Functional Characterization of the Influence of Human RGS1 (hRGS1) on G Protein-Coupled Receptor Signaling in *Saccharomyces cerevisiae*

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Abstract

Regulators of G protein signaling (RGS) are proteins that negatively regulate G protein-coupled receptor signaling. Although a conserved core domain is necessary and sufficient for their GTPase accelerating protein (GAP) activity, many RGSs possess C- and N-terminal protein-binding motifs that augment GAP activity and participate in other cellular regulatory mechanisms.

Human RGS1 functionally complements a *Saccharomyces cerevisiae* mutant lacking the RGS homologue Sst2p. We demonstrate that deletion of the N-terminus or RGS domain negatively affects this ability in Sst2p-deficient strains, whereas deletion of the C-terminal 10 residues of RGS1 does not. Coexpression of the N-terminus and RGS domains restores complementation of Sst2p to that of wild type. The conservative replacement of sequential residues spanning the N-terminus of RGS1 causes little loss of function. These results suggest that the N-terminal and RGS domains of RGS1 function in concert to effect signaling and that the C-terminal 10 residues of RGS1 are not required for this activity. Further, residues present in the N-terminus are not highly conserved suggesting that overall structure, rather than individual residues or motifs, may be important for function.

Résumé

Les (RGS) constituent une famille de protéines qui contrôlent de façon négative la signalisation des récepteurs couplés aux protéines G (RCPG). Ils possèdent tous un domaine RGS conservé qui est nécessaire et suffisant à leurs fonction GTPase accélératrice (GAP) sur les protéines G. Certains RGSs ont en leurs extrémités C- et N-terminals des motifs structuraux qui servent à lier d'autres protéines, à augmenter leur activité GAP et qui participent à d'autres mécanismes régulatoires intracellulaires.

La RGS1 humaine (RGS1) peut complémenter fonctionellement son homologue Sst2p dans la levure *Saccharomyces cerevisiae* déficiente pour ce gène. Nous avons démontré que l'ablation de la region N-terminal ou du domaine RGS, mais non celle des 10 derniers acides aminés de la region C-terminal de RGS1, atténue cette capacité. La coexpression du domaine N-terminal avec le domaine RGS restitue cette capacité chez les mutants Sst2p au niveau retrouvé dans la souche sauvage. Des remplacements séquentiels d'acides aminés conservatives couvrant la séquence N-terminal de RGS1 n'ont pas d'éffet sur sa fonction. Ainsi nous postulons que le N-terminus et le domaine RGS de RGS1 agissent en commun dans la signalisation et que les 10 derniers résidus de la partie C-terminal de RGS1 ne sont pas nécessaires pour cette activité. En outre, les résidus de la partie N-terminal de RGS1 sont faiblement conservés. Il semble donc que l'ensemble de la structure de la partie N-terminal de RGS1 plutôt que ses résidus ou motifs individuels soit critique pour sa fonction.

Dedication

To my parents, Claudia and Bill

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Preface: Contributions of Authors

In accordance with the guidelines for thesis submission, this thesis consists of a literature review (Chapter 1) and an appended manuscript (Chapter 2). With respect to the appended manuscript, all experiments therein were performed by W. Somerville, with the following exceptions: the p426GAL1-EGFP expression plasmid as well as ATG-GFP, RGS1-GFP, N-term-GFP and Box-GFP expression constructs were constructed by J. Kong, all western blot analyses were performed by W. Song, and yeast strains were constructed by M. Greenwood.

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Chapter 1: Literature Review

1.1 The G Protein-Coupled Receptor (GPCR) Family

The ability of an organism to respond to external stimuli depends on intracellular signal transduction and, in higher organisms, on intercommunication between component cells and tissues. Many extracellular stimuli such as hormones, growth factors, and neurotransmitters, as well as physical signals such as photons and odorants are transmitted to the interior of the cell by ligand-specific receptors located at the plasma membrane. One such group of receptors, the G protein-coupled receptors (GPCRs), directs responses to a large number of extra cellular ligands through coupling to a heterotrimeric G protein of the form $G\alpha\beta\gamma$. The importance of this protein family is underscored by its high degree of conservation throughout evolution, and by the fact that it constitutes as much as 0.5% of the human genome (153).

1.1.1 Structure

G protein-coupled receptors are characterized by the presence of seven transmembrane (TM) helical domains designated TM1 to TM7, three extracellular loops and three cytoplasmic loops connecting the transmembrane segments, as well as N-terminal extracellular and C-terminal intracellular domains (4) (Figure 1a). A disulfide bridge formed between two conserved cysteine residues in extracellular loops 1 and 2, as well as extensive hydrogen bonding of residues within and between different TMs are believed to be important for TM packing and for the restriction of predicted TM conformation (96, 135). The GPCR family can be further divided into five subgroups determined by the length and function of their N- and C-terminal domains and intracellular loops (14).

1.1.2 Ligand Binding and Receptor Activation

The ligand-binding pockets present within different GPCRs are almost as diverse as the agonists with which they bind. For example, biogenic amines such as epinephrine

and norepinephrine bind to the TM core of the receptor exclusively (137). Peptide hormones such as glucagon and calcitonin bind to the N-termini of their cognate receptors, and the resulting receptor-ligand complex then contacts the extracellular loops of the receptor (177). The glycoprotein hormones LH, FSH, and TSH also bind to the N-termini of their respective receptors. Here however, the resulting receptor-ligand complexes induce a conformational change in the receptor such that contact is made with one of its TM domains (96).

Kinetic studies of agonist binding performed in several experimental systems have shown that GPCRs exist in several states: 1) a resting state which couples to G-protein upon addition of agonist, 2) a pre-coupled receptor-G-protein complex with high affinity for agonist, and 3) a highly inactive state unable to couple to agonist (127). The existence of the second state may explain the high basal activity noted for some of these receptors (68). Receptor activation subsequent to ligand binding appears to result in molecular rearrangements that change the spatial relationships between TMs and thus the conformation of the receptor in the plasma membrane (96). Indeed, peptide analogs of the individual TMs of the chemokine receptors CXCR4 and CCR5 act as specific receptor antagonists, presumably by competitively blocking TM interactions and interfering with conformational changes required for activation (181).

1.1.3 GPCR-G Protein Coupling

Heterotrimeric G proteins are localized to the inner surface of the plasma membrane and are composed of an α-subunit that has high guanine nucleotide affinity noncovalently bound to a $\beta\gamma$ dimer (64)(Figure 1a). There are twenty-three known G protein α subunits that can be further divided into four subfamilies, $G_s\alpha$, $G_i\alpha$, $G_q\alpha$ and $G_{12/13}\alpha$, on the basis of sequence similarities (59). Seven G protein β isoforms and twelve γ isoforms have been identified to date (52, 86), making possible the formation of a large number of unique heterotrimers (139).

In the inactive state, the α-subunit binds guanosine diphosphate (GDP). Activation by ligand binding triggers a reorganization of GPCR TMs thereby making accessible critical residues in the cytoplasmic loops that interact with the G protein heterotrimer (17). This results in the exchange of GDP for guanosine triphosphate (GTP) on the Gα–subunit, activation of the G protein, and its dissociation into constituent Gα and βγ subunits (64)(Figure 1a). The mutation of residues in the β-subunit that interact with the α-subunit impairs GTP/GDP exchange suggesting that Gβ stabilizes a Gα conformation necessary for this exchange to occur (76).

There is increasing evidence that cellular localization plays as great a role in receptor-G protein coupling as does interaction between specific residues on the respective proteins. Instead of existing as freely mobile molecules within the plasma membrane, GPCRs may act as scaffolds targeting G proteins and other signaling molecules to specific membrane domains (62, 126, 149). Proper targeting of G proteins to these domains appears to require N-terminal myristoylation and subsequent palmitoylation of the Gα-subunit as well as interaction with Gβγ (58, 121, 195, 196).

1.1.3.1 Specificity of GPCR-G Protein Coupling

Localization to membrane complexes may contribute to receptor-G protein specificity; limited mobility may result in receptors being confined to interact with a distinct subset of G proteins (126). Evidence that this spatial arrangement of G proteins in membranes contributes to signaling specificity has come from studies of embryonic stem cells deficient in the extracellular matrix binding protein β1 integrin. As a result of this deficiency, these cells have an altered distribution of $G_i\alpha$ that causes a defect in muscarinic receptor signaling but has no effect on β adrenoceptor signaling (13).

The diversity of potential G protein heterotrimers, made possible by the large number of Gβ and γ isoforms with which Gα can bind, may also play a role in the specificity of G proteins for the receptor. One study that used recombinant proteins

consisting of invariant $G_s\alpha$ or $G\gamma_2$ subunits complexed with various isoforms of Gβ, illustrated that differences in coupling to both receptors (β_1-adrenergic or A_{2a} adenosine) and to effectors (adenylyl cyclase 1 or 2) were dependent on the Gβ isoform used (119).

1.1.4 Initiation of Downstream Signaling Events

1.1.4.1 Signaling Dependent on the GPCR-G Protein Interaction

The interaction between GPCR and G protein causes a conformational change in the G protein such that the GDP binding sites in the β6/α5 loop are lost and GDP is released (17). Once released, GDP is replaced by GTP resulting in destabilization of the heterotrimer, activation of the G protein, and its dissociation into component α- and βγ-subunits (Figure 1a). Depending on which receptor and/or subunit type is activated, the liberated α and βγ subunits can then effect many different downstream signaling events.

For example, Gα activation may stimulate or inhibit adenylyl cyclase ($G_s\alpha/G_i\alpha$) (118), stimulate phospholipase Cβ (PLCβ) ($G_q\alpha$) (118), or promote ion exchange or cytoskeletal rearrangement ($G_{12/13}\alpha$) (126). The same Gα-subunit may activate different second messengers depending on the receptor that activates it; coupling of the α_{1A}- adrenergic receptor to $G_q\alpha$ leads to phosphatidylinositol-3 kinase (PI3-kinase)-/protein kinase B (PKB)- independent activation of p70 S6 kinase (5), whereas $G_q\alpha$ coupling to the bombesin receptor stimulates protein kinase D (PKD) (199).

Liberated Gβγ-subunits act on a variety of downstream effectors such as PLCβ and adenylyl cyclase (179). In addition, Gβγ is able to activate G-protein-responsive inwardly rectifying potassium channels (GIRKs) in response to acetylcholine stimulation of muscarinic receptors (104), and to inhibit voltage-dependent neuronal calcium channels downstream of γ-aminobutyrate (GABA), opioid, and adrenergic receptors (200). Gβγ subunits also modulate the c-Jun N-terminal kinase (JNK) cascade through the binding of Rho family proteins such as Rac, cdc42 and Rho (34),

and can induce stress fiber formation in quiescent HeLa cells in a Rho-dependent fashion (187).

Many ligands acting through GPCRs elicit mitogenic responses in a variety of cell types. In yeast cells, the free Gβγ-subunit (Ste4p-Ste18p) activates the pheromone-stimulated mitogen activated protein kinase (MAP kinase) pathway (192). In carbachol-stimulated NIH 3T3 cells, Gβγ activates PI3-kinaseβ in a tyrosine phosphorylation-independent manner (124), and Gβγ subunits have been shown to interact directly with the catalytic subunit, p110, of the γ isoform of PI3K *in vitro* (111). In NG-108 neuronal cells, insulin-like growth factor-1 (IGF-1) stimulation of IGF-1 receptor (IGF-1R) results in the activation of a $G_i\alpha$ protein associated with this receptor, the release of Gβγ subunits, and activation of the MAP kinase pathway (75). Since cross-talk has been demonstrated between the PI3K and MAP kinase pathways (150), and these data demonstrate cross-talk between tyrosine kinase receptors and heterotrimeric G protein pathways, there exists a potential mechanism by which Gβγ subunits could regulate mitogenic signaling in mammalian cells.

1.1.4.2 Signaling Independent of G Protein or Receptor

Agonist stimulation of GPCRs also results in the activation of G protein-independent signaling pathways. This phenomenon may be in part due to the presence of structural motifs known to participate in protein-protein interactions, such as PDZ (*P*SD-95/*d*lg/ *Z*01 homology) and Src homology-2 and -3 (SH2 and SH3) domains, in many GPCRs (14, 74, 115, 157).

Several receptor-independent activators of G proteins have also been identified. In particular, two mammalian proteins designated AGS2 and AGS3 (Activators of G protein Signaling) have been shown to activate the *Saccharomyces cerevisiae* pheromone response pathway in the absence of receptor (138, 180).

1.2 Control of GPCR Signaling

1.2.1 The Importance of Regulation

The importance of regulating the activity of GPCRs is underscored by the number of disorders that can be attributed to aberrant GPCR signaling (59). Spontaneously occurring mutations have been identified that affect both receptors and G proteins. For instance, mutations that produce constitutively active thyroid-stimulating hormone receptors have been observed in both human thyroid adenoma and carcinoma suggesting an association between GPCR function and neoplasia (118). The genetic lack of $G_s\alpha$ results in Albright's hereditary osteodystrophy, the manifestations of which are due to a lack of response to parathyroid hormone by the parathyroid (158).

Pathogenic conditions exist that influence the ability of a G protein to effect nucleotide exchange or to bind receptor thus affecting downstream signaling. Cholera toxin, released by the bacterium *Vibrio cholerae*, ADP ribosylates a side chain of $G_s\alpha$ resulting in the arrest of GTP hydrolysis and the maintenance of an active G protein (161). Pertussis toxin released by the bacterium *Bordetella pertussis* (the causative agent of whooping cough) ADP ribosylates $G_i\alpha$, uncoupling receptors in the respiratory epithelium from this G protein (49).

The large number of pathophysiological conditions that result from abnormalities in G protein signaling is such that, approximately 60% of prescription drugs currently marketed act on GPCR signal transduction systems (73).

1.2.2 Control at the Level of the GPCR

1.2.2.1 Desensitization

The continued exposure of GPCRs to agonist leads to the rapid attenuation (minutes to hours) of receptor responsiveness, a process known as desensitization (20, 62, 71, 185). The current understanding of this phenomenon is largely derived from studies of the photoreceptor rhodopsin and from the β_2-adrenergic receptor (β_2AR). Desensitization of rhodopsin has been observed to occur within less than one second

following light stimulation, a mechanism that prevents a single flash of light from being seen as constant illumination (156). For the β_2AR, a rapid loss of agonist-stimulated adenylyl cyclase activity occurs when desensitized cells are rechallenged with agonist (106). Although desensitized receptors retain the ability to bind agonist, their ability to interact with and activate heterotrimeric G proteins is reduced (162, 178).

The process of desensitization involves several mechanisms and is initiated through the phosphorylation of key C-terminal receptor residues by specific protein kinases. Agonist-specific desensitization is mediated via phosphorylation of the receptor by G protein receptor kinases (GRKs) (18, 33, 78, 113). Heterologous desensitization is a non-agonist-specific process whereby both unoccupied and agonist-occupied receptors are phosphorylated by the second messengers protein kinase A (PKA) and protein kinase C (PKC) produced by receptor activation of $G_s\alpha$ and $G_q\alpha$ respectively (110, 164). Once phosphorylated, GPCRs bind the protein arrestin which competes with agonist and induces a conformational change in the receptor that disrupts the receptor-G protein interaction, thus terminating signaling (24).

The continued exposure of phosphorylated receptor to agonist promotes its translocation from the cell surface to intracellular vesicles, resulting in loss of ligand binding sites and decreased affinity for agonist (106). This process is accomplished by β-arrestins, which bind with high affinity to clathrin and act as adaptors promoting endocytosis of phosphorylated GPCRs via clathrin-coated vesicles (70, 81, 107, 129, 159).

1.2.2.2 Resensitization

The process of GPCR internalization may also play a role in the recovery of receptor from desensitization. β_2-adrenergic receptor responsiveness to isoproterenol is restored within 15-30 minutes following agonist withdrawal, and is associated with the return of receptors to the cell surface (110). This phenomenon, termed resensitization, has been shown to require receptor internalization, as β_2-adrenergic receptor mutants that are unable to internalize, do not recover from desensitization

upon agonist removal (51, 198). Once internalized, the receptors are dephosphorylated by a phosphatase that is active only at the acidic pH present in endosomal vesicles. If this favorable pH is altered, for example by treatment with NH_4Cl, the receptor-phosphatase interaction, and both receptor dephosphorylation and resensitization are prevented (105, 140, 141).

1.2.2.3 Downregulation

The modes of GPCR control mentioned above are associated with acute desensititization and redistribution of the receptor without change in total receptor number. A third mode of GPCR control occurs through receptor downregulation and degradation hours to days after prolonged exposure to agonist (32). Downregulation of the β_2-adrenergic receptor has been shown to occur not only via receptor proteolysis, but also by agonist-induced decreases in receptor mRNA levels (65, 97, 183, 185).

1.2.3 Control at the Level of the G Protein

1.2.3.1 Posttranslational Modifications, Transcriptional control and Protein-protein Interactions

GPCR signaling can also be controlled at the level of the G protein itself. There is evidence that post-translational modifications of G proteins subunits such as phosphorylation (63), myristoylation/palmitoylation (12, 42, 175, 176) and ADP-ribosylation (49, 114, 161) can regulate signaling downstream of GPCRs. The agonist-dependent regulation of G protein mRNA levels (1, 72, 123) as well as the interaction of G proteins with second messengers such as PLC, calcium and Ras-GTPase-activating protein (GAP) have also been documented as having a regulatory role (100, 112, 116).

1.2.3.2 Inherent Gα GTPase Activity

The predominant and most efficient mechanism by which the inactivation of heterotrimeric G proteins is accomplished is via a GTPase activity inherent to the Gα- subunit. Hydrolysis of Gα-bound GTP shifts the equilibrium in favor of heterotrimeric subunit reassociation thus terminating signaling (46) (Figure 1b). For

$G_s\alpha$, the intrinsic rate of GTP hydrolysis *in vitro* corresponds to the rate of adenylyl cyclase deactivation in membranes with both events having half lives of approximately 15seconds (152). Other G protein systems have been shown to display similar GTP hydrolysis rates *in vitro* and yet demonstrate much faster signaling deactivation half lives *in vivo*. In the visual transduction system for example, the Gα-subunit of transducin ($G_t\alpha$) has an *in vitro* GTP hydrolysis rate of approximately 15s, whereas the response to a brief pulse of light stimulation has been shown to terminate at approximately 100 milliseconds *in vivo* (189). Further studies provided evidence that the rate of $G_t\alpha$-catalyzed *in vitro* GTP hydrolysis might occur as quickly as the physiological response, suggesting that a factor responsible for accelerating the GTPase activity of the Gα subunit may have been lost upon initial $G_t\alpha$ purification (2, 94).

1.2.3.3 GTPase Activating Proteins (GAPs)

The discrepancies observed between *in vitro* and *in vivo* G protein deactivation lead to a search for proteins that could accelerate Gα GTP hydrolysis. The existence of GTPase-Activating Proteins, or GAPS, had previously been demonstrated to accelerate the GTPase activity of the monomeric GTP-binding protein p21Ras (15, 184) and so it was postulated that GAPs might also exist for the heterotrimeric G proteins. Indeed, both PLCβ1 and the γ subunit of the retinal cGMP phosphodiesterase (PDEγ) have been shown to exhibit GAP activity for $G_q\alpha$ and $G_t\alpha$ subunits respectively (3, 184).

1.3 Regulators of G Protein Signaling (RGS)

1.3.1 Initial Discovery and Description

A group of proteins that act as GAPs for Gα subunits, known as regulators of G protein signaling (RGS), were recently identified. These proteins act by functionally inactivating the active GTP-bound form of the heterotrimeric G protein Gα-subunit thus limiting the lifetime of receptor-activated G proteins (9, 29, 191).

The first evidence for the existence of these proteins came from studies using haploid mutants of the yeast *Saccharomyces cerevisiae* that were super-sensitive to pheromone-induced cell cycle arrest mediated by the G protein-coupled receptor Ste2p (25). Genetic analysis later revealed that the normal function of one of the mutated gene products, now known as Sst2p, was to inhibit pheromone-induced mating response through binding to the yeast G protein α subunit Gpa1p (45, 108). Similar gene products were later discovered in *Caenorhabditis elegans* (EGL-10) and *Aspergillus nidulans* (FLBA) that were found to be negative regulators of their respective G protein α-subunits FadA and GOA-1, and to share sequence homology with the yeast Sst2p within a 120 amino acid region (101, 109). Simultaneously, it was demonstrated that a mammalian RGS, Gα-interacting protein (GAIP), bound to $G_{i3}\alpha$ subunits *in vitro* via a 125 amino acid core domain (40). This domain was found to be homologous to that found in Sst2p, EGL-10, FLBA as well as to two previously cloned human genes GOS8 (166) and BL34/1R20 (90), now known as RGS2 and RGS1 respectively.

The RGS protein family is defined by the presence of this 120 to 130 amino acid core domain, referred to as the RGS box or domain, which has been shown to be necessary and sufficient for their *in vitro* GAP activity (193).

1.3.2 Structure and Function

1.3.2.1 Gene Structure

Recently, the complete gene structure and chromosomal positions of all murine and human RGSs was published and substantiated the previous sub grouping of these proteins based on amino acid sequence (152, 167) (Figure 2). The length of these genes varies from 4.1 kb (RGS1) to 123 kb (RGS12) and generally reflects the number of exons present as well as protein size. Some mammalian RGSs such as *AXIN1* and *AXIN2* lack introns within the RGS domain, whereas others such as RGS 9 and RGS12 have as many as three. Interestingly, the chromosomal location of *AXIN2* is proximal to that of $G_{13}\alpha$ suggesting that the expression of the two might be coordinately regulated allowing AXIN2 to control this Gα-subunit (167). In addition,

the gene encoding GAIP or RGS19 is closely linked to and shares a promoter with the opioid-receptor-like gene ORL1 (93).

1.3.2.2 The RGS Domain

Currently, approximately 30 RGS-containing proteins have been identified, 25 of which are mammalian proteins that can be grouped into five or six subfamilies according to sequence homology within the RGS box (167) (Figure 2). Several non-mammalian RGSs, such as *S.cerevisiae* Sst2p as well as *A. nidulans* FLBA and *C. elegans* C29H12.3, contain a "split RGS" domain separated by intervening amino acid residues (45, 101, 109) (Figure 3). Although the function of these intervening residues is as yet unknown, the observation that mammalian RGS2, RGS4, RGS1, RGS8, RGS5 and RGS16 have the ability to complement a defective pheromone response in yeast lacking the *SST2* gene (30, 47, 133) (30, 102, 155) suggests that these residues do not play a crucial role in the activity of these proteins. In contrast, both *C. elegans* EGL-10 and *S.cerevisiae* Rgs2 have an RGS domain that more closely resembles their mammalian counterparts (101, 188)(Figure 3).

1.3.2.3 The Mechanism and Consequences of RGS GAP Activity

The mechanism by which RGSs are able to augment GTP hydrolysis by the Gα subunit was elucidated through the use of G protein α-subunits that were artificially locked in active (αGTPγS), inactive (αGDP), or transition (αGDP-AlF_4^-) states. The affinity of RGS proteins for both αGDP and αGTPγS was relatively weak, whereas many RGS proteins displayed high affinity binding to αGDP-AlF_4^- indicating that RGSs may exert their GAP activity by stabilizing this transition state (29, 103, 143, 191, 194) (9).

The corroboration of this notion came when the crystal structure of the RGS4/G_{i1}αGDP-AiF_4^- complex was described in 1997(182) revealing that the key RGS4 residues participating in this interaction resided within its RGS domain. Analysis of residues within "switch" regions of G_{i1}α participating in the RGS4 interaction, suggested that they would be stabilized by this interaction thus allowing GTP hydrolysis to occur more rapidly (193). Indeed, analysis of the structure of

$G_{i1}\alpha$-GDP-AlF_4^- with and without RGS4 suggests that the switch regions become more rigid in the presence of RGS4 trapping the Gα-subunit in a transition state conformation that facilitates the attack of a water molecule on the γ phosphate of GTP (54, 182).

Although residues of RGS4 do not make direct contact with the GTP substrate, Asn^{128} has been shown to play a critical role in its GAP activity via interactions with Gln^{204} and Glu^{207} of $G_{i1}\alpha$ (145). The mutation of this asparagine residue to alanine results in an RGS4 protein that cannot bind to αGDP-AlF_4^- and consequently retains only 4% of the GAP activity of wild-type RGS4 (174).

In vitro experiments have demonstrated that the RGS domain is necessary and sufficient for the GAP activity of RGS4, RGS10 and GAIP (143), as well as for RGS1, RGS2, RGS8, RGS9, RGS11, RGS12, RGS16, Ret-RGS and RGSZ1 (193). Evidence for their ability to regulate G protein signaling *in vivo* came with the observation that RGS1, RGS2, RGS3 and RGS4 could inhibit the activation of the MAP kinase pathway mediated by interleukin 8 (IL-8)(53). Since their discovery, RGSs have been shown to be involved in the negative regulation of signaling mediated by all GPCRs studied to date (22, 38, 59, 193). Interestingly, an RGS that interacts with a steroid (estrogen) receptor was identified recently, although the functional significance of this association is unclear (91).

1.3.2.4 Selectivity of RGSs for Gα-subunits

Although many RGS proteins have been shown to be GAPs for members of the $G_i\alpha$ and $G_q\alpha$ subfamilies, some show preferences towards different substrates. One example is GAIP, or RGS19, which is relatively non-selective towards $G_i\alpha$ members and shows weak $G_q\alpha$ GAP activity (190). Although closely related structurally to GAIP, RGSZ1 and RET-RGS1 are highly selective towards $G_z\alpha$ relative to other $G_i\alpha$ members and show little $G_q\alpha$ GAP activity (190). The substitution of serine for asparagine at a position analogous to N128 in RGS4 has been proposed to be the basis of the ability of RGSZ1 and RET-RGS1 to act as GAPs for $G_z\alpha$ (152).

RGS2 acts as a $G_q\alpha$-specific GAP in single-turnover GTPase assays and as a GAP for $G_i\alpha$ in reconstituted vesicle steady-state assays (92). However, results obtained *in vivo* in transfected cells support the former result suggesting that the higher sensitivity of the steady state assay may detect a non-physiological activity of RGS2 (84). In contrast, RGS4 is promiscuous, demonstrating little preference for different $G_i\alpha$ or $G_q\alpha$ subunits as substrates (9, 10, 80). Although structurally different from the yeast RGS Sst2p (Figures 2 and 3), RGSs 1, 2, 4, 5, 8 and 16 also demonstrate promiscuity acting as GAPs for the yeast Gα-subunit Gpa1p and negatively regulating the pheromone response pathway (11, 30, 83, 102, 133, 155).

Early studies indicated that RGSs were unable to act as GAPs for $G_s\alpha$ subunits (9, 10, 191). However, an RGS (RGS-PX1) was recently discovered that both acts as a GAP for $G_s\alpha$ *in vitro* and inhibits its signaling (measured as a decrease in cAMP production) *in vivo* in HEK293 cells (60). Although its *in vitro* GAP activity was not assessed, a truncated version of RGS3 (RGS3T), containing only the C-terminus, was found to inhibit $G_s\alpha$-mediated adenylyl cyclase activity and increases in intracellular cAMP in transfected baby hamster kidney cells (26). In addition, a recent report indicates that RGS2 reduces *in vivo* cAMP production in odorant-stimulated olfactory epithelium membranes, independently of its GAP activity on the Gα-subunit, by inhibiting the activity of adenylyl cyclase II (169).

Taken together, these results suggest that RGS selectivity may be dependent both on its structure and context of the assay system used to measure its activity. Indeed, studies of RGS function in mammalian cells often demonstrate greater selectivity of RGSs for their G protein targets than have *in vitro* assays of GAP activity or yeast pheromone assays. Although RGS1, RGS2 and RGS16 are structurally similar and have all been shown to interact with $G_q\alpha$ *in vitro* (19, 92, 197), only RGS16 inhibits Platelet activating factor receptor (PAF)/$G_q\alpha$-mediated activation of p38 MAP kinase in CHO cells (202). In the same study, only RGS1 preferentially inhibited PAF/$G_i\alpha$-mediated MAP kinase activation, although both RGS1 and RGS16 bind $G_i\alpha$ *in vitro* (10, 125).

The greater selectivity of RGS proteins for Gα targets in mammalian cells than is seen either in yeast or in vitro, suggests that interactions with other factors may be responsible for determining the signaling pathways that they regulate.

1.3.2.5 The GPCR and RGS Specificity

Recent reports have implicated the GPCR itself as being a critical determinant in modulating the specificity of RGSs for different Gα-subunits (36, 77, 168, 197, 201). Using a yeast-based system, we have recently investigated the role of GPCRs in modulating RGS function (102). Our results demonstrate that, in the presence of the endogenous yeast GPCR Ste2p, RGS1, RGS2, RGS5 and RGS16, differentially inhibit signaling in the rank order of activity RGS1 > RGS16 > RGS2 > RGS5. However, when the human somatostatin receptor 5 (SST_5) is expressed in the same cells, all four RGSs completely abolish SST_5-mediated signaling indicating that these RGSs serve as better GAPs for the yeast Gα-subunit Gpa1p when it is activated through SST_5.

In addition, evidence obtained from gene knockout experiments suggests that individual RGSs may be involved in the regulation of signaling mediated through specific GPCRs. In transgenic mice lacking RGS9-1, both rod cell GTP hydrolysis and the recovery of rod photoreceptors to stimulation were slower than in wild type mice (28). In RGS2-deficient mice, T lymphocyte proliferation and interleukin 2 production were reduced as was the synaptic development and basal electrical activity of CA1 neurons in the hippocampus (132).

There is evidence that the presence of motifs in addition to the RGS domain may allow some RGSs to selectively regulate different GPCRs that use the same G proteins. RGS4 blocks $G_q\alpha$-mediated PLC-β activation by m3 muscarinic cholinergic, cholecystokinin and bombesin receptors in murine pancreatic acinar cells (197). However, RGS4 inhibits muscarinic receptor signaling far more (approximately 30-fold) than cholecystokinin signaling, with bombesin signaling demonstrating an intermediate sensitivity to RGS4. This selectivity is likely not a function of differential $G_q\alpha$-receptor coupling as the same patterns of receptor sensitivity were observed in cells from wild-type mice as well as in cells lacking one

or more classes of $G_q\alpha$ subunits (197). Instead, the selective action of RGS4 for these receptors has been shown to be a function of the RGS domain acting in concert with its N- and C-terminal flanking regions (201).

1.3.2.6 RGSs as Effector Antagonists

As well as acting as GAPs, some RGS proteins behave as effector antagonists. For example, in studies using NG-108 cell membranes, RGS4, RGS2 and GAIP inhibited bradykinin-mediated stimulation of PLCβ in the presence of $G_q\alpha$-GTPγS through the blockade of overlapping binding sites for RGS on the Gα–subunit and PLCβ (80, 85). In addition, RGS3 has been shown to bind to Gβγ *in vitro* and to inhibit its activation of PLCβ both *in vitro* and *in vivo* (163).

Recently, we have shown that RGS1, RGS2, RGS5 and RGS16 are able to inhibit signaling in the presence of a mutant Gα-subunit that is unable to interact with RGSs, suggesting that these RGSs may interact with a downstream component of the signaling pathway (102).

1.3.2.7 Other Domains, other Functions

Some RGSs, such as RGS1, RGS2 and RGS4, consist predominantly of an RGS domain whereas others such as Sst2p, RGS3, RGS12, and RGS14 possess N- and C-terminal extensions that share little homology suggesting that they may perform distinct functions (Figure 2). In many cases, these domains provide targets for localization and may confer regulatory functions by selectively associating RGSs with components of specific signaling pathways.

1.3.2.7.1 PDZ domains and membrane localization

PDZ domains, which bind to C-terminal (A/S)T-X(L-V) motifs in target proteins and organize membrane-bound protein complexes, have been identified in two RGSs (35). RGS12 binds via its N-terminal PDZ domain (Figure 2) to the interleukin-8 receptor CXCR2 (171), and the C-terminal PDZ-binding motif of GAIP (Figure 2) binds to the PDZ domain of GIPC (GAIP Interacting C-terminus), which co-localizes with several transmembrane proteins including the glucose 1 transporter (21, 39). Thus, PDZ domains present in these RGSs may serve to localize them to particular signaling complexes with membrane proteins including GPCRs. The lack of this

domain in other members of the family would therefore preclude them from this interaction, further contributing to the selectivity of response.

1.3.2.7.2 DEP and GGL domains; membrane localization and Gα selectivity

Mammalian RGS6, RGS7, RGS9, RGS11, and EGL-10 from *C. elegans* contain DEP (Dishevelled, EGL-10, Plekstrin) domains at their N-termini that contribute to protein- membrane localization (Figures 2 and 3) (142). These RGSs also contain a domain having homology with the $G\gamma_5$ subunit referred to as GGL (G protein gamma-like) that enables these proteins to bind selectively to $G\beta_5$ subunits (Figure 2) (172). Interestingly, the binding of RGS6, RGS7, and RGS11 to $G\beta_5$ subunits has been shown to confine the GAP activity of these RGSs to the $G_o\alpha$-subunit (144, 172).

In the visual transduction system, RGS9 is a GAP for the Gα subunit of transducin ($G_t\alpha$); this activity being contingent upon the association of $G_t\alpha$ with its effector PDE-γ(117). When complexed with the long splice variant of $G\beta_5$ ($G\beta_5L$), RGS9 has a decreased affinity for free active transducin compared to the $G_t\alpha$-PDE-γ complex (170). Because transducin is the only G protein present in photoreceptors, the ability of RGS9 to discriminate between free and effector-bound forms through its association with $G\beta_5$, confers a degree of specificity to this signaling pathway.

1.3.2.7.3 GoLoco Motifs and $G_{i/o}\alpha$ signaling

RGS12 and RGS14 proteins contain a $G_{i/o}\alpha$-Loco (GoLoco) motif that interacts specifically with $G_i\alpha$, stabilizing its inactive GDP-bound form thus inhibiting its rate of GDP/GTP exchange (Figure 2) (99). Whereas RGS12 and RGS14 act as GAPs for both $G_i\alpha$ and $G_o\alpha$ subunits (89), this GoLoco motif enables them to selectively stabilize only the inactive state of $G_i\alpha$. Thus, the presence of these two independent interaction sites represents a mechanism by which these RGSs could coordinate G protein signaling through selective interaction with Gα subunit types present in the same cell.

1.3.2.7.4 The N-terminus and RGS translocation

There is evidence that the N-termini of certain RGS proteins is involved in their translocation to specific sites following GPCR activation and further, that this

redistribution is crucial for their GAP activity. In the absence of pheromone, the yeast RGS Sst2p resides in the cytoplasm but is translocated to the plasma membrane upon addition of mating factor where it can then act as a GAP for Gpa1p (45). Similarly, RGS3, RGS4, RGS16, and RGS8 are restricted to the cytosol in the absence of Gα activation, but translocate to the plasma membrane upon activation of Gα by agonist, or upon expression of a constitutively active mutant form of Gα (27, 55, 57, 155). Deletion of the N-terminus of RGS3 prevents both its translocation and the attenuation of $G_{q/11}\alpha$-mediated MAP kinase activation (57), and the expression of an N-terminal RGS4 deletion mutant in yeast restricts this protein to the cytoplasm where it can no longer inhibit the yeast pheromone response (173). Similar observations have been made with RGS8, which, without its N-terminus, is unable to localize to the membrane and fails to inhibit yeast pheromone response or to desensitize G protein-gated potassium channels (155). Recently, the N-terminus of RGS2 was shown to target the latter to the plasma membrane in response to activated $G_q\alpha$, and to be required for attenuation of the yeast pheromone response (83).

As is the case for G proteins, lipid modifications of RGS N-termini have been shown to play a role in membrane targeting. The N-terminus of RGS1 contains several *N*-myristoylation motifs that, when myristoylated, anchor RGS1 to the plasma membrane (43). Other RGSs such as GAIP and RGS17 possess a cysteine-rich region. Palmitoylation of these cysteine strings has been proposed to be a mechanism that mediates membrane anchoring and trafficking, and that favors protein-protein interactions (22). Indeed, it has been suggested that palmitoylation of this region in GAIP may be involved in anchoring it to clathrin-coated vesicles (37), and a palmitoylation-deficient RGS16 mutant was shown to be ineffective at attenuating $G_i\alpha$ and $G_q\alpha$ signaling pathways (56).

RGS2, RGS4, RGS5 and RGS16 possess an N-terminal 33 amino acid region rich in basic amino acid residues called the polybasic track (PBT). Amino acids 7 to 32 constitute the core of this domain which, together with hydrophobic residues, forms an amphipathic α-helix that has been shown to be essential for the membrane

association of these RGSs (11, 27, 83, 173). Cysteine palmitoylation of the PBT appears to be dispensable for the attenuation of the yeast pheromone pathway as palmitoylation-deficient mutants of RGS4 and RGS16 are still membrane-anchored and can inhibit pheromone signaling (27, 56). However, the deletion or point mutation of residues within the 33 amino acid PBT region of either protein, impairs both membrane anchorage and attenuation of the yeast pheromone response (27, 173). These results suggest that elements in the PBT other than palmitoylation sites are responsible for membrane localization and regulation of the yeast pheromone response by RGS4 and RGS16. Interestingly, removal of the PBT of RGS4 abolishes its ability to selectively activate $G_q\alpha$-coupled receptors (see section 1.3.2.5) indicating an additional role for this domain in receptor selectivity (197).

1.3.3 The Control of RGS Proteins

1.3.3.1 Transcriptional Control

RGS mRNAs have been found in all tissues and cell types examined to date (41). Certain RGSs are differentially expressed; RET-RGS1 mRNA is restricted to the retina (61), and the mRNA for RGS1 is expressed only in lymphocytes (128). Other RGSs, such as GAIP and RGS3, are ubiquitously expressed (40, 160).

Some RGS mRNAs are constitutively expressed whereas the expression of others is dependent on cellular context and/or stimuli. For example, RGS4 and RGS7 are constitutively expressed in the rat brain (69), RGS7 is constitutively expressed in PC12 cells (136), and RGS4 is constitutively expressed in pancreatic acinar cells (197). A significant number of experiments have demonstrated that the basal cellular level of RGSs such as these serves to limit the responsiveness of the GPCR to agonist (31, 67, 95).

In contrast, the levels of other RGSs mRNAs are altered in response to developmental stimuli. Such is the case for GAIP mRNA, which has been shown to decrease during the differentiation of enterocytes (131), and for RGS8 mRNA, which is induced during the neuronal differentiation of an embryonal carcinoma cell line (154). More recently, microarray analysis and cDNA library screening revealed that

the expression of RGS5 mRNA was repressed during *in vitro* capillary morphogenesis (7).

The expression of the yeast RGS Sst2p is induced in response to mating pheromone and serves in a negative feedback loop to inhibit G protein-mediated signaling (48). The upregulation of many RGSs has also been demonstrated to cause feedback inhibition of mammalian G protein coupled signaling pathways (82, 90, 98, 128, 165, 166, 186, 193). For example, PAF stimulation of a B lymphoma cell line results in enhanced RGS1 mRNA expression, which in turn attenuates MAP kinase signaling in these cells (53).

The levels of some RGS mRNAs are altered in certain pathophysiological conditions. For instance, the expression of RGS1, RGS7 and RGS16 is upregulated in sepsis (133, 134), whereas decreased RGS4 mRNA levels are seen in the prefrontal cortex of patients with schizophrenia (120). Transgenic mice that overexpresses RGS4 develop cardiac hypertrophy owing to a reduced cardiac response to acute volume overload (151). Finally, RGS mRNA levels may be altered in response to medications used to treat pathophysiological conditions. The administration of haloperidol, commonly used in the treatment of psychiatric disorders, has been shown to cause an increase in the level of RGS2 mRNA in rat striatum (148).

1.3.3.2 RGS Splice Variants

The existence of splice variants of several RGSs may confer a further degree of control upon G protein signaling. A truncated version of RGS3 (RGS3T) containing only the C-terminus has been described which, unlike the native protein, inhibits $G_s\alpha$-mediated adenylyl cyclase activity (26). The RGS9 gene gives rise to two alternatively spliced mRNAs with different C-termini. One splice variant, RGS9-1, is expressed in the retina and acts as a transducin-specific GAP (79). The other, RGS9-2, is 191 amino acids longer and is expressed in the brain where it negatively regulates μ-opioid receptor-$G_{i/o}\alpha$-coupled signaling (146).

Recently, it was demonstrated that RGSZ1 and Ret-RGS, expressed in brain and retina respectively, are splice variants of the RGS20 gene (6). Because both proteins

are $G_z\alpha$ GAPs, differential splicing of one gene accounts for the tissue-specific expression of RGSs that act on the same Gα-subunit.

1.3.3.3 Posttranslational Modifications

Several studies have demonstrated that the phosphorylation of RGS proteins can positively or negatively affect their activity or prevent their degradation. Phosphorylation of GAIP by MAP kinase in human intestinal HT-29 cells stimulates its GAP activity towards $G_{i3}\alpha$ (130). Conversely, the phosphorylation of a conserved serine in RGS3 and RGS7 allows binding of 14-3-3 proteins resulting in decreased *in vitro* and *in vivo* GAP activity towards $G_{i1}\alpha$ (8). Epidermal growth factor receptor-mediated tyrosine phosphorylation of RGS16 enhances its inhibition of $G_i\alpha$-dependent MAP kinase activation (44). Phosphorylation of RGS10 by cAMP-dependent PKA has no effect on the GAP activity of this RGS *per se*, but rather causes its translocation from the plasma membrane to the nucleus (23). Finally, PKC-mediated phosphorylation of RGS2 causes an inhibition of its GAP activity towards $G_{11}\alpha$both *in vitro* and in COS7 cells (36).

Interestingly, the phosphorylation of the yeast RGS Sst2p by the yeast MAP kinase Fus3p has no affect on its ability to attenuate the pheromone response, but instead appears to decrease its rate of degradation presumably by stabilizing its proline,glutamine,serine,threonine-rich (PEST) domain(66). Sst2p is also endoproteolytically processed to produce N- and C-terminal fragments that are unable to inhibit pheromone signaling (88). That this process requires an intact signaling pathway suggests that it may represent a mechanism of negative feedback control on Sst2p function

The posttranslational lipid modifications that have been postulated to play a role in the membrane localization and activity of certain RGS family members were mentioned earlier (see section 1.3.2.7.4).

1.3.4 The Subfamily of Small RGSs

1.3.4.1 Structure

The small RGSs constitute a subgroup of low molecular weight RGSs that includes RGS1, RGS2, RGS4, RGS5, RGS8, and RGS16. All members of this group have short N- and C-terminal domains lacking defined functional motifs present in other RGSs (Figure 2). One common feature shared by several members of this family is an amphipathic α-helical domain, formed by a PBT together with a cluster of hydrophobic amino acids which, as mentioned earlier, contain residues that appear to be necessary for membrane localization and for the ability to attenuate yeast pheromone signaling mediated by RGS2, RGS4, RGS5 and RGS16 (11, 27, 83).

1.3.4.2 Mutational Analysis of RGS4 Reveals Functional Domains

As mentioned earlier, RGS4 can selectively inhibit signaling via the $G_q\alpha$-coupled muscarinic cholinergic, cholecystokinin and bombesin in a selective manner dependent on the presence of the RGS box and its N- and C-terminal flanking regions (197). Deletional analysis has shown that the RGS box of RGS4 acts as a GAP *in vitro* and is able to inhibit $G_q\alpha$-initiated, PLC-β-mediated calcium release when expressed in pancreatic acinar cells (201). However, the activity of the RGS box was not receptor-selective or as potent as that seen with native RGS4. When the C-terminal RGS box-flanking sequence of RGS4 was deleted, the potency of $G_q\alpha$ inhibition was reduced approximately 100-fold. Whereas an N-terminal peptide consisting of amino acids 1 to 33 of RGS4 was able to inhibit $G_q\alpha$-mediated calcium release with the same potency and receptor selectivity demonstrated for RGS4, deletion of the N-terminal sequence flanking the RGS box reduced inhibitory potency and receptor selectivity to that of the RGS box alone. These results suggest that the N-terminal domain of RGS4 confers receptor selectivity and that both it and the C-terminal flanking region act synergistically with the RGS box to increase inhibitory potency.

The inhibition of full-length RGS4, but not that of the N-terminal peptide, was overcome by the addition of activated $G_q\alpha$-subunits ($G_q\alpha$-GTPγS) suggesting that the

latter may act as an effector antagonist, blocking the access of the Gα-subunit to its effector PLCβ (201).

1.3.4.3 RGS1

The transcript for RGS1 was first identified in a subtractive cDNA screen of phorbol ester-treated human B lymphocytes (90). RGS1 impairs PAF-mediated increases in intracellular Ca^{++} in human B lymphocytes as well as the migration of human and murine B cells in response to the chemokine SDF-1 (stromal-derived factor-1)(122, 147). RGS1 is constitutively expressed in human monocytes where it is membrane localized and acts as a $G_i\alpha$ and $G_q\alpha$ GAP, thereby inhibiting signaling via the chemoattractant receptors C5a, fMLP and LTB4 (43).

Like many other members of the small RGS subgroup, RGS1 is able to inhibit the pheromone pathway in yeast lacking Sst2p (133). Pheromone-induced activation of the yeast MAP kinase cascade depends on the release of Ste4p-Ste18p (Gβγ) from the Gα-subunit Gpa1p (50). *In vitro* studies using recombinant proteins have illustrated that RGS1 does not bind mammalian Gβγ subunits (191), suggesting that it is unlikely that RGS1 directly prevents the interaction of Gβγ with downstream effectors. In addition, although both Sst2p and RGS4, are able to inhibit the yeast pheromone response initiated at the receptor, they are unable to do so when signaling is initiated by the addition of Ste4p-Ste18p (53). Taken together, these results suggest that the inhibition of pheromone signaling in the presence of these RGSs is most likely due to their GAP activity towards the Gα-subunit. Because they augment GTP/GDP exchange, RGSs enhance the production of Gα-GDP, which has a high affinity for Gβγ, thereby favoring the reassociation of the heterotrimer and reducing the amount of free Gβγ available for interaction with downstream effectors.

1.4 Objectives and Results of This Study

1.4.1 Introduction

Although many studies have investigated the contributions made by the N- and C-terminal domain regions of other small RGSs to their GAP activity, little is known

about what contributions these regions make to the activity of RGS1. In Chapter 2, we describe an extensive mutagenesis-based approach aimed at elucidating which domains and/or residues are critical to the activity of RGS1. In addition, we have expressed RGS1 in the context of a yeast G protein-coupled pheromone receptor pathway lacking receptor and/or G protein α-subunits in order to determine whether interactions of RGS1 other than those involving the Gα-subunit might contribute to its inhibition of this pathway. Although evidence obtained *in vitro* (see section 1.3.4.3) suggests that RGS1 does not bind Gβγ, we provide evidence that RGS1 is able to inhibit signaling mediated by this subunit *in vivo*.

1.4.2 Point and Deletion Mutations of RGS1

Initially, we construct point mutants in which three conserved residues within the RGS domain of RGS1 were individually mutated. We then expressed these mutants in a yeast strain lacking Sst2p, and assessed their affects on GPCR signaling mediated by the pheromone receptor using the halo assay (53). We show that the mutation of two conserved residues (F94A and Y124F) has no affect on the ability of RGS1 to inhibit pheromone signaling, whereas the mutation N137A greatly diminishes its activity establishing a role for this residue in RGS1 function.

Subsequently, we produced mutants containing the N-terminus of RGS1, the RGS box, and all but the C-terminal 10 residues of RGS1, expressed these proteins as GFP-fusions in yeast lacking Sst2p and assessed their function by halo assay. Interestingly, the expression of either the N-terminus or RGS box alone yielded proteins that were able to complement Sst2p, albeit to a lesser extent than wild type in the halo assay, suggesting that N-terminus as well as the RGS box of this protein are important to its function. Indeed, when expressed together, the two mutants produced an inhibition of pheromone response that more closely resembled wild type, consistent with results seen in mutagenesis studies of RGS4 suggesting that these two domains work in concert to produce the affects on signaling demonstrated by native RGS1 (201). In contrast, the expression of RGS1 lacking the C-terminal 10 residues had no effect on pheromone response suggesting that these residues are not required for the effects on signaling assayed in our system.

1.4.3 Mutagenesis by Conservative Segment Exchange

In order to determine which amino acids within the N-terminus of RGS1 might be important for its activity, we sequentially mutated the entire N-terminal region (amino acids 1 to 75) 3 or 4 residues at a time using Conservative segment Exchange (CSE)(87). This method consists of exchanging 3 or 4 amino acids at a time for residues having similar properties. Glycine and proline residues were not changed due to the possible influence that their replacement might have on tertiary protein structure. The conservative nature of these amino acid exchanges is expected to reveal the importance of groups of residues to the function of RGS1. These mutants were again expressed as GFP-fusions in yeast, and their function was assessed by halo assay.

With the exception of two, all 20 RGS1 CSE mutants were expressed at similar levels. One mutant was undetectable by immunoblot and had activity resembling that of the vector control, whereas another exhibited a slight increase in expression and was more active than wild type hRGS1. It is possible that in the latter cases, the mutations have affected negatively and positively, respectively, the stability of this protein *in vivo*. Importantly, we demonstrate that it is the overall structure of RGS1, rather than specific residues or motifs, that is important to its function.

1.4.4 Expression of RGS1 in a GPCR Pathway Lacking Receptor and /or Gα-subunit

Having determined that the N-terminal domain of RGS1 is important for its function, we undertook to express wild type RGS1, as well as mutants containing either its N-terminus or RGS box, in the context of a GPCR pathway lacking receptor and/or Gα-subunit. These experiments allowed us to investigate whether or not RGS1 could directly inhibit signaling mediated by the Gβγ-subunit and further, which domains might be required for this activity. We used two yeast strains both lacking the *SST2* gene as well as those encoding the Gα-subunit (*GPA1*) and *FAR1* (responsible for mediating the growth-inhibitory effect of pheromone) and containing an integrated *FUS1*-LacZ reporter gene. One strain also lacked the gene encoding the

yeast pheromone receptor *STE2*. Cells having receptor were then transformed with constructs expressing vector, RGS1, or Gpa1p, or were cotransformed with RGS1 and Gpa1p, whereas cells lacking the receptor were transformed with these constructs as well as those expressing the RGS1 N-terminus and RGS box. The resulting transformants were then grown overnight in galactose-containing medium in the absence of agonist and assayed for basal β-galactosidase activity as a measure of Gβγ-mediated signaling and induction of *FUS1*-LacZ transcription.

In cells having receptor, the expression of Gpa1p decreased basal β–galactosidase activity 2-fold, whereas full-length RGS1 was unable to inhibit basal signaling. When expressed together however, these proteins caused a decrease in signaling not observed with either protein alone. These results are consistent with a model wherein Gpa1p acts to sequester Gβγ thus inhibiting its signaling, while RGS augments the activity of Gpa1p and assists in maintaining the G protein in its inactive, heterotrimeric state.

In cells lacking receptor, basal β-galactosidase activity was increased 2-fold in the presence of vector alone which is not unexpected since it has been reported that the yeast receptor Ste2p is able to inhibit G protein activation in the absence of ligand (16). Interestingly, contrary to the results obtained in receptor-expressing cells, in the absence of receptor, the expression of either Gpa1p or full-length RGS1 alone resulted in a 3-fold decrease in β-galactosidase production. As in cells having receptor, the coexpression of Gpa1p and full-length RGS1 decreased signaling further. In the absence of Gpa1p, the expression of either the N-terminus or RGS box of RGS1 resulted in only modest decreases in β–galactosidase activity.

These results indicate that RGS1 is capable of not only acting as a GAP for the Gα-subunit but as an effector antagonist as well, further adding to the documentation of this role of RGSs discussed in section 1.3.2.6. That RGS1 is capable of inhibiting Gβγ-mediated signaling only in the absence of the receptor suggests that the receptor, when present, may act to inhibit the RGS1-Gβγ interaction. In addition, these data suggest that an intact RGS1 is required to inhibit signaling via Gβγ.

1.5 Figures

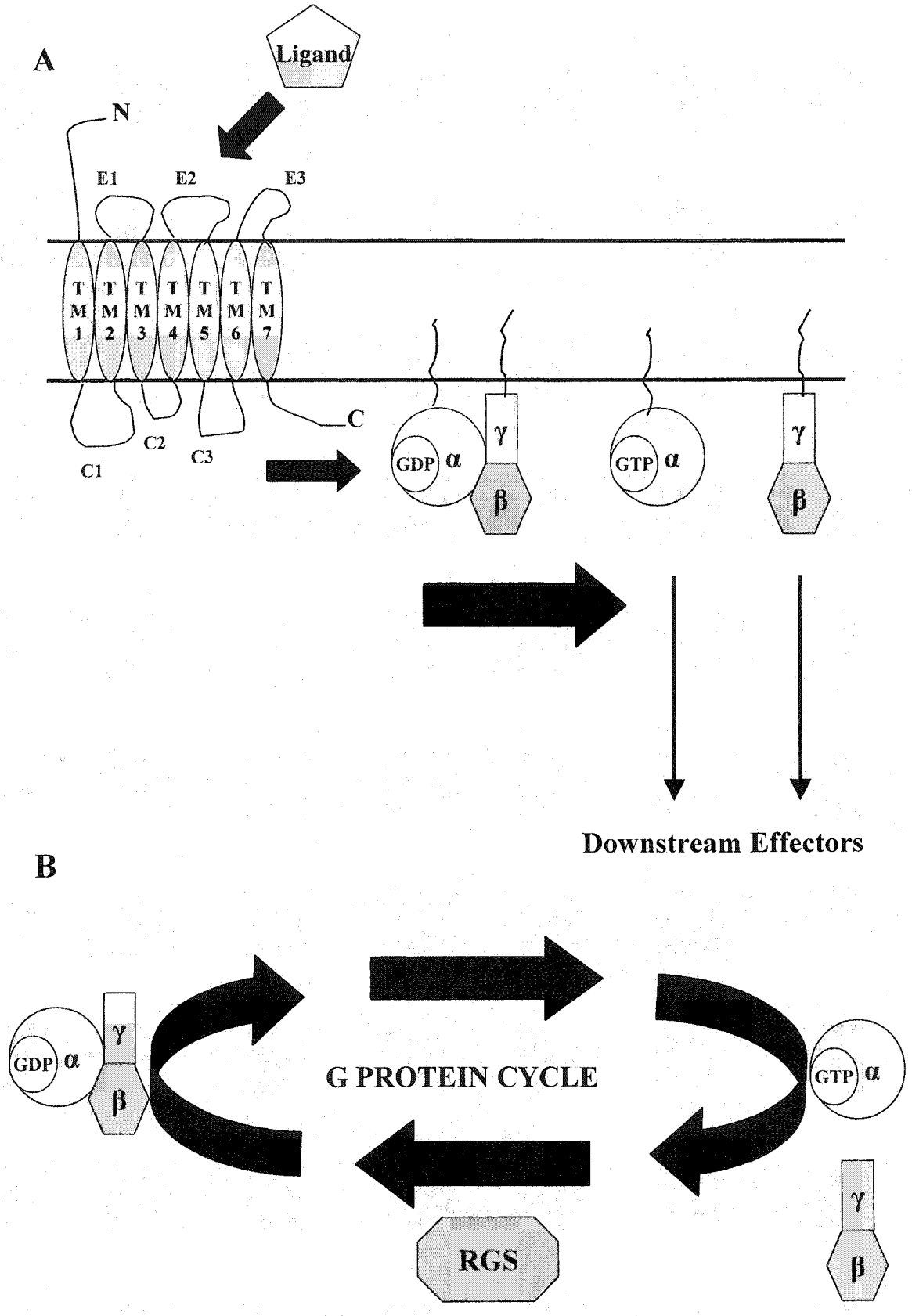
1.5.1 Figure 1a) GPCR Structure, Activation and Coupling to Heterotrimeric G Protein

The GPCR consists of seven transmembrane domains (TM1-7), three extracellular loops (**E1-3**), three cytoplasmic loops (**C1-3**), and N-terminal extracellular (**N**) and C-terminal (**C**) cytoplasmic domains. 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 Gα-subunit and activation of the G protein. Activation results in the dissociation of the G protein into constituent Gα- and Gβγ-subunits which are then able to activate downstream effectors (see text).

Figure 1b) The G Protein Cycle

Activation of the heterotrimeric G protein as a result of ligand binding to the GPCR results in GDP/GTP exchange, dissociation into Gα- and Gβγ-subunits and activation of downstream effectors. Deactivation of G protein-mediated signaling occurs predominantly via the inherent GTPase activity of the Gα-subunit, which causes the exchange of GTP for GDP, and subsequent reassociation of the G protein heterotrimer. The RGS protein family contributes to G protein deactivation by stabilizing the GTP/GDP transition state thus favoring heterotrimer reassociation (see text)

(Adapted from Bockaert et al. 1999 *EMBO J* 18:1724)

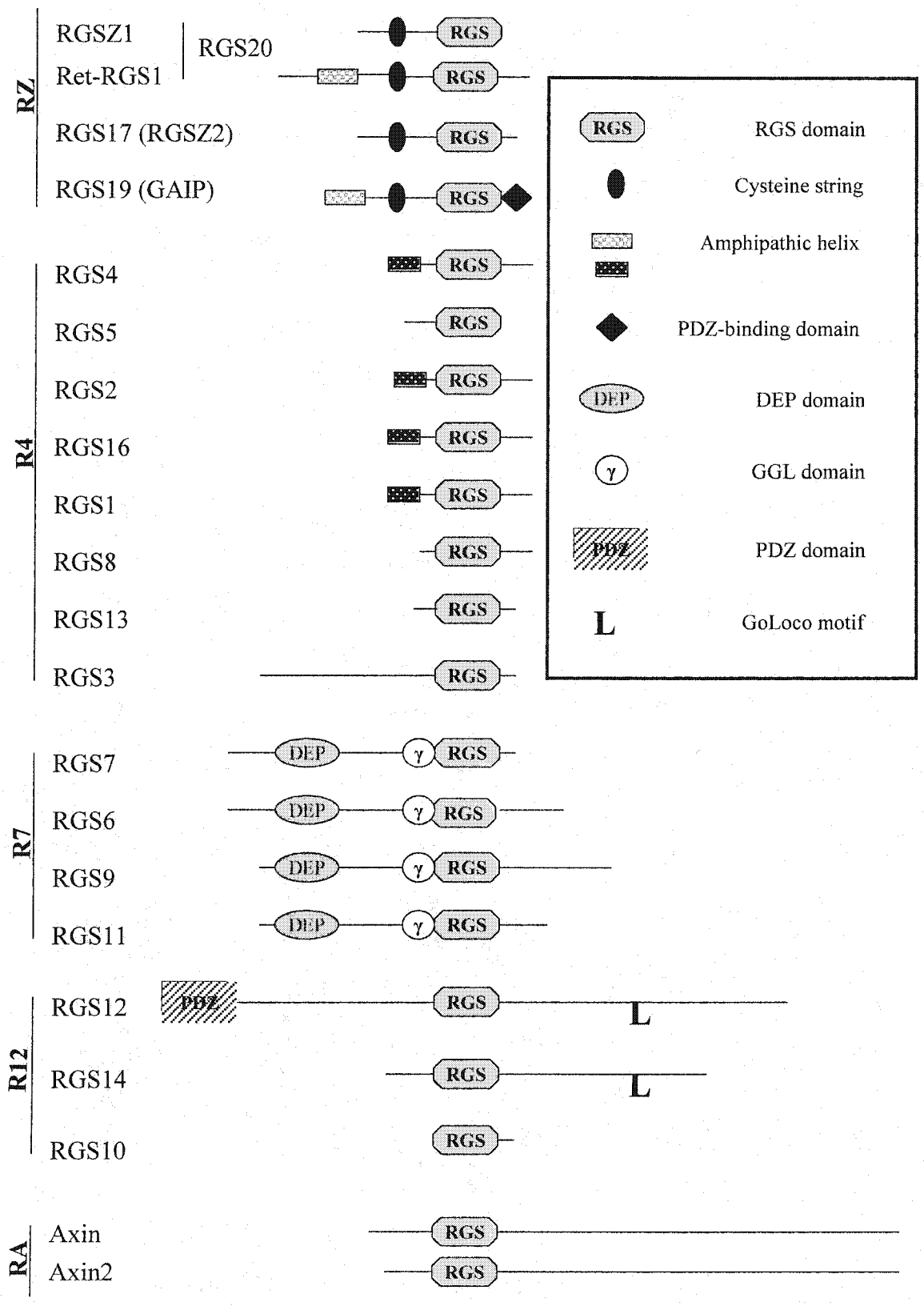

A
Ligand
N
E1
E2
E3
TM1
TM2
TM3
TM4
TM5
TM6
TM7
C
C1
C2
C3
GDP
α
γ
β
GTP
α
γ
β
Downstream Effectors
B
GDP
α
γ
β
G PROTEIN CYCLE
GTP
α
γ
β
RGS

1.5.3 Figure 2: The Mammalian RGS Family

Sequence identities within the RGS domain establishes five mammalian subfamilies: RZ, R4, R7, R12, and RA. Sequences shown are human except RGS8 (rat), RGSZ2 (mouse), and RET-RGS1 (bovine). Members of subfamilies are also homologous in regions flanking the RGS domain shown as labeled motifs within each protein's structure (see legend). Functions of these domains are discussed in the text. Abbreviations: PDZ, PSD95/DLg/Z01 homolgy; DEP, Dishevelled, EGL-10, Pleckstrin; GGL, Gγ-like; GoLoco, $G_{i/o}\alpha$-Loco.

(Adapted from: Wilkie et al. 2002. *Genomics* 79:178)

Mammalian RGSs

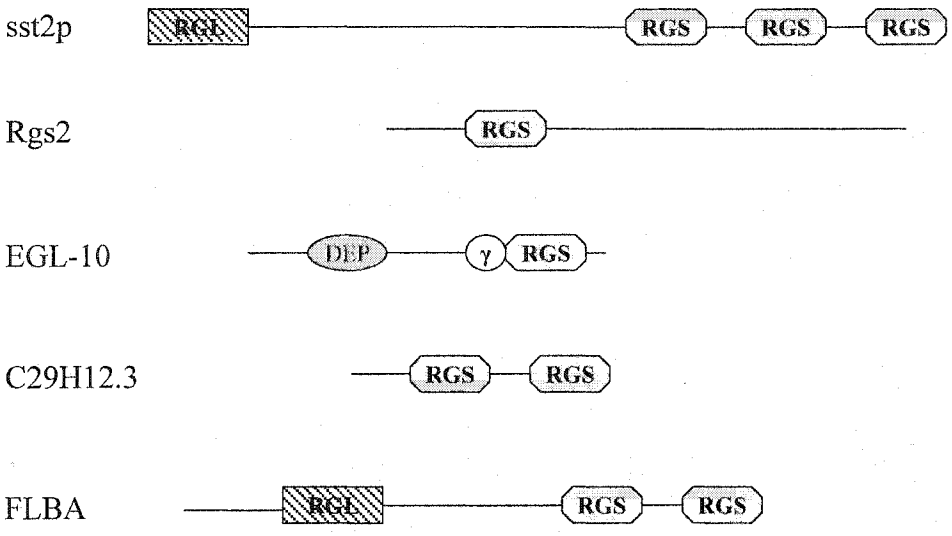


1.5.4 Figure 3: Non-mammalian RGSs

Five non-mammalian RGSs are shown: Sst2p and Rgs2 (*S. cerevisiae*), EGL-10 and C29H12.3 (*C. elegans*), and FLBA (*A. nidulans*). Many other *C. elegans* proteins exist but are not shown, and no *Drosophila* proteins are shown. Domains discussed in the text are shown as labeled motifs within the protein's structure (see legend). Abbreviations: DEP, Dishevelled, EGL-10, Pleckstrin; GGL, Gγ-like.

(Adapted from: De Vries et al. 2000. *Annu. Rev. Pharmacol. Toxicol.* 40:238, Thevelein et al. 1999. *EMBO J* 18:5579, Koelle and Horvitz 1996. *Cell* 84:120)

Non-mammalian RGSs



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Chapter 2: The N-terminal Non-RGS domain of Human Regulator of G-protein Signaling 1 Contributes to its Ability to Inhibit Pheromone Receptor Signaling in Yeast.

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2.1 Summary

Regulators of G protein signaling (RGS) are a family of proteins that interact with G-proteins to negatively regulate G protein-coupled receptor (GPCR) signaling. In addition to a conserved core domain that is necessary and sufficient for their GTPase activating protein (GAP) activity, RGSs possess N- and C- terminal motifs that confer distinct functional differences. In order to identify the role of the non-RGS region of human RGS1, we have characterized a series of fusions between RGS1 and GFP in a yeast mutant lacking the RGS-containing *SST2* gene. Using halo assays, we demonstrate that an RGS1-GFP fusion inhibits GPCR signaling in yeast while GFP fusions containing either the N-terminus $RGS1^{1-75}$,or the sequence containing the RGS box $RGS1^{76-197}$ produce proteins that retain partial RGS1 activity. RGS1 function could be largely restored by co-expressing both $RGS1^{1-75}$ and $RGS1^{76-197}$ GFP fusions. These results suggest that the sequences of the N-terminal and the RGS box of RGS1 function in concert to inhibit signaling. Analysis of a series of mutants spanning the entire N-terminal non-RGS region of RGS1 produced by Conservative Segment Exchange mutagenesis showed little loss of function in yeast. This suggests that the overall structure of the N-terminal region of RGS1 rather than specific motifs or residues is required for its function. Using yeast strains harboring a Gβγ-responsive *FUS1-LacZ* reporter gene, we also demonstrate that RGS1 enhances the ability of the *GPA1*-encoded

Gα-protein to inhibit basal signaling in the absence of receptor agonist. Further, RGS1 can inhibit basal Gβγ-mediated signaling, but only in the absence of a functional *STE2*- encoded GPCR. Taken together, our results suggest that RGS1 is a multifunctional protein involved in regulating multiple aspects of GPCR signaling.

2.2 Introduction

Regulators of G-protein Signaling (RGS) serve as GTPase Accelerating Proteins (GAPs) for the Gα-subunit of heterotrimeric G-proteins (1-4). As such, RGSs regulate G-Protein Coupled Receptor (GPCR) signaling by limiting the lifetime of the receptor-activated GTP-bound form of Gα-proteins (5). Indeed, it follows that overexpression of a number of RGSs inhibits GPCR mediated signaling (6-15). Induction of RGS gene expression in response to a variety of stimuli including GPCR agonists, DNA damage, and cytokines such as Tumor Necrosis Factor α (TNFα), indicates that they are dynamic regulators of GPCR signaling (8,10,16-22). Gene knock-out experiments demonstrate that RGSs also serve a housekeeping function to limit basal GPCR mediated responses (6,8,9,23). It is therefore not surprising that alterations in the levels of RGSs have been implicated in mediating altered GPCR responses in a number of pathophysiological conditions (24-29). These are just a few reasons that the development of specific inhibitors of RGSs is actively being pursued (28,29).

RGS refers to a conserved 120-130 amino acid domain that has been shown to be sufficient for the GAP activity of RGSs (30-33). *In vitro* biochemical assays as well as *in vivo* experiments have shown that whereas RGSs can interact with all classes of Gα-proteins, individual RGSs appear to have preferences for distinct classes of Gα-subunits (3,11,34-36). The non-RGS regions of RGS proteins diverge significantly in size and sequence (37,38). On this basis, RGS proteins have been classified into a number of different sub-groups (37,39,40). RGSs can also be classified into 2 groups based on their overall size. Many of the large RGS-containing proteins have identifiable functional sequence motifs such as PDZ, GGL and DEP domains that

confer specific functions. The small RGSs (ca. 200 residues), including RGS1, RGS2, RGS4, RGS5, and RGS16 have little in the way of defined functional motifs with the exception of an amphipathic α-helix and an RGS domain. Nevertheless, many of the small RGSs has been shown to be involved in interacting with other proteins (15,41-47). For example, RGS4 has been shown to be significantly more potent at inhibiting carbachol-mediated responses than those mediated by bombesin and cholecystokinin even though all three agonists activate the same G-protein (45) and this selectivity has been shown to require its N-terminal non-RGS region. The existence of a trimeric complex involving the receptor, the Gα-protein and the RGS could explain the observed selectivity of this small RGS (48). In addition, there is increasing evidence that non-RGS sequences directly interact and inhibit some effector enzymes. For example, the N-terminal non-RGS portion of RGS2 appears to be required for its ability to inhibit adenylyl cyclase (15).

The pheromone response pathway of the yeast *Saccharomyces cerevisiae* is an extensively characterized GPCR-mediated signaling cascade that consists of a single GPCR (Ste2p), Gα-protein (Gpa1p), Gγ-protein (Ste4p), Gβ-protein (Ste18p) and an RGS (Sst2p) (49). The relative simplicity of this pathway is in stark contrast to those seen in mammals where most cells express multiple GPCRs, G-proteins and RGSs. For example, in the heart at least 13 different GPCRs, 9 Gα-subunits and at least 10 different RGSs are expressed (50-53). There are a number of $G_{s\alpha}$-coupled GPCRs such as the β_1- and β_2 adrenergic receptors as well as $G_{q\alpha}$-coupled GPCRs for endothelin and Angiotensin II that have positive ionotropic effects, whereas some $G_{i\alpha}$-coupled GPCRs such as muscarinic receptors mediate negative ionotropic effects. Integration of the myriad of GPCR cardiac signaling events is a complex phenomenon that remains poorly understood (54). The fact that mammalian genes encoding different components of the GPCR signaling pathway can be functionally expressed in yeast makes this organism an attractive model organism in which to delineate both the structure and function of GPCRs and RGSs. As well, the relative simplicity of this GPCR signaling pathway allows us to study individual RGSs in isolation in an *in vivo* system (10,46,55-59).

RGS1 is a small, 197 residue protein that was first identified in activated B cells where it may function to regulate their directed migration (10,18). Although RGS1 expression has a limited tissue distribution, it has been shown to be inducible in response to TNFα and to be up-regulated in the heart of a porcine model of sepsis (18,25). Although the N-terminal region of RGS1 does have an amphipatic helix that is present in many small RGSs, the function of its N-terminus remains largely uncharacterized. We have previously shown that RGS1 can functionally inhibit GPCR signaling in yeast. Here we have used yeast to characterize a series of mutants in order to determine the function of the N-terminal region of RGS1. We demonstrate that this region is required for full RGS1 activity, that it can independently inhibit signaling in the absence of the RGS box, and that its overall structure is important to its function. Further, we demonstrate that only an intact RGS1 protein can inhibit Gβγ-mediated signaling in the absence of the yeast pheromone receptor.

2.3 Experimental Procedures

2.3.1 Plasmids

Plasmids p425GAL1-EGFP and p426GAL1-EGFP were constructed to facilitate the expression of EGFP (red-shifted variant of GFP) fusions in yeast. These plasmids were constructed by first amplifying by PCR a 719 bp fragment containing the coding sequence of EGFP using pEGFP-N3 (Clontech) as template, Vent Polymerase and the following forward 5'-CTAAGCTTCTGCAGTCGACGGTACCGCGG-3' and reverse 5'-CTCTCGAGGCGGCCGCTTTACTTGTACAGCTC-3' oligonucleotides as previously described (25,46). The primers were designed so that the EGFP PCR product contained unique 5'*Hin*dIII site and 3'*Xho*I sites. The blunt ended PCR product was first cloned into the *Eco*RV site of pBluescript (Stratagene) for analysis and then subcloned as a *Hin*dIII-*Xho*I fragment into the polylinker region of the yeast expression plasmids p425GAL1 and P426GAL1 (60). A multiple cloning site (MCS) at the 5' end of EGFP that contains a number of unique restriction endonuclease sites serves to facilitate the construction of gene fusions in p425GAL1-EGFP and p426GAL1-EGFP, and the presence of the *GAL1* promoter allows the expression of galactose-inducible EGFP fusions. The presence of separate auxotrophic markers, *LEU2* for p425GAL1-EGFP and *URA3* for p426GAL-EGFP allowed for the selection of both plasmids in the same yeast cell (61).

The control vectors p425GAL1-ATG-EGFP and p426GAL1-ATG-EGFP which serve to express EGFP alone, were constructed as described above except that the sense primer 5'-CAAGCTT[CGCAAACA](ATG)TCTGCAGTCGACGGTACCGCGGGCCCGGA-3') contained a yeast Kozak [CGCAAACA] sequence followed by a translational initiation codon (ATG) in the same translational reading frame as EGFP in order to ensure the efficient translation of these mRNAs in yeast. In order to generate a human (h) RGS1-EGFP gene fusion, the structural portion of hRGS1, including its translational initiation codon and its yeast Kozak sequence, but lacking the translational stop codon, was amplified by PCR using p423GAL1-hRGS1 (25) as a

template with the sense 5'ACACTAGTCAAACAATGCCAGGAATGTTCTTCTCTGCTAAC-3', and antisense 5'-ACAAGCTTGCTTTAGGCTAGCCTGCAG-3' oligonucleotide primers. Similarly, the DNA fragment encoding residues $RGS1^{1-68}$ was amplified by PCR using the sense 5'ACACTAGTCAAACAATGCCAGGAATGTTCTTCTCTGCTAAC-3' and antisense 5'-CACGAAGCTTGCATTACTTCAGCAGCAGAAAGTACATC-3' oligonucleotides while the DNA encoding the RGS box, $RGS1^{68-197}$, was amplified using sense 5'-ACACTAGTCAAACAATGCAATGGTCTCAATCTCTGGAAAAACTTCTT-3' and antisense 5'-ACAAGCTTGCTTTAGGCTAGCCTGCAG-3' primers. Unique 5'*Spe*I and 3'*Hin*dIII restriction endonuclease sites were introduced at the ends of the RGS1 PCR products. These sites were used to clone the PCR products into the unique *Spe*I and *Hin*dIII restriction endonuclease sites in p425GAL1-EGFP. The nucleotide sequences of the RGS1-EGFP, $RGS1^{1-68}$ (N-Term-GFP) and $RGS1^{68-197}$ (Box-GFP) gene fusion predicts that both portions are in the correct translational reading frame to generate GFP protein fusions.

The previously described plasmid p414GPA1 was used for the expression of *GPA1* (46). The *GPA1* gene is expressed under the control of its own promoter and mimics the endogenous gene centromeric plasmid (57).

2.3.2 Conservative Segment Exchange Mutagenesis of the N-terminus of RGS1

Conservative Segment Exchange (CSE) mutagenesis of RGS1 was performed using the technique of overlap extension PCR essentially as previously described (62,63). Mutagenic oligonucleotides contained silent restriction sites that served to identify the different mutants as well as unique 5'*Spe*I and 3' *Hin*dIII restriction endonuclease sites which were used to clone the mutants in frame at the 5' end of EGFP in p426GAL-EGFP for expression in yeast.

2.3.3 Yeast

2.3.3.1 Strains and Media

The *Saccharomyces cerevisiae* strain BY4741-6055 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sst2Δ::G418*^{R}) used for the halo assays was obtained from Research Genetics. Strains MMY9 (*MATa his3 leu2 trp1 ura3 can1 gpa1Δ::ADE2 sst2Δ::ura3Δ far1Δ::ura3Δ fus1::FUS1-HIS3 LEU2::FUS1-LacZ*) and RP2 (*MATa his3 leu2 trp1 ura3 can1 gpa1Δ::ADE2 sst2Δ::ura3Δ far1Δ::ura3Δ fus1::FUS1-HIS3 LEU2::FUS1-LacZ ste2Δ::G418*^{R}) were used to assess the ability of RGS1 to inhibit Gβγ-mediated signaling (46,57). Yeast cells were routinely grown on synthetic minimal media consisting of Yeast Nitrogen Base (YNB) containing 2% glucose supplemented with the appropriate amino acids and bases (61). Plasmids were introduced into yeast using lithium chloride as described (64). The resultant transformants were selected and maintained by the omission of the appropriate supplements from the growth media (leucine for p425GAL1, uracil for p426GAL1 and tryptophan for p414 vetors) (60). Glucose was replaced with 2% galactose and 2% raffinose to induce *GAL1* dependent expression.

2.3.3.2 Halo Assays

Halo assays were performed essentially as previously described (25). Briefly, cultures of the different transformants were grown to saturation in YNB media supplemented with the appropriate nutrients and 2% glucose. An aliquot of the cultures (200 µl) was diluted in sterile ddH_2O (1.8 ml) and 2 ml of pre-warmed (55^0C) 1% agar was added. The cells were then quickly mixed and plated onto YNB nutrient agar plates supplemented with 2% galactose and 2% raffinose. Sterile filter disks containing α-factor (1000 or 3000 pmoles) were then placed onto the top agar and the plates were incubated at 30^0C for 3 to 4 days.

2.3.3.3 β-galactosidase Activity

β-galactosidase activity was determined using permeabilized cells and *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate (61). The amount of ONPG cleaved

was determined spectrophometrically (OD_{420}) and activity was normalized to culture density (OD_{600}).

2.3.4 Western Blot Analysis

Aliquots of exponentially growing cultures were harvested, washed in sterile ddH_2O and stored at $^{-}80^{0}C$. Soluble protein was subsequently extracted by treating an equal number of cells with NaOH (0.1M, $25^{0}C$, 3 min) followed by heating (3 min at $95^{0}C$) in SDS-PAGE loading buffer (0.06 M Tris-HCl pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol and 0.0025% bromophenol blue) (65). Protein samples were separated by SDS-PAGE (12% acrylamide; 37.5 acrylamide: 1 N,N'-methylenbisacrylamide) and transferred to Nitrocellulose membrane (6). Equal loading was confirmed by visualizing an identical gel stained with coomassie blue (46). The membrane was challenged with a rabbit anti-GFP antisera or anti-goat anti-RGS1 antisera (Santa Cruz Biotechnology) for 2h at room temperature in a Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20. After washing, the blot was incubated with an HRP-conjugated goat anti-rabbit IgG antisera for 1h at room temperature in 5% milk and 0.1% Tween 20 in Tris-buffered saline. Signals were subsequently developed using chemiluminescent luminol reagent followed by exposure to X-ray film (Kodak X-Omat) as described (Santa Cruz Biotechnology).

2.4 Results and Discussion

2.4.1 The N-terminal non-RGS portion is required for full RGS1 activity

The yeast RGS- containing protein Sst2p regulates signaling from the *STE2* encoded α-factor receptor by modulating the GTPase activity of Gpa1p (49,66). Since mammalian RGSs can functionally replace the *SST2* gene, their expression in yeast serves as a relatively simple and efficient bioassay for the analysis of RGS structure and function (10,25,46,58,67). Although the RGS box of RGS1 is known to have GAP activity, the potential role of its non-RGS sequences remains unknown.

In order to examine the role of the non-RGS N-terminal region as well as the RGS box, we constructed and expressed a series of RGS1-GFP fusions in yeast (Fig. 1A). One fusion, RGS1-GFP, contains all 197 residues of RGS1. The second fusion, N-Term-GFP, contains the N-terminal non-RGS 75 residues of RGS1 while the third fusion, Box-GFP, contains the RGS box of RGS1 consisting of residues 76 to 197. Construct ATG-GFP serves as a control as it expresses GFP alone. All fusions as well as the control ATG-GFP were individually cloned into the multicopy vector p425GAL1, placing their expression under the control of the galactose inducible yeast promoter *GAL1* (60). In addition, Box-GFP was also cloned into p426GAL1 in order to be able to express both it and the N-Term-GFP in the same yeast cell. This is possible given that p425GAL1 has the *LEU2* gene as a selectable marker while the *URA3* gene is the selectable marker for p426GAL1. The different plasmids were introduced into the yeast strain BY4741-6055 and the halo assay was used to assess the ability of the GFP fusions to inhibit GPCR signaling. This assay is based on the fact that stimulation of the yeast GPCR Ste2p with its agonist α-factor leads to the inhibition of growth (8,25). Essentially, an aliquot of a freshly saturated culture, grown in glucose, is spread onto a nutrient agar plate containing galactose. A filter disk containing 1000 pmoles of α-factor is deposited on the surface of the agar and the cells are then allowed to grow for the 2 to 3 days. The degree of growth inhibition around the filter disk, for a given dose of agonist is proportional the level

of receptor activation. A large zone of growth inhibition was observed with control cells that express GFP (ATG-GFP) alone (Fig. 1B). In contrast, cells expressing RGS1-GFP were completely resistant to the growth inhibitory effects of α-factor. These results are similar to what has been previously observed with RGS1 alone, and indicate that GFP either alone or fused to RGS1 does not interfere with signaling or RGS function (10,25,67). Although cells expressing N-Term-GFP were sensitive to the growth inhibitory effects of α-factor, they were not as sensitive as control cells expressing GFP alone. Expression of the Box-GFP fusion conferred a much greater resistance to α-factor than the N-Term-GFP. Even though the RGS1 box alone was efficient at inhibiting Ste2p signaling, it was clearly less efficient than the intact RGS1 protein. Cells expressing both N-Term-GFP and Box-GFP fusions were more resistant to α-factor than cells expressing either fusion alone. Western blot analysis using anti-GFP antisera indicated that all 3 GFP fusions accumulated at similar levels in exponentially growing cells (Fig. 1C). These results suggest that the N-terminus plays a role in the ability of RGS1 to inhibit GPCR signaling and further, that RGS1 differs from other small RGSs that show a dramatic reduction in their ability to inhibit signaling without their N-terminus. For example, the expression of an N-terminally deleted form of RGS2 failed to inhibit Ste2p signaling in yeast (67). In this case, the RGS2 box was shown to be non-functional because it failed to be recruited to the plasma membrane thus preventing it from interacting with the GPCR signaling machinery. Similarly, the RGS box of RGS4 was found to be 10^4 times less efficient at inhibiting carbachol-evoked Ca^{++} potentials than intact RGS4 (45). Not only were the N-terminal 33 residues of RGS4 required for its ability to inhibit GPCR signaling, but its presence was also shown to enable it to selectively inhibit some but not all GPCRs. In contrast, the ability of the RGS4 box to inhibit signaling was indiscriminate with respect to the receptor. This suggests that the N-terminus of RGS4 interacts with specific GPCRs, which serves to bring the RGS box in close proximity to the Gα-protein. This receptor specificity has now been documented for a number of other RGSs (48). For instance, using yeast as a model system, we have previously demonstrated that mammalian RGSs are significantly more efficient at

inhibiting the heterologously expressed mammalian receptor for somatostatin than the endogenous receptor for α-factor (46).

2.4.2 Mutational analysis of the N-terminal region of RGS1

As a first step towards undertaking a detailed mutational analysis of the N-terminal region of RGS1, we generated and analyzed point mutations within the RGS box of RGS1. Three separate residues in the RGS box of RGS1 namely F94, Y124 and N137, were chosen because they are conserved in a number of RGSs and their potential role in RGS1 had not been addressed (3,4). Furthermore, N137 of RGS1 corresponds to residue N128 of RGS4, that has previously been shown to be critical for its GAP activity, making it likely that this residue would also be important for RGS1 function (68). The mutants $RGS1^{F94A}$, $RGS1^{Y124F}$ and $RGS1^{N137A}$ as well as wild type RGS1 were cloned into the yeast expression vector p426Met25 (Fig. 2A). The individual plasmids were transformed into the *sst2Δ* yeast strain BY4741-6055 and their ability to inhibit Ste2p mediated signaling was assessed using the halo assay (Fig. 2C). As expected, cells harboring the control plasmid p426Met25 were sensitive to the growth inhibitory effects of α-factor as evidenced by a large zone of growth inhibition. In contrast, cells expressing RGS1 showed very little growth inhibition in response to α-factor. Similar results were obtained with both the $RGS1^{F94A}$ and $RGS1^{Y124F}$ mutants indicating that these residues are not critical for RGS1 function. The moderate zone of growth inhibition exhibited by the mutant $RGS1^{N137A}$ indicates that it has a reduced ability to inhibit Ste2p-mediated signaling. Western blot analysis using anti-RGS1 antisera indicates that RGS1 as well as the RGS1 mutants accumulate to similar levels in yeast. Taken together these results indicate that residue N137 is required for RGS1 function and demonstrate that this yeast bioassay can be used as in the functional analysis of RGS1 mutants.

In an attempt to identify functionally important residues within the N-terminal region of RGS1 we constructed a series of mutants using the technique of Conservative Segment Exchange (CSE) mutagenesis (62,63). In this approach, 3 to 8 amino acids are replaced by different but chemically similar residues. This technique

is useful in that it allows the systematic evaluation of the role of large stretches of amino residues using relatively few mutants. One limitation of the technique is the inability to evaluate glycine, cysteine and proline residues since there are no chemically similar alternatives for these residues. The N-terminal non-RGS region of RGS1 was divided into groups of 3 or 4 residues to generate 20 CSE mutants. The amino acid changes for each mutant, sequentially identified as NT1 to NT20, are shown under the corresponding wild type sequence of RGS1 (Fig. 3). These mutants were constructed as GFP fusions in plasmid p426GAL1-EGFP and expressed in yeast strain BY4741-6055 and their ability to inhibit GPCR signaling was then assessed using the halo assay. Their level of expression was determined by western blot analysis using anti-GFP antisera. The zones of growth inhibition induced by 3000 pmoles of α-factor were measured and compiled for all 20 mutants (Fig. 4A). The zone of growth inhibition exhibited by cells expressing RGS1 was arbitrarily established as 1, while the zone of no growth surrounding cells expressing GFP alone was proportionally calculated to be 1.7. Using this assay, the mutants could be classified into 4 different groups. The results of the halo assays as well western blot analysis of one representative member of each mutant group is shown (Fig. 4B and C). Mutant NT2-GFP represents the largest group of mutants. These mutants exhibited a halo size between 1 and 0.8 indicating that their activity was essentially the same as,or slightly more efficient than, that of the wild type RGS1-GFP fusion. As well, the level of protein expressed in yeast from this class of mutants was the same as wild type RGS1. One mutant, NT1-GFP, showed a reduced ability to inhibit Ste2p mediated growth arrest exhibiting a halo size of 1.4. Western blot analysis indicated that this mutant failed to accumulate to detectable levels. The halo size of a single mutant, NT16-GFP was 0 suggesting that its activity was greater than RGS1. However western blot analysis indicated that this mutant accumulated to higher levels than wild type RGS1 perhaps explaining its apparent increase in potency. Wild type RGS1 has a half-life of around 12 hours in yeast (W. Song and MT. Greenwood, unpublished). A mutationally-induced increase or decrease in stability is a possible cause for the observed increase in the level of NT16-GFP and NT1-GFP respectively.

Finally, the mutants NT10- and NT14-GFP exhibited a halo size of 0.7, indicating that they may be slightly more efficient than RGS1. Western blot analysis indicates no significant difference in the protein level of these mutants compared to RGS1. Given that RGS1 is not an endogenous protein, these mutants may be more efficient at inhibiting signaling in yeast because they are more adept at interacting with the yeast GPCR machinery. The observation that most of the NT-RGS1 mutants exhibited activity similar to the wild type protein suggests that the overall structure of the N-terminal region of RGS1, rather than specific motifs or residues, is required for its function.

2.4.3 The C-terminal Non-RGS is not Required for RGS1 Function

In addition to having non-RGS sequences located at their N-terminus, most small RGSs also contain non-RGS sequences at their C-terminus (1,4). These C-terminal sequences are not conserved and vary in size from 28 residues in RGS4 to 2 residues in RGS5. RGS1 has a C-terminal non-RGS sequence of unknown function that consists of 10 residues. To determine if this region is important to its function, we have constructed and analyzed an RGS1 mutant lacking its C-terminal 10 residues. When expressed and assayed for function, this mutant displayed a halo size of 0.8 (Fig. 4A) suggesting that this region is not required for its ability to inhibit GPCR signaling.

2.4.4 RGS1 Requires Gpa1p to Inhibit Receptor- Independent Gβγ Activation

In yeast, responses mediated by the GPCR Ste2p are initiated by the Gβγ subunit whereas the Gα-protein Gpa1p serves to sequester the Gβγ subunit and to inhibit basal signaling in the absence of GPCR stimulation (49). To examine the influence of Gpa1p on RGS1 function, we used yeast strain MMY9 as we have previously demonstrated that mammalian RGS1, 2, 5 and 16 can inhibit GPCR signaling when expressed in this strain (46,57). In addition to lacking the *SST2* gene, this strain lacks the *FAR1* gene required to mediate the growth inhibitory effects of α-factor and

contains an integrated *FUS1*-LacZ reporter gene that is activated by the Gβγ-subunit. Since MMY9 cells also lack the *GPA1* gene, Gβγ-mediated activation of the FUS1-LacZ reporter is constitutive. The use of this strain therefore allowed us to determine whether RGS1 could inhibit Gβγ-mediated signaling. Saturated cultures of MMY9 cells expressing the different GFP fusions were inoculated into galactose containing media and grown at 30^0 C for 6 h whereupon the basal level of *FUS1*-promoted β-galactosidase activity was determined. In the absence of both *GPA1* and *SST2* genes, we detected over 500 U of β-galactosidase activity in control MMY9 cells expressing GFP alone (Fig. 5). The basal level of activity decreased 2-fold in cells expressing the *GPA1* gene from plasmid p414CenGPA1. Co-expression of RGS1-GFP along with *GPA1* resulted in a further decrease in *FUS1*-promoted β-galactosidase activity. In the absence of Gpa1p, RGS1-GFP was unable to inhibit basal Gβγ signaling. These results are consistent with the notion that Gpa1p serves to sequester Gβγ thus inhibiting basal signaling while RGS1 inhibits Gβγ signaling by serving as a GAP for Gα thereby assisting in maintaining the G-protein in the inactivate, heterotrimeric state (69).

2.4.5 RGS1 Inhibits Basal Gβγ Signaling in the Absence of Ste2p and Gpa1p

The yeast GPCR Ste2p has been reported to inhibit heterotrimeric G-protein activation in the absence of ligand. Although the yeast RGS Sst2p serves to assist both Ste2p and Gpa1p in maintaining low basal signaling, it is not known if RGS can inhibit signaling in the absence of both the receptor and Gpa1p. We therefore used yeast strain RP2, an isogenic *ste2Δ* derivative of MMY9, to examine the ability of RGS1 to inhibit basal signaling in the absence of the yeast receptor and Gα-subunit. Basal *FUS1*-promoted β-galactosidase activity was increased 2-fold in RP2 cells expressing GFP alone as compared to MMY9 cells (Fig.5). Expression of either *GPA1* or RGS1-GFP resulted in a ca. 3-fold decrease in basal *FUS1*-LacZ activation while co-expression of both *GPA1* and RGS1-GFP resulted in a further decrease in β-galactosidase activity. These results indicate that RGS1 is capable of inhibiting Gβγ

signaling but only in the absence of Ste2p, suggesting that the receptor may act to prevent RGS from interacting with Gβγ. In the absence of Gpa1p, expression of either N-Term-GFP or Box-GFP resulted in modest decreases in Gβγ signaling. Although Box-GFP was found to be a potent inhibitor of Ste2p-mediated activation of G-proteins (Fig. 1B), this assay indicates that an intact RGS1 is required to inhibit signaling via Gβγ. A number of recent reports have suggested that the GPCR, G-protein and small RGS form a complex that serves to regulate signaling (41-43,45-47). Our results support this model (48) and further suggest that RGSs may play an even more diverse role in regulating multiple aspects of GPCR signaling. Our observation that RGS1 can inhibit Gβγ signaling in the absence of receptor suggests that RGSs may be involved in the regulation of signaling following a decrease in the level of cell surface receptors (i.e. agonist-mediated internalization of GPCRs). The ability to inhibit Gβγ is not common to all RGSs since the yeast Sst2p as well as mammalian RGS4 and RGS10, have been shown not to inhibit Gβγ signaling (49,70). However, a recent report has demonstrated that RGS3 is capable of inhibiting Gβγ-mediated signaling in cells overexpressing $G\beta_1$ and $G\gamma_2$ and a 145 amino acid sequence containing part of the N-terminal non-RGS region and part of the RGS sequence has been shown to be involved in mediating this ability of RGS3 (70). Our results also suggest that the N-terminus as well as the RGS box are required for the ability of RGS1 to inhibit Gβγ signaling (Fig. 5). The RGS3 region identified corresponds to residues 21 to 165 on RGS1. An alignment of the these 2 sequences reveals a high degree of sequence identity between the RGS portions in this region (52%) while no significant sequence identity is observed in the non-RGS portions of RGS1 and RGS3 (not shown). Nevertheless, the non-RGS portions of both proteins contain a number of similarly spaced positive (Arg and Lys) residues that may contribute to their ability to inhibit Gβγ signaling. A number of large RGSs including RGS6, 7, 9 and 11 have a GGL domain that has been shown to allow these RGSs to interact Gβ-subunits (38,40). However, analysis of the RGS1 amino acid sequence does not reveal any apparent Gβγ-interacting motifs within this protein. Given that our results suggest that the absence of the Ste2p receptor is required in order to

observe the RGS1-mediated inhibition of Gβγ, it would be interesting to determine if GPCR overexpression could abrogate this effect.

In summary, we have demonstrated that the RGS box and the N-terminal non-RGS region of RGS1 are capable of inhibiting Ste2p-mediated signaling in yeast but to a lesser extent than the intact RGS1. The analysis of Conservative Segment Exchange mutants spanning the entire N-terminal non-RGS region of RGS1 suggests that the overall structure of the N-terminal region of RGS1, rather than specific motifs or residues, is required for its function. Finally, we have demonstrated that RGS1 can inhibit basal Gβγ signaling in yeast but only in the absence of the receptor Ste2p . Due to the relative simplicity of the pheromone response pathway, yeast is an ideal model organism in which to delineate the function and the structure of mammalian RGSs.

2.5 Acknowledgments

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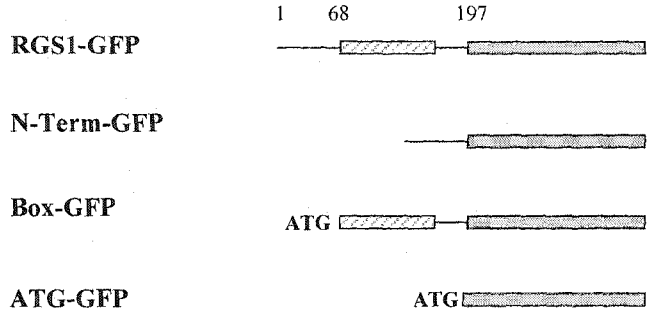
2.6 Figures

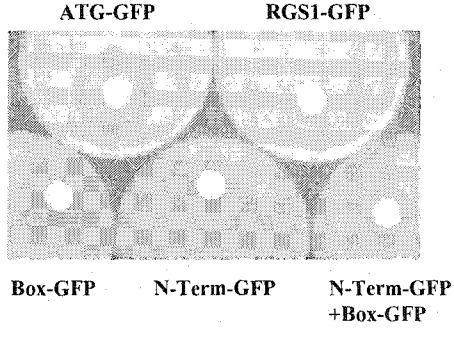
2.6.1 Figure 1: Construction and Analysis of RGS1 deletions

The entire coding sequence of RGS1 as well as the N-terminal and RGS box sequences of RGS1 were amplified by PCR and cloned into p426GAL1-EGFP to generate RGS1-GFP fusions. *A*, schematic representation of the RGS1-GFP fusions. The RGS sequence within RGS1 is shown as a hatched box while its N- and C-terminals are shown as lines. The GFP sequence common to all fusions is shown as a shaded box. The RGS1-GFP consists of the entire 197 residues of RGS1 fused to GFP, N-Term-GFP contains the N-terminal 68 residues of RGS1 fused to GFP while Box-GFP consists of residues 68 to 197 fused to GFP. The fusions were cloned into p426GAL1-EGFP for expression in yeast. ATG-GFP serves as a control for the expression of GFP. *B*, halo assays were performed with 1000 pmoles of α-factor using the yeast strain BY4741-6055 lacking its endogenous RGS containing gene *SST2* and harboring p426GAL1-EGFP plasmids expressing the different RGS1-GFP fusions. *C*, an equal amount of protein extract prepared from exponentially growing cultures of yeast transformants expressing the different RGS1-GFP fusions were analyzed by Western blot using an anti-GFP antisera. An arrow indicates the location of the N-Term-GFP fusion. The origin of the multiple bands that are present in the cells expressing N-Term-GFP is not known. Similar results were obtained in 3 separate experiments.

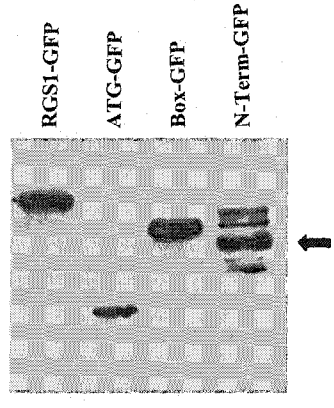
A


1
68
197
RGS1-GFP
N-Term-GFP
Box-GFP
ATG
ATG-GFP
ATG

B


ATG-GFP
RGS1-GFP
Box-GFP
N-Term-GFP
N-Term-GFP
+Box-GFP

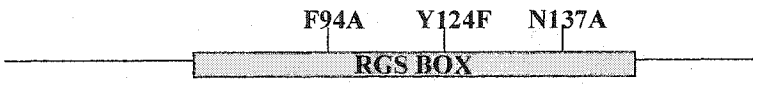
C


RGS1-GFP
ATG-GFP
Box-GFP
N-Term-GFP

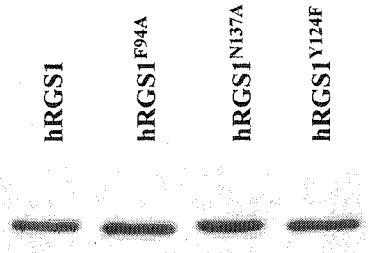
2.6.2 Figure 2: Functional Analysis of RGS1 Point Mutants in Yeast.

A two-step PCR based procedure was used to generate RGS1 point mutants. *A*, schematic representation of the 197-residue RGS1 protein showing the location of the amino acid residues that were altered within the conserved RGS motif (shaded box). Mutant as well as wild type RGS1 clones in plasmid p426Met25 were introduced and functionally analyzed in the Sst2Δ yeast strain BY4741-6055. *B*, western blot analysis using a specific anti-RGS1 antisera was performed using extracts prepared from cells expressing wild type hRGS1 or from cells expressing the F94A, Y124F or N137A RGS1 mutants *C*, halo assays using 3000 pmoles of α-factor were performed in order to assess the function of the RGS1 mutants. Yeast cells lacking RGS (vector) show a large zone of growth inhibition induced by α-factor. In contrast, cell expressing RGS1 (hRGS1) are completely resistant to the growth inhibitory effects of agonist (no clear zone). The halo assays of the 3 RGS1 point mutants F94A, Y124F and N137A are also shown. .

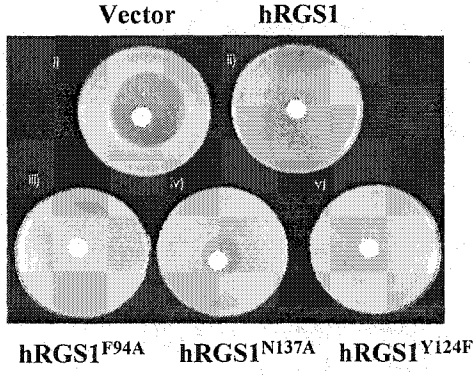
A


F94A
Y124F
N137A
RGS BOX

B

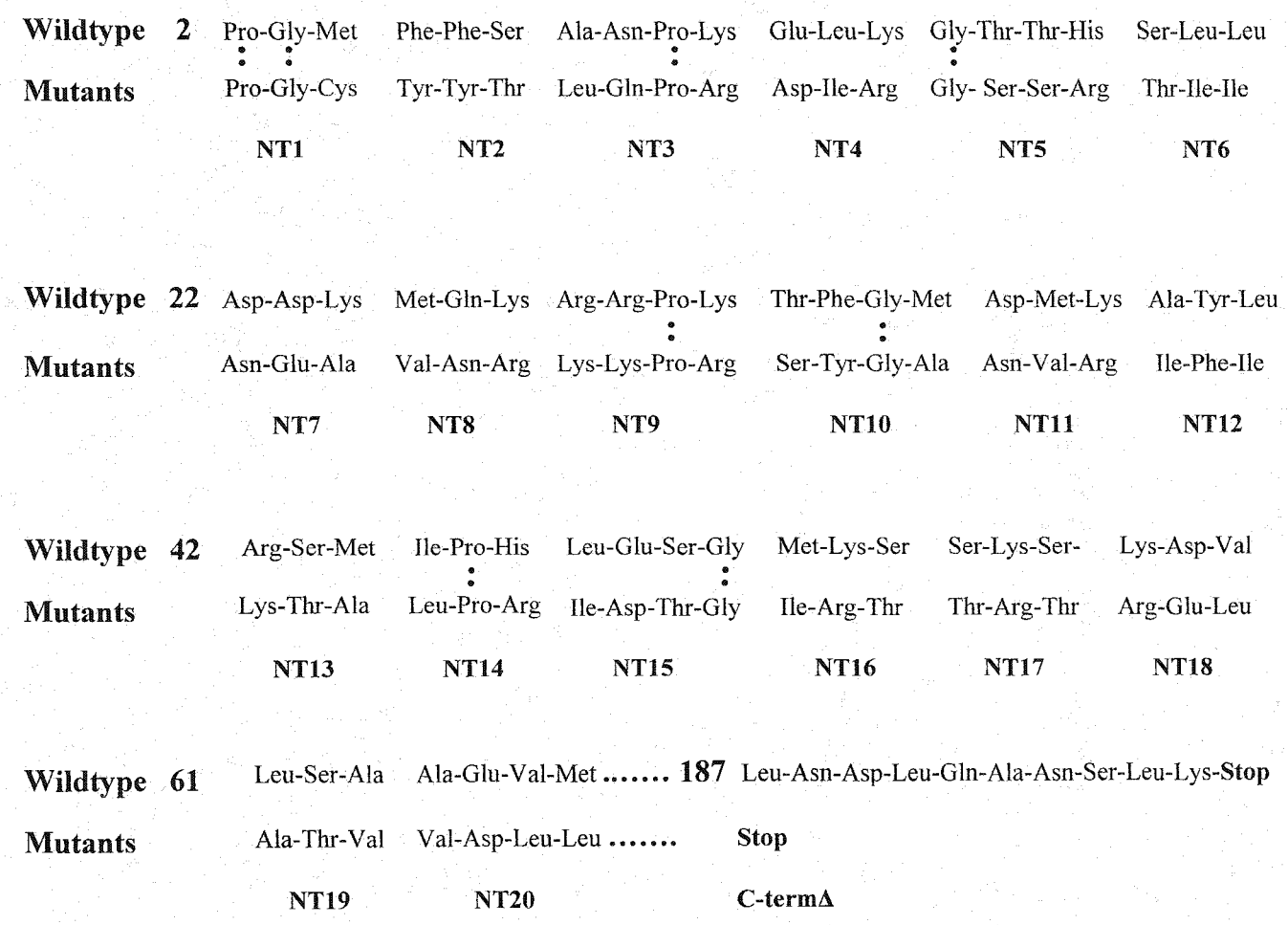

hRGS1
hRGS1F94A
hRGS1N137A
hRGS1Y124F

C


Vector
hRGS1
hRGS1F94A
hRGS1N137A
hRGS1Y124F

2.6.3 Figure 3: Sequences of RGS1 CSE Mutants

The amino acid sequence of the N-terminal non-RGS region of RGS1 beginning with residue number 2 is shown as Wildtype. The sequence of the corresponding CSE mutants, that are numbered as NT1 to NT20, are shown aligned with wild type RGS1. The sequence of wild type non-RGS C-terminal sequence beginning at residue 187 is also shown. A translational stop codon was introduced at residue 187 in order the RGS1-C-TermΔ mutant.


Wildtype 2
Pro-Gly-Met
Phe-Phe-Ser
Ala-Asn-Pro-Lys
Glu-Leu-Lys
Gly-Thr-Thr-His
Ser-Leu-Leu
Mutants
Pro-Gly-Cys
Tyr-Tyr-Thr
Leu-Gln-Pro-Arg
Asp-Ile-Arg
Gly- Ser-Ser-Arg
Thr-Ile-Ile
NT1
NT2
NT3
NT4
NT5
NT6
Wildtype 22
Asp-Asp-Lys
Met-Gln-Lys
Arg-Arg-Pro-Lys
Thr-Phe-Gly-Met
Asp-Met-Lys
Ala-Tyr-Leu
Mutants
Asn-Glu-Ala
Val-Asn-Arg
Lys-Lys-Pro-Arg
Ser-Tyr-Gly-Ala
Asn-Val-Arg
Ile-Phe-Ile
NT7
NT8
NT9
NT10
NT11
NT12
Wildtype 42
Arg-Ser-Met
Ile-Pro-His
Leu-Glu-Ser-Gly
Met-Lys-Ser
Ser-Lys-Ser-
Lys-Asp-Val
Mutants
Lys-Thr-Ala
Leu-Pro-Arg
Ile-Asp-Thr-Gly
Ile-Arg-Thr
Thr-Arg-Thr
Arg-Glu-Leu
NT13
NT14
NT15
NT16
NT17
NT18
Wildtype 61
Leu-Ser-Ala
Ala-Glu-Val-Met 187
Leu-Asn-Asp-Leu-Gln-Ala-Asn-Ser-Leu-Lys-Stop
Mutants
Ala-Thr-Val
Val-Asp-Leu-Leu .....
Stop
NT19
NT20
C-termΔ

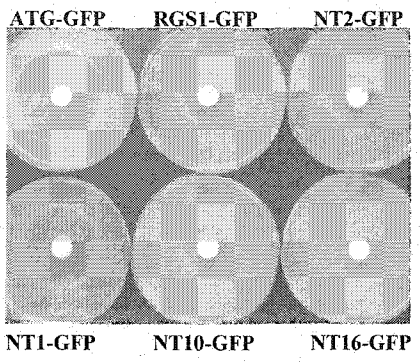
2.6.4 Figure 4: Functional Analysis of RGS1 CSE Mutants

Plasmids expressing CSE mutants as fusions with GFP were introduced into the Sst2Δ yeast strain BY4741-6055 and the ability of the resulting tranformants to inhibit GPCR signaling was assessed using the halo assay. *A*, a compilation of the relative sizes of the zone of growth inhibition produced by 3000 pmoles of α-factor for CSE mutants expressed in BY4741-6055. The halo surrounding cells expressing RGS1-GFP was arbitrarily set at 1.0 so that for cells expressing ATG-GFP, the halo size corresponded to 1.7, whereas a halo size less than 1.0 indicated a smaller halo than that obtained for wild type RGS1. *B*, representative halo assays of the 4 different classes of CSE RGS1 mutants. The halo assay from yeast cells expressing GFP (ATG-GFP) alone is shown compared to cells expressing wild type RGS1 (RGS1-GFP) and to four different RGS1 CSE mutants NT-2-GFP, NT-1-GFP, NT-10-GFP and NT-16-GFP. *C*, western blot analysis was performed using anti-GFP anti-sera on the same representative CSE mutants that were analyzed by halo assay in B. Extracts were prepared from galactose-induced exponentially growing cultures of BY4741-6055 yeast cells expressing RGS1 (RGS1-GFP) as well as the four CSE mutants.

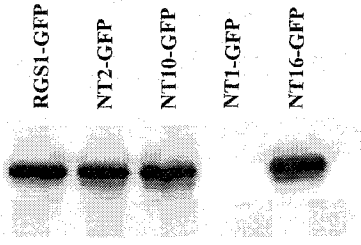
A

IDENTITY	HALO SIZE*
RGS1-GFP	1.0
ATG-GFP	1.7
NT1-GFP	1.4
NT2-GFP	1.0
NT3-GFP	0.9
NT4-GFP	0.9
NT5-GFP	0.9
NT6-GFP	0.9
NT7-GFP	0.9
NT8-GFP	0.9
NT9-GFP	0.9
NT10-GFP	0.7
NT11-GFP	0.9
NT12-GFP	0.8
NT13-GFP	1.0
NT14-GFP	0.7
NT15-GFP	0.9
NT16-GFP	0.0
NT17-GFP	0.9
NT18-GFP	0.9
NT19-GFP	0.8
NT20-GFP	0.9
C-termΔ-GFP	0.8

B

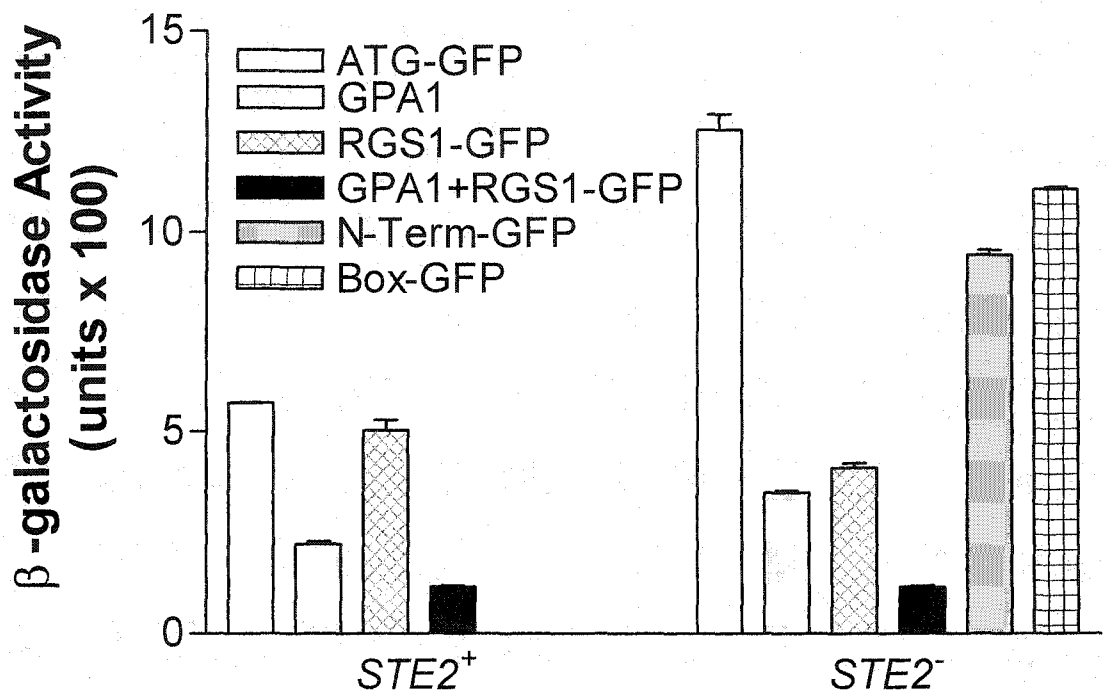


C



2.6.5 Figure 5: Inhibition of Basal FUS1-LacZ Reporter Activity by RGS1

β-galactosidase activity was determined from a variety of transformants of the yeast strain MMY9 (*STE2*$^{+}$, left side) expressing the yeast pheromone receptor Ste2p as well as the isogenic strain RP2 (STE2^{-}, right side) lacking this receptor. These cells were transformed with plasmids expressing GFP alone (ATG-GFP), Gpa1p (GPA1), RGS1 (RGS1-GFP), the N-terminus of RGS1 (N-Term-GFP) or the RGS box of RGS1 (Box-GFP) as detailed in the figure. β-galactosidase activity was determined in triplicate samples obtained from galactose grown cultures of the different transformants. Data represent the mean ± SEM of triplicate assays and are typical of three independent experiments.


β-galactosidase Activity
(units x 100)
15
10
5
0
ATG-GFP
GPA1
RGS1-GFP
GPA1+RGS1-GFP
N-Term-GFP
Box-GFP
STE2+
STE2-

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Chapter 3: Future Directions

In this report we have established that both the RGS and Non-RGS N-terminal domains of RGS1 are important to its activity when this protein is heterologously expressed in yeast. It would be of interest to examine the localization patterns of mutants expressing GFP tagged fusions of both the N-terminus and RGS box of RGS1 using confocal microscopy. Based on evidence obtained from such localization studies with RGS3 (3), RGS4 (7), RGS8 (6), RGS2 (5), and RGS16 (1), in which the N-terminus of these proteins was responsible for membrane targeting, we might expect RGS1lacking the N-terminus to be confined to the cytosol to a greater extent than the wild type. In contrast, we would expect the protein containing the N-terminus of RGS1 to be targeted to the plasma membrane. The same mutants representing the various subgroups of Conservative Segment Exchange Mutants whose function was assessed by halo assay could also be examined in much the same way. The localization patterns of these mutants, especially those that exhibited increased or decreased activity in the halo assay might provide some clues as to the origin of their functional differences.

We have also established that full length RGS1 is able to inhibit signaling mediated by the yeast Gβγ-subunit Ste4p-Ste18-p, in the absence of receptor. It would be of interest to determine whether RGS1 acts to directly inhibit Gβγ itself, or whether RGS1 prevents the interaction of Gβγ with one or both of its immediate downstream effectors the Ste5p scaffold protein or the activator of Ste11p (yeast MAP kinase-kinase-kinase), Ste20p(2, 4). However, the investigation of these interactions is complicated by the fact that the interruption of any of the four components of the yeast MAP kinase cascade mentioned above, would result in the complete abrogation of the pheromone response pathway, making it impossible to determine whether or not RGS1 has had an effect on the system. However, one could determine the activity of RGS1 in an assay in which either Gβγ, Ste5p, or Ste20p were overexpressed. For example, if the inhibition of Gβγ-mediated signaling by RGS1 was prevented by the overexpression of Ste5p, but not Ste20p or Gβγ, it would

indicate that RGS1 exerts its inhibition at the level of Ste5p, for instance by preventing its interaction with members of the MAP kinase cascade for which it is a scaffold.

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