suggests that NGF may serve to selectively potentiate the signalling responses of GPCRs that are negatively regulated by RGS4. RGS levels are also upregulated by a variety of agonists for non-GPCR receptors. For example, RGS9 levels are modulated in the nucleus accubens by estrogen, RGS2 levels are modulated by NMDA in the striatum, tumor necrosis factor α (TNFα) induces RGS1 in B cells and RGS16 in leukaemia cells, and as mentioned above, NGF decreases RGS4 levels in cultured neuronal PC12M cells [142, 150–152]. This suggests that the upregulation of specific RGSs may play a role in the cross-talk that occurs between GPCRs and other classes of receptors.

Regulation of RGS protein levels by selective degradation

Given that an increase in the levels of RGS proteins has profound effects on GPCR signalling and physiological responses, it stands to reason that removal of the stimuli leading to increased RGS expression will require a reduction in the levels of RGS proteins in order to return the system to baseline. This appears to be the case in yeast, since the half-life of the Sst2p RGS is dynamically regulated by receptor stimulation [21]. A number of RGSs are known to be regulated by a variety of post-translational modifications such as palmitoylation and phosphorylation, which serve to modulate RGS localization, potency and stability [81, 82, 153–157]. Despite a few studies [21, 154], very little is known about the possible role that selective degradation may play in the regulation of RGS proteins. Mumby's group has recently shown that levels of the endogenously expressed R4 RGS4 protein in the neuronal PC12M and pituitary AtT20 cells are elevated following treatment of the cells with proteosome inhibitors [149]. This suggested that selective protein degradation serves to regulate the basal levels of RGS4. The process is selective, since the level of the endogenously expressed R7 RGS7 protein in PC12M cells remained unaltered even after 6 h of treatment with proteosome inhibitor. Experiments using cycloheximide to block total protein synthesis were used to show that RGS4 protein has a short half-life of just under 1 h. Surprisingly, the authors found that stimulating cells with a variety of GPCR agonists did not alter the degradation rate of RGS4 protein. It remains to be determined whether the rapid and selective degradation of RGS proteins is an important aspect of the mechanisms regulating RGS levels in mammalian cells. Given that the levels of RGS proteins play an important part in modulating the strength of GPCR responses, it will also be important to determine the mechanisms and the physiological stimuli that lead to altered RGS levels. In the case of RGS4, it was found that a non-GPCR agonist, NGF, actually resulted in a decrease in the levels of both the RGS4 mRNA and protein. This suggests that a decrease in the levels of a given RGS may be mediated by multiple mechanisms.

Ubiquitin is often involved in the selective degradation of individual proteins [59]. One pathway involving selective ubiquitin-mediated degradation of individual proteins is called the N-end rule. This pathway involves the selective degradation of proteins based of the amino acid that is present at its N-terminus. Most protein synthesis in eukaryotes is initiated by a start codon (ATG) that codes for methionine [158]. Thus, most proteins begin with a methionine residue at their N-terminus. In many cases the initiator methionine is removed and the second amino acid actually represents the N-terminal residue in the mature protein. Using reporter constructs having all possible amino acids at the 2nd position, the half-live of the same protein having different N-terminal residues was thus determined [59]. It was later found that proteins bearing Asp, Glu or Cys residues at their N-terminus were selectively degraded after the addition of an Arg residue to their N-terminus by the Ate1-encoded arginine transferase. Animals lacking the Ate1 gene die of cardiovascular defects, suggesting that the N-end pathway substrates that require ATE1 arginylation must be degraded to allow normal development to proceed [159]. Recently, both RGS4 and RGS5 were shown to be the first mammalian proteins to be selectively degraded by the ATE1 and ubiquitin dependent N-end rule pathway [160, 161]. Thus, the selective degradation of R4 RGS proteins is likely to play a variety of as yet unknown physiological roles.

Altered RGS expression is associated with pathophysiological conditions having aberrant GPCR responses

A number of pathophysiological conditions are associated with altered responses from multiple GPCRs. For example, hyperresponsive GPCR responses are seen in pregnancy-induced hypertension (preeclempsia) [162], in the rejected transplanted heart [163], while hyporesponsive GPCRs are observed in sepsis [164], congestive heart failure [165, 166] and in the drug-addicted individual [167]. Sepsis is a complex syndrome that is often triggered by an inappropriate immune response to bacterial infection. If left untreated, sepsis can lead to

hypotension, decreased cardiac response and eventually cardiovascular shock. The hypotension persists despite the fact that the levels of a number of vasoactive and cardio-stimulatory GPCR agonists are elevated. The condition is associated with refractive responses of multiple different GPCRs despite the fact that there is little loss of cell surface receptors. We have previously carried out a degenerate RT-PCR screen in the hearts of a porcine model of sepsis and have thus identified both RGS1 and RGS16 as being upregulated [168]. Similarly, Wieland's group found that R4 RGSs, including RGS16 and RGS4 were upregulated in the septic heart [70, 169]. They further demonstrated that overexpression of RGS16 was sufficient to attenuate endothelin-1 signalling in the heart. Given that increased levels of many RGSs diminish multiple GPCR responses, these studies suggest that altered RGS levels may play a role in the refractive hypotension that occurs in sepsis. Sepsis is associated with an increase in the levels of a number of pro-inflammatory cytokines, such as interleukins 1 and 6, tumor necrosis factor α and interferon γ. These cytokines exert multiple physiological effects, including decreasing cardiac function [70]. It was found that interleukin 1β was sufficient to increase RGS16 levels in cultured cardiomyocytes. This suggests that understanding the mechanisms regulating RGS gene expression will be important in understanding complex syndromes such as sepsis.

Altered RGS levels have been associated with a number of other pathophysiological conditions as well as genetic syndromes, including Bartter's/Gitelman's syndrome, the failing heart, schizophrenia, Parkinson's disease and Alzheimer's disease [70, 170–175]. A complete description of all the syndromes associated with altered RGS levels is beyond the scope of this review, so the reader is invited to examine a number of excellent recent reviews on the subject [42, 68, 69, 176, 177]. Many of these reviews also discuss the potential usefulness of therapeutics aimed at modulating RGS function. In addition, strategies aimed at developing inhibitors of mammalian RGSs using yeast cells have been described [62].

The role of endogenous RGSs

Overexpression of a variety of different RGSs in cultured cells suggests that signalling responses from most if not all GPCRs can be inhibited by RGSs. Although there is some functional overlap, there nevertheless appears to be a good deal of selectivity for individual RGSs. The selectivity of members of the small R4 RGS subfamily seems to be related in part to their ability to interact with and stimulate the GTPase activity of specific subsets of Gα-proteins that are coupled to specific GPCRs. Alternatively, selectivity may also reside in the ability of RGSs to specifically block GPCR activated G-proteins from activating specific effectors [48–50]. Although the concept that RGSs show selectivity has been demonstrated by overexpression studies using heterologous cell lines, studies using endogenous systems will be required to determine the *in vivo* specificities of the different RGSs. A variety of approaches have been used to decipher the *in vivo* specificity of endogenously expressed RGSs. Gene knock-down techniques are widely used to decrease the levels of specific endogenously expressed transcripts, including a number of RGSs [178]. For example, the role of endogenously expressed RGSs in cultured rat smooth muscle cells was examined by ribozyme-mediated knock-down experiments [134]. A decrease in RGS5 mRNA levels caused an increase in Angiotensin II/AT_{1a} receptor-mediated responses, while a decrease in RGS3 mRNA caused an increased in carbachol/M3 receptor-induced responses. These responses were specific to RGS5 and RGS3 since they were not observed with the knock down of other RGSs, including RGS2 and 7.

The overexpression of RGS-resistant G_α mutants has also served as a useful strategy to identify the involvement of endogenously expressed RGSs in regulating specific GPCR responses [90, 179–182]. The RGS-resistant G_α mutant was first identified in a screen of mutant yeast cells that display a supersensitive responsive phenotype upon stimulation of the Ste2p GPCR [180]. One such mutant was found to be a Gly^{302} to Ser substitution in the coding region of the G_α-encoded *GPA1* gene. The pheromone-supersensitive phenotype of yeast expressing the Gly^{302} to Ser *GPA1* mutant was found to be due to a decrease in the ability of the G_α mutant to bind to the Sst2p RGS. As a result, the GTPase activity of the G_α mutant could not be stimulated by Sst2p. The *GPA1* mutant retained its other normal functions since it maintained its intrinsic GTPase activity and its ability to be activated by the GPCR. Mutations of the analogous Gly residue to Ser in mammalian $G_{q\alpha}$ and $G_{i1\alpha}$ genes resulted in similarly RGS-resistant G-protein mutants [180, 181]. Overexpression of a G_α^{sst} mutant in cultured cells has been shown to allow normal GPCR signalling responses that cannot be blunted by overexpression of RGSs. For example, stimulation of the heterologously expressed serotonin 5-HT_{2C} receptor in cells overexpressing wild-type $G_{q\alpha}$ protein resulted in the typical increase in intracellular calcium [180]. This response could be blunted by overexpressing RGS7. Overexpression of the same receptor with the RGS-resistant G_α^{sst} mutant could not abolish the serotonin-mediated increase in calcium. In this case, overexpression of RGS7 did not attenuate the response to serotonin. A number of studies have employed these G_α^{sst} mutants to explore the role of endogenously expressed RGSs for a variety of different GPCRs and physiological processes [90, 179, 183, 184]. For example, the expression of an RGS-resistant $G_{q\alpha}^{sst}$ mutant was used to demonstrate that endogenously expressed RGSs in C6 glioma cells serve to regulate opi-

oid-mediated $G_{o\alpha}$ inhibition of adenylyl cyclase and increases in ERK1/2 activation [179].

One of the most powerful techniques to evaluate the function of a specific gene involves the generation of mice gene knockout models. At present, knockout animals have been described for Rgs1, Rgs2, Rgs4, Rgs9, Rgs14 and all the GRKs [113, 128, 185–189]. Analysis of these animals confirms that the loss of individual RGSs leads to distinct and specific effects. Since all the RGS genes are knocked out, the observed phenotypes may not be due to the loss of RGS domains. This is especially true for the RGS9, RGS14 and GRK knockouts, since these proteins contain multiple domains (Fig. 2). For example, the hyperresponsive phenotype due to the loss of Grk genes is likely due to a decrease in agonist-mediated desensitization of GPCRs due to the loss of the receptor kinase domain found in the GRKs [128, 190]. Nevertheless, knockout of the smaller R4 RGS genes may lead to clues as to the importance of the RGS domain in regulating different GPCR responses. For example, the importance of RGS1 in mediating chemokine receptor signalling that was previously observed in cultured B cells was confirmed in Rgs1 knockout animals [142, 191]. Surprisingly, no specific cellular or physiological defect has been described in animals lacking RGS4 [186]. RGS4 has been shown to be upregulated by morphine and injection of RGS4 in the rat locus coeruleus diminishes opioid receptor responses [192]. In spite of these observations, there is no apparent defect in opioid signalling responses in Rgs4 null animals [186]. Similarly, the previously described association of RGS4 with schizophrenia has not been observed in psychological tests performed on Rgs4 null mice [186]. Compensatory mechanisms and different genetic backgrounds may nevertheless explain the apparent negative results obtained with Rgs4 null animals [186]. Finally, analysis of Rgs2 knockout animals suggests that this $G_{q\alpha}$-specific RGS is involved in a variety of processes including T cell activation and regulation of GPCRs, such as the Angiotensin II AT_1 receptor, that are involved in controlling blood pressure [189, 193–195]. Despite the fact that Rgs2 is a small RGS protein of the R4 subfamily, the observed phenotypes may not be exclusively due to the loss of RGS2 GAP activity, since the N-terminal non-RGS region of this protein can serve to inhibit GPCR responses by acting as an effector antagonist [51]. Rgs null animals will nevertheless serve as excellent models to examine their *in vivo* functions. For example, since RGS4 is expressed in the heart and its levels are increased in response to sepsis and heart failure [169, 174, 175, 196], assessment of the potential cardiac defects in Rgs4-deficient mice will be of interest.

As an alternative strategy to gene knockout, Garzón's group has developed antisense-based methodologies to knock down *in vivo* gene expression in the central nervous system of the mouse [197–199]. Knock down of different RGSs was used to assess their role in regulating tolerance and desensitization of opioid receptors in the CNS [197, 199] and to show the specificity of RGS function. For example, knock down of either RGSZ1 or GAIP served to increase μ-opioid receptor but not δ-opioid receptor responses [198].

In conclusion, although the number of RGS-containing proteins as well as their functional roles has expanded in recent years, the major function of a significant subset of these proteins continues to be related to their ability to regulate GPCR signalling. All the available evidence suggests that the mechanisms regulating the levels and the specificities of these different RGS proteins are part of a complex regulatory network involved in modulating the timing, intensity and duration of GPCR signalling. Given that GPCRs are involved in practically all aspects of normal and altered physiological processes, defining the role of RGSs will have broad implications.

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