

RGS Proteins: Lessons from the RGS9 Subfamily

running title: RGS Proteins

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- I. Discovery of photoreceptor GAP and RGS protein family.
 - A. GTPase Acceleration in Phototransduction.
 - B. The recognition of RGS proteins as ubiquitous G protein GAPs.
- II. Some Outstanding Problems in RGS Protein Function and Structure
 - A. Problem of RGS specificity
 - B. Problem of role of additional domains
 - C. RGS specificity can be conferred through cell-type specific expression by alternative RNA processing.
 - D. Problem of membrane targeting
 - E. Problem of regulation & modulation of GAP activity- how is the correct timing achieved and varied according to cellular conditions ?
- III. Contributions to solving these problems from RGS9 and its subfamily of RGS proteins.
 - A. Intrinsic selectivity of RGS domains towards various G_{α} may account for some specificity but is unlikely to account for most of it.

- B. RGS specificity can be conferred through cell-type specific expression by transcriptional control.
 - C. RGS specificity can be conferred through cell-type specific expression by alternative RNA processing.
 - D. RGS specificity can be conferred through cell-type and organelle-specific stability by requisite association with another subunit (e.g. $G\beta 5-L$)
 - E. RGS specificity can be conferred through membrane targeting by domains outside the catalytic domain.
 - F. RGS specificity can be conferred through specific enhancement or inhibition by effectors.
 - G. Knockouts
 - F. RGS specificity can be conferred through specific enhancement or inhibition by effectors.
- IV. Future Prospects

References

RGS proteins enhance the time resolution of G protein signaling cascades by accelerating GTP hydrolysis of $G\alpha$ subunits of heterotrimeric G proteins. RGS9-1, a photoreceptor-specific RGS protein, is the first vertebrate member of this sizeable family whose physiological function in a well-defined G protein pathway has been identified. It is essential for normal sub-second recovery kinetics of the light response in retinal photoreceptors. Understanding this role allows RGS9-1 to serve as a useful model for understanding how specificity and regulation of RGS function

are achieved. In addition to the catalytic RGS domain, shared among all members of this family, RGS9-1 contains several other domains, which are also found in a closely related subset of RGS proteins, the RGS9 subfamily. One of these domains, the GGL (G β like) domain has been identified as the attachment site for G β ₅ proteins which act as obligate subunits for this subfamily. Results from RGS9-1 and other subfamily members suggest that specificity is achieved by cell-type-specific transcription, RNA processing, and G β ₅-dependent protein stabilization. In addition, membrane localization via specific targeting domains likely plays an important role.

I. Discovery of photoreceptor GAP and RGS protein family.

The RGS family of genes encode GTPase accelerating proteins (GAPs) for heterotrimeric G proteins. These genes are ubiquitous in eukaryotes, and in metazoans display considerable variety, with close to 50 different kinds now known. A subset of these, which we call the RGS9 subfamily, has been identified in vertebrates, nematodes, and insects, and is found primarily, although not exclusively, in neural tissue or at neuromuscular junctions. It is named the RGS9 subfamily after the photoreceptor-specific RGS9-1, which was the first RGS protein to make its presence known in biochemical assays of GTP hydrolysis, and the first mammalian member of the RGS family to have its physiological function clearly identified. Its identification and characterization represent the coming together of two major lines of research: the effort in several laboratories to understand the molecular mechanisms for rapid recovery from visual excitation, and the effort of a different set of laboratories to understand the

functions of the RGS family whose existence began to be recognized in 1995 and 1996. Several very useful reviews of RGS proteins have been published recently [1-3], and these provide excellent overviews. Our focus here is on RGS9 and its closest relatives, in the hope that our understanding of the function of RGS9-1 in phototransduction can provide some useful insights into how other RGS proteins might function and be regulated.

A. GTPase Acceleration in Phototransduction.

When vertebrate photoreceptor cells respond to light, they must do so with a high degree of temporal resolution, to allow detection of motion and rapid changes in scene. It has been known since the 1930's, from psychophysical studies [4, 5], that changes in intensity occurring in less than 100 ms can be detected and processed by the human retina and visual system. By the early 1990's a combination of exquisite electrophysiological studies and careful biochemical characterization had made it possible to account for all the essential features of the rapid activation of rods and cones using the measured or estimated physicochemical properties of a small set of well-characterized protein molecules [6]. These included, in addition to rhodopsin or the closely related cone pigments, heterotrimeric G proteins called transducins or G_t , cGMP phosphodiesterases (PDE), and cGMP-gated cation channels. In either rods or cones, a total of nine polypeptides appear to be sufficient to account for the activation kinetics observed by electrophysiology.

In recent years, the inactivation phase of the light response has received increasing attention. It contrasts with the activation phase in two important ways: it is much slower than the activation phase, and therefore rate-limiting for the temporal

resolution of photodetection, and it is more complicated than activation, involving a larger set of polypeptides and second messengers. Because phototransduction is G protein-mediated, it has been widely taken for granted that GTP hydrolysis by G_t would be important for inactivation, and experiments with hydrolysis-resistant GTP analogues seemed to confirm that this is the case, at least for responses to bright flashes of light, and possibly for dim flashes as well [7-9]. However, biochemical experiments with G_t made it clear that by itself, the α subunit of G_t hydrolyzes GTP much too slowly to account for recovery kinetics observed in intact photoreceptors. Amphibian rods recover with a time constant of about 2 s, while mammalian rods recover about ten times as fast (see reviews by [6, 10]). Cones recover even faster than rods. Purified G_α proteins hydrolyze bound GTP on a time-scale of tens of seconds, in general (see references in [11, 12]), and $G_{t\alpha}$ is no exception.

This discrepancy led several groups to search for GTPase accelerating proteins with activities towards G_α similar to those of GAPs that had been identified for Ras and other small monomeric G proteins [13], and at least one group to look for GTPase-independent mechanisms for cascade inactivation [14]. Using crude membrane preparations, evidence was found first for a dependence on membrane concentration of single-turnover GTP hydrolysis by G_t [15-17], and second, for an enhancement of this hydrolysis by the effector subunit PDE γ [18]. Subsequent work [12] revealed that a tightly membrane-associated protein (now known to be the RGS9-1/ $G\beta_{5L}$ complex), distinct from PDE and its subunits accounted for the GTPase accelerating activity. Further, it was found that PDE γ on its own had no effect on GTP hydrolysis by G_t ,

despite its formation of a tight complex with $G_{t\alpha}$ -GTP, but that it dramatically enhances the activity of the membrane-bound GAP [19, 20].

B. The recognition of RGS proteins as ubiquitous G protein GAPs.

Shortly after these discoveries, the RGS family came to the forefront when it was noted [21-23] that a domain present in a protein involved in regulating pheromone responses in yeast, Sst2p [24], was present in a number of proteins in mammals, *C. elegans*, [21] and *aspergillus nidulans* [25]. The observation that these proteins acted as negative regulators of G protein signaling in yeast and nematodes led to the suggestion [21] that they may function as GTPase accelerators, and to the name, Regulator of G protein Signaling, or RGS, for the shared domain. The demonstration [26] that indeed RGS proteins can accelerate G_{α} -GTP hydrolysis has been followed by a flurry of activity in this field. Soon after, it was recognized that a member of this GAP family, RGS9-1, was expressed with high specificity in photoreceptor cells, and could account for the G_t GAP activity in rods, and probably cones as well [27, 28]. More recently, the role of two non-RGS domains and of $G_{\beta 5}$ proteins that appear to act as obligate subunits have been identified for the RGS9 subfamily, as described below.

II. Some Outstanding Problems in RGS Protein Function and Structure

A. Problem of RGS specificity

One of the puzzling features of RGS protein function has been the observation that many different RGS domains can accelerate GTP hydrolysis by many different G_{α} , including members of the G_i , G_o , G_t , $G_{q/11}$, and G_z classes of G_{α} . Outside of an apparently

complete lack of activity by all tested RGS protein towards $G_{s\alpha}$, and by most towards $G_{12\alpha}$ and $G_{13\alpha}$, there seems to be little specificity built into the G_{α} -RGS domain interaction. Of course different RGS- G_{α} pairs do show different affinities and catalytic efficiencies when these are carefully quantified (see discussion below), but these are relatively small differences compared, for example, to the discrimination which receptors in the same signaling pathways [29-31] display towards various potential ligands. Because it is hard to account for the extensive diversity within the RGS family unless each member is assumed to have specific physiological roles in specific cell types, additional mechanisms for conferring specificity seem likely. In the case of the RGS9 subfamily, it is clear that this is the case, as discussed below.

B. Problem of role of additional domains

An additional puzzle for understanding RGS proteins is the role of the domains of various sizes outside the RGS domain that are found in these proteins. Two examples are the fungal RGS proteins, Sst2p from budding yeast [24], and the FlbA gene product from *Aspergillus nidulans* [25]. Less than 20% of their structures consist of the RGS domain. Of the remaining nearly 600 amino acids of Sst2p, approximately 300 form a domain identified as homologous to Ras-GAP [32], and both the Ras-GAP and RGS domains are necessary to complement the pheromone hypersensitivity phenotype of *sst2*.

A number of metazoan RGS proteins also have multiple domains (Figure 1). The RGS12 splice variants contain up to 1447 amino acids, with an active RGS domain, an active PDZ (PSD-95, Disc-large, and ZO-1) domain, a C-terminal PDZ binding domain, and a phosphotyrosine binding (PTB) domain [1] in addition to over 1000 amino acid residues of unknown function [33]. RGS3 [23] has a large domain N-terminal to the RGS

domain, whose function is unknown, but whose sequence suggests its involvement in coiled-coil protein-protein interactions. The subfamily containing GAIP, RGSZ1 [34], and RetRGS-1 [35] have cysteine-rich sequence elements proposed to be sites of palmitoylation [36], and GAIP has a C-terminal PDZ-domain binding peptide as well [37]. Axin [38], an inhibitor of Wnt signaling important in axis development, includes an RGS domain within its nearly 1000 amino acid sequence, in addition to a domain homologous to the Dishevelled protein (DIX), and a domain that recognizes glycogen synthase kinase 3 (GSK-3) [39]. The p115 Rho GEF protein has a domain which catalyzes guanine nucleotide exchange by the small GTP binding protein Rho in a $G_{13\alpha}$ -dependent way [40], but in addition has an RGS domain that stimulates GTP hydrolysis by $G_{13\alpha}$ and $G_{12\alpha}$ [41]. The protein kinase anchoring A protein D-AKAP2 [42] has been proposed to contain an RGS domain in addition to its recognition domain for the regulatory subunit of cAMP-dependent protein kinase.

One of the identifying characteristics of the RGS9 subfamily, apart from sequence similarity within the RGS domain, is a common set of domains in the N-terminal portion of these proteins. Two of these display homology to non-RGS proteins: the G_{γ} -like, or GGL domain [43], and the DEP domain [44]. The former is named for its similarity to G protein γ subunits, and the latter for the first three proteins in which it was recognized: *Dishevelled* (a *Drosophila* protein involved in *wingless* signaling), EGL-10 (a *C. elegans* RGS protein of the RGS9 subfamily) and pleckstrin. The conservation of these domains suggests they have important regulatory roles in RGS function, but for the most part these remain poorly understood. An important exception is the role of the GGL domain in the RGS9 subfamily, as discussed below.

C. Alternative RNA Processing, and Functions of Splice Variants

Diversity in RGS proteins is evident not only in the numerous genes encoding them found in higher organisms, but also in the different mRNA products of these genes resulting from alternative processing. Alternative mRNAs or cDNAs have been identified for RGS9 [45-47], RGS11 [43], RGS7 (Wei He, unpublished observations), RGS6 [48], RGS12 [33], RGS3 [21, 23], RGS2 [49], GAIP [22], p115GEF [50]. A number of these have been determined to give rise to alternative protein products, suggesting important roles for this diversity of RNA processing events, but we are just beginning to understand what some of these roles might be. Figure 2 shows our current understanding of alternative RNA processing in the RGS9 subfamily.

D. Problem of membrane targeting

G proteins are, in general firmly attached peripheral membrane proteins. When GAP activity was detected in photoreceptor membranes, it was found to be so tightly bound as to behave in many ways like an integral membrane protein [12]. Analysis of the sequences of RGS proteins have failed to support the idea of their having transmembrane domains, with the possible exception of RET-RGS1 [35]. Several RGS proteins have been localized to specific subcellular membranes, such as GAIP on clathrin-coated vesicles [51]. Therefore, important questions remain regarding the mechanisms of membrane binding and targeting of RGS proteins to those membranes where they are needed.

E. Problem of regulation & modulation of GAP activity- how is the correct timing achieved and varied according to cellular conditions ?

Because every other aspect of G protein signaling seems to be subject to multiple levels of regulation, it would be surprising if the same were not true of the GAP activity of RGS proteins. Excessive constitutive GAP activity towards any G_{α} would be likely to preclude signaling through that G protein, while insufficient GAP activity at the right place and the right time could allow the signal to continue for tens of seconds, which is already known to be incompatible with certain rapid responses. For example, when G protein regulated K^{+} channels (GIRK channels) are co-expressed with muscarinic acetylcholine receptors, but without RGS proteins, the responses are greatly slowed. However, co-expression of RGS proteins accelerates the kinetics of the response to more nearly physiological rates [52-56]. Obviously a fine tuning of protein levels, or of protein activity is needed for RGS proteins, and one of the more important questions in this field is how this regulation is achieved.

III. Contributions to solving these problems from RGS9 and its subfamily of RGS proteins.

A. Intrinsic selectivity of RGS domains towards various G_{α} may account for some specificity but is unlikely to account for most of it.

Of course one possible mechanism for specificity in RGS signaling is through different affinities of each RGS protein for different G_{α} . RGS proteins, in general, accelerate the GTPase kinetics of most G proteins; however, several studies (for example, [57-59]) have compared the potency of different RGS proteins for a particular G_{α} and found significant differences in relative potency. Similarly, many studies have compared the GTPase acceleration levels of many different G_{α} subunits to a single RGS protein in hopes of

identifying its cellular partner (for example, [34, 60]). It is tempting to assume that the most effective RGS protein (determined either by highest maximal velocity, lowest effective concentration for GAP activity, or binding affinity to the AlF_4^- transition state analogue) for a given G protein indicates the relevant physiological partner in the cell. However, results from RGS9-1 indicate that this mode of specificity does not necessarily point to the relevant RGS: G_α pair operative in cells. An instructive example comes from comparing results from the GAIP/RGSZ1/Ret-RGS1 subfamily with those from the RGS9 subfamily. RGSZ1 and GAIP potently accelerate GTPase rates for $G_{Z\alpha}$ (>400-fold) as compared to $G_{i\alpha}$ or $G_{o\alpha}$ [34, 57, 61]. Nonetheless, RGSZ1 does still accelerate GTPase for $G_{o\alpha}$ and $G_{i\alpha}$, stimulating their hydrolysis rates approximately 2.5-fold at an RGS concentration of only 21 nM. If the activities of different RGS proteins are compared by stimulation of $G_{Z\alpha}$ GAP activity, RGSZ1, RET-RGS1, and GAIP have much greater $G_{Z\alpha}$ GAP activity than RGS4. However, RGS4 is nonetheless an effective GAP for $G_{Z\alpha}$, stimulating its hydrolysis about 8-fold at an RGS concentration of only 12 nM [62] in one report, and demonstrating readily measurable $G_{Z\alpha}$ GAP activity at only 300 pM in another [34]. Based on these comparisons, alone, the RGSZ1 family proteins would appear to be the physiological GAPs for $G_{Z\alpha}$ in cells due to this *in vitro* specificity. However, the case of $G_{t\alpha}$ and RGS9-1 argues that *in vitro* selectivity alone does not necessarily indicate biological specificity. The activity of the recombinant RGS domain of RGS9 towards its cognate G_α protein $G_{t\alpha}$ [27, 63] is much lower than either the activity of RGS4 towards $G_{Z\alpha}$ or the activity of RGSZ1 family members towards some $G_{i\alpha}$ family members. Moreover, of all RGS proteins tested on $G_{t\alpha}$, which now

include RGS9 [27, 63, 64], RGS6, RGS7, RGS11 (Wei He, unpublished observations), RGS4, GAIP [58], and RGS16 [65, 66], the most potent GAP appears to be RGS16, or possibly RGS4. However, it is clear that neither RGS16 nor RGS4 is the physiological partner for $G_{t\alpha}$. In fact, most of the RGS proteins tested with $G_{t\alpha}$ for GAP activity are more potent than RGS9; it is the combination of tissue-specific expression, subcellular localization, and co-factor potentiation that confer specificity to the RGS9-1- $G_{t\alpha}$ pair in photoreceptors. Thus, even in the unusual case (*e.g.* for the RGSZ1 subfamily) where RGS- G_{α} coupling shows striking specificity *in vitro*, caution must be exercised in drawing conclusions about *in vivo* function. Intrinsic differences in activity or affinity must be considered only one of many factors governing specificity of biological function. Moreover, results with RGS9 as well as other RGS proteins make it clear that activity in native membranes, with all domains properly folded and all necessary protein (and possibly lipid) co-factors in place, activity of an RGS protein towards a particular G_{α} can be dramatically different from what is observed for the same RGS- G_{α} pair when both are examined as purified recombinant proteins.

B. RGS specificity can be conferred through cell-type specific expression by transcriptional control.

One of the first features of RGS family specificity that was examined was the pattern of RNA expression in different tissues. For example, RGS7, RGS9, RGSZ1, RGS8 and RGS4 were found to have expression largely restricted to the central nervous system [21, 23, 27, 53, 61], while others are found in multiple tissues, each displaying its own pattern of specificity. Relatively few studies have addressed expression patterns at the level of specific cell types, although detailed immunohistochemical analyses have been carried

out on RGS7 [67] and RGS9 [28, 47]. From studies of expression carried out so far, it is obvious that expression of different RGS genes is tightly controlled, and varies from cell type to cell type, and varies with time as well. A functional role for temporal regulation of transcription has been most clearly documented for the SST2 gene in budding yeast. Its transcription is increased in response to mating pheromone, providing a mechanism for desensitization. There is also evidence for temporal regulation or regulation in response to stimuli [68-73] of RGS gene expression in mammals. In mammals the clearest example of functional specificity conferred by cell-type specificity of transcription is the RGS9 gene. It is transcribed almost exclusively in only two tissues, the brain and the retina, with some low level expression also detectable in the lung. Moreover, within the brain expression is only found in a small subset of neurons, primarily restricted to the striatum [27, 45, 47, 74, 75]. Within the retina it is expressed only in rod and cone photoreceptors [27, 28, 47]. A clear correlation with function comes from the finding that the only phenotype detected so far in mice with their RGS9 genes inactivated is a dramatic slowing of recovery in the light responses of rod photoreceptors [76].

C. RGS specificity can be conferred through cell-type specific expression by alternative RNA processing.

The RGS9 gene provides another example of cell-type specific regulation, with even greater selectivity. Northern analysis reveals that the RGS9 mRNA species produced in the striatum is considerably shorter (2.5 kb in rodents, cattle, and humans) than the ones produced in the retina (8.5-9.5 kb) [27, 45-47, 75]. Sequence analysis of the cDNA and the genes themselves reveals that these different mRNAs result from alternative

polyadenylation which leads to differential exon usage, and to different proteins being encoded by the brain and retinal messages. All of the consequences of these different protein structures are not yet known, but there is evidence for functional differences in down regulation of G protein pathways in transient transfection assays ([45] and the C-terminal domain, which includes the divergent residues, has been implicated in membrane binding of RGS9-1 (Wei He, unpublished observations).

D. RGS specificity can be conferred through cell-type and organelle-specific stability by requisite association with another subunit (e.g. $G\beta_{5-L}$)

Obviously confining an RGS protein to a particular cell type or sub-cellular organelle can provide a measure of specificity, because it can only interact with those $G\alpha$ with which it is in close proximity. As discussed above, some cell type specificity can be achieved at the mRNA level, but there is some evidence that selection can occur at the level of protein translation, stability or trafficking as well. One means for achieving this is by interaction with another protein, such as a requisite subunit. Two such subunits have been identified, both products of the same gene and homologous to G protein β subunits. These are $G\beta_5$ and $G\beta_{5-L}$, two splice variants differentially expressed in different cell types. $G\beta_5$ is found in numerous cell types in the nervous system and elsewhere, while $G\beta_{5-L}$ is found exclusively in photoreceptor cells [77]. When a weak similarity was noted between a domain of RGS11 (now called the GGL domain) found also in other RGS9 subfamily members, and G protein γ subunits, the hypothesis was proposed and tested that these might bind to $G\beta$ subunits. Heterologous expression studies [43, 48, 78] demonstrated that this interaction could occur with high affinity and specificity for $G\beta_5$.

and endogenous RGS7 and RGS9-1 in the retina were found to be tightly bound to G β 5 [79] and G β 5-L [80], respectively. Mice lacking the RGS9 gene produce no functional G β 5-L despite the presence of normal levels of mRNA encoding it [76]. The simplest explanation is that RGS9-1 is required for proper folding or stability of G β 5-L. The converse appears to be true as well. In Sf9 cells, RGS9-1 cannot be expressed in stable active form without co-expression of G β 5-L. Likewise, attempts to express bovine RGS9-1 as an EGFP fusion in *Xenopus laevis* using transgenesis and a *Xenopus* opsin promoter produced no detectable RGS9-1 until a G β 5-L transgene construct was introduced simultaneously (Xue Zhang, unpublished observations). Thus it would appear that even if RGS9-1 mRNA were produced in an inappropriate cell type, it would be unlikely to generate a large amount of functional stable protein, unless G β 5-L (or possibly G β 5) were expressed there as well. A recent study on kinetics of G-protein regulated potassium channels [56] confirms that G β 5 enhances the activity of either RGS7 or RGS9.

E. RGS specificity can be conferred through membrane targeting by domains outside the catalytic domain.

One potential mechanism for achieving specificity is to target RGS domains to sites of action where specific signaling proteins are found, typically on membrane surfaces.

There is some evidence that domains specific for certain RGS proteins, or subsets of RGS proteins, help target them to membranes, or at least allow them to bind the membranes tightly. In the case of RGS9-1, limited proteolysis reveals that specific removal of a C-terminal 3 kDa peptide abolishes tight binding to rod outer segment membranes (Wei He,

unpublished observations). An intriguing possibility, not yet tested, is that the alternative RNA processing that gives rise to different C-terminal domains in brain RGS9-2 and retinal RGS9-1 confers different membrane anchoring specificity on the two tissue-specific protein products. The alternative C-terminal domain of RGS9-2 is rich in proline residues, which might play a role in recognizing tethering proteins in the brain.

This C-terminal domain of RGS9-1 is unique among RGS proteins, so it seems likely that different domains are responsible for membrane targeting in other RGS proteins.

RGS-GAIP [51] and RGSZ1 [34], which are also membrane bound in cells, have been proposed to be tethered via palmitoylation of clusters of cysteine residues near their N-termini. RGSZ1 brain binds membranes in brain very tightly, and is resistant to detergent and high ionic strength. The N-terminal 380 amino acids of RGS3 seem to be important for translocating it from the cytosol to the plasma membrane upon agonist stimulation [81]. Although endogenous RGS4 in NG108 cells is predominantly soluble [82], and the same is true for RGS4 or RGS16 transfected into mammalian cells [83, 84], in yeast expressing RGS4 [85] and RGS16 [84], they are bound to membranes. Their palmitoylation (at either of two cysteine residues) was not required for membrane association in either yeast and mammalian cells. It has been proposed that an amphipathic helix N-terminal to the RGS domain mediates their membrane membrane binding [84]. There are probably many additional motifs for RGS protein-membrane recognition that remain to be determined.

F. RGS specificity can be conferred through specific enhancement or inhibition by effectors.

There are apparently at least three modes by which effectors can modulate GAP activity of RGS proteins. In one mode, they can substitute for the RGS protein, directly accelerating GTP hydrolysis by binding to G_{α} in the absence of RGS. Phospholipase $C\beta$ (PLC) has been shown to do this [86], and there is some evidence that adenylyl cyclase may exert a similar effect on $G_{s\alpha}$ [87]. Alternatively, effectors may inhibit RGS action. In the case of PLC, there appears to be competition with RGS domains for binding to $G_{q\alpha}$ [59, 88, 89], so that the effector must apparently be displaced in order for the RGS domain to bind. The physiological role of this antagonism is unclear.

For the other effector for which these interactions have been analyzed, the $PDE\gamma$ subunit of cGMP PDE, it appears that inhibitory action can be exerted in a ternary complex involving G_{α} -GTP, $PDE\gamma$, and the RGS domain [58, 65]. $PDE\gamma$ exerts an inhibitory effect in this mode on all RGS domains tested except for RGS9. These include RGS4, GAIP, RGS16, [58, 65].

In contrast, $PDE\gamma$ stimulates the activity of the RGS protein with which it is found in nature: RGS9-1 of photoreceptor cells [19, 27, 90]. Structure-function studies have revealed that part of the specificity of the $PDE\gamma$ effect is encoded in the RGS domain itself, and part of it is conferred by interactions with regions of the GAP outside of the RGS domain proper; specifically, the GGL domain, and its complex with $G_{\beta 5-L}$ play an important role in inhibiting RGS9-1 GAP activity in the absence of $PDE\gamma$ and in facilitating enhancement of GAP activity when $PDE\gamma$ is added. These effects of $PDE\gamma$ on RGS9 are very robust *in vitro*, but require free $PDE\gamma$ - i.e., $PDE\gamma$ not in its usual state of

tight complexation with the catalytic PDE $\alpha\beta$ unit. How this activity of PDE γ is made available in intact photoreceptors remains a mystery.

G. Knockouts

Gene inactivation is a powerful approach to determining physiological function, and indeed the first clues to RGS domain function came from genetic studies of mutants in *S. cerevisiae* [24, 32, 91] and *C. elegans* [21]. More recently, genetic approaches have revealed the function of additional RGS proteins in *S. cerevisiae* [92], *S. pombe* [93], *C. elegans* [93], and *Drosophila melanogaster* [94]. Gene inactivation in mammals is somewhat more difficult, and usually requires starting with the gene, and then discovering the phenotype. This method has been very valuable in elucidating the functions of some G α proteins [95]; however, the results have sometimes been difficult to interpret at first, because of redundancy of function in some cases [96], or unexpected subtlety of phenotype in others [97, 98]. On the other extreme, genes whose deletion leads to lethality early in embryogenesis (e.g. as observed for the Fused gene encoding the RGS protein Axin, [99]) also pose difficulties in analysis. Because there are so many RGS proteins, redundancy or subtlety of a phenotype can be expected to present even greater problems in analysis of RGS gene knockouts. Results from inactivation of the RGS9 gene in mice provides an illustrative example [76]. Based on earliest reports of expression of this gene in the brain, one might have looked at brain development or obvious behavioral traits and concluded that there was no phenotype of RGS9 knockouts. Examination of retinal morphology, or of sensitivity to light would have led to the same conclusion. Only careful recording of the kinetics of the recovery phase of the photoresponse and specific biochemical assays revealed the striking phenotype of these

mice: slowed GTP hydrolysis for a specific G protein, $G_{t\alpha}$, and slowed recovery of photocurrents [76]. A complementary study showed that replacement of the gene for the effector subunit that stimulates RGS9-1 GAP activity, PDE γ , with a mutated version producing a defective protein also leads to slowed recovery [100]. The most obvious phenotype in this case was a profoundly reduced sensitivity of rods to light, and the careful kinetic analysis which revealed the slowed recovery was guided by the prior biochemistry [101]. As results are obtained from knockouts of additional RGS genes in mammals, it will be important to have biochemical data on the activity of endogenous RGS proteins in specific cell types to guide analysis of the phenotypes.

H. Regulation likely involves multiple other proteins:

In the short time that RGS proteins have been the objects of serious scrutiny, several proteins with which they interact, either through the RGS domain or through other domains have been identified. Based on what is known about other key regulators of signal transduction, and based on the presence of domains whose most likely functions involve protein-protein interactions, it seems reasonable to expect that many more proteins will be found to interact with and regulate (or be regulated by) RGS proteins. Likely candidates are PDZ-domain containing proteins, or proteins with PDZ binding C-termini, protein kinases and phosphatases, receptors, and calcium binding proteins. The search for such novel interactions is underway in several laboratories and is already bearing fruit. For example, RGS7 has been found to interact with polycystin, and with components of the proteasome pathway [102].

IV. Future prospects.

The field of RGS proteins is still in its early stages. Perhaps the biggest "gap" in our understanding is uncertainty about the physiological roles of the individual proteins containing RGS domains. The keys to filling in this gap will be: 1) To focus on biochemical and cell biological studies of endogenous RGS proteins in the tissues and cells in which they are normally expressed. Heterologous expression, and especially overexpression, in tissue culture cells can provide useful information, but can be very misleading with respect to functions in specific pathways. Studies of expression patterns do not solve the problem, but they can help to guide such studies by providing candidate genes and proteins for further studies in particular cells; much more needs to be done in the area of cataloguing these patterns. However, it needs to be kept in mind that as potentially potent catalysts, RGS proteins do not have to be present at high levels to exert large effects on signaling. 2) To study effects of gene inactivation. To date, studies of mutants with interesting phenotypes whose mutated genes turn out to encode RGS proteins have been among the most informative. In the future, it seems likely that some of the biggest advances in understanding RGS domain function in mammals will come from gene inactivation in mice.

Another area of focus will be on structure and function of domains outside the RGS domains. How the different modules fit together in three dimensions, and how they work together to regulate signaling remain some of the most intriguing mysteries in this field.

Finally, discovery of new interactions between RGS proteins and other regulatory proteins will be a very active area of research in the next few years.

References

Figure Legends

Figure 1. Variety of domain structures in RGS-domain-containing proteins. RGS: ~125 a.a. conserved catalytic domain defining RGS proteins. DEP: DEP domain; R14: a domain of homology between RGS12 and RGS14; R4: a domain of homology between RGS4 and RGS5; R9: a domain of unknown function common to the RGS9 subfamily; PDZ: PDZ domain; DIX: domain of Axin with homology to a domain in Dishevelled outside DEP domain; GGL: G protein γ -like domain. PTB, phosphotyrosine-binding domain. The domain structure of RGS6 is very similar to that of RGS7, and the domains of RGS11 and EAT-16 103] are very similar to those of RGS9. RGS9-2 contains an alternative C-terminal domain of 205 a.a..

Figure 2. Alternative RNA processing of RGS9 subfamily members. Gene structures (RGS9, RGS11, RGS6) are for human genes [47] and likely alternative processing schemes are based on human (RGS9, RGS11 and RGS6), or mouse (RGS6, RGS7) cDNAs. The GenBank accession numbers retrieved are as follows: human RGS11 genomic sequence(Z69667); human RGS6 genomic sequence (AC005993, AC005477, AC005157 and AC005533); human RGS11 cDNAs (AB016929, AF035153 and AF035154); human RGS6 cDNAs (AF156932, AF107620, AF107619, AF073921 and AF073920).

Footnotes

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	RGS		R14	
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 RGS14

PDZ		PTB		RGS		R14	
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 RGS12

	RGS				DIX
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 AXIN

DEP		R9		GGL		RGS	
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 EGL-10

DEP		R9		GGL		RGS	
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 RGS9-1

} RGS9 Subfamily

DEP		R9		GGL		RGS	
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 RGS7

R4		RGS	
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 RGS4

						RGS	
--	--	--	--	--	--	-----	--

 RGS3

						RGS	
--	--	--	--	--	--	-----	--

 RGS2

100

 Scale

