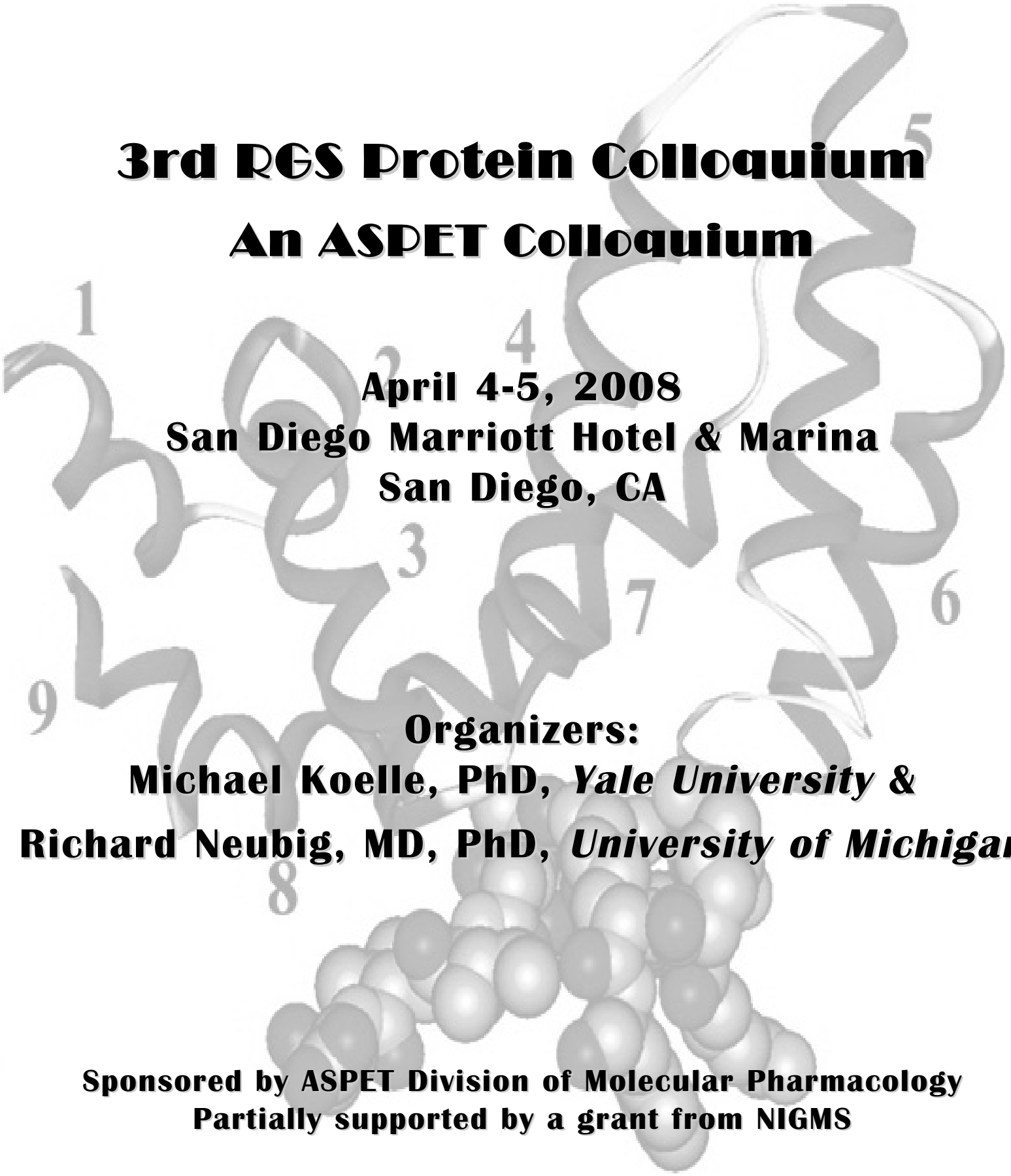
3rd RGS Protein Colloquium

An ASPET Colloquium



April 4-5, 2008
San Diego Marriott Hotel & Marina
San Diego, CA

Organizers:
Michael Koelle, PhD, *Yale University* &
Richard Neubig, MD, PhD, *University of Michigan*

Sponsored by ASPET Division of Molecular Pharmacology
Partially supported by a grant from NIGMS

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Day1
Friday, April 4

1:00 pm Welcome from the meeting organizers

1:15 pm **Session 1 - RGS Protein Structure/Function**

John Tesmer, *University of Michigan*
Roles of RGS proteins and RGS homology domains in signaling scaffolds

John Sondek, *University of North Carolina, Chapel Hill*
Gβ5/R7 proteins: the other Gβγ dimer

Abstract Talk: Zhe Chen, *University of Texas Southwestern Medical Ctr*
Crystal structures of a RhoGEF-RGS: Galpha-13 complex at various GTP hydrolysis states

3:00 pm Coffee break

3:30 pm **Session 2 - RGS7 Family RGS Proteins**

Kendall J. Blumer, *Washington University School of Medicine*
R7BP: A palmitoylation-regulated membrane anchor and shuttling protein for the RGS7 family and G□5

Kirill Martemyanov, *University of Minnesota*
Macromolecular complexes of RGS9 – master regulators of G protein signaling in retina and striatum

Vladlen Slepak, *University of Miami*
Structure and function of Gbeta5-R7 complexes: 10th anniversary

Short Abstract Talk: Rebecca Roof, *University of Michigan*
Inhibitors of RGS4 identified in a one-bead, one-compound peptide library

5:15 pm Posters in Marriott Hall 3

3rd RGS Protein Colloquium

Day 2
Saturday, April 5

7:30 am — Continental breakfast

8:00 am — **Session 3 - Interactions of RGS Proteins**

John R. Hepler, *Emory University*
Is RGS14 a novel integrator of G protein and MAPkinase signaling cascades important for hippocampal-based learning and memory?

Andrew Tinker, *University College, London*
The molecular basis of the pleiotropic effects of RGSs in the regulation of G protein gated K+ channels

Short Abstract Talk: Osamu Saitoh, *Nagahama Inst Bio-Science & Technology*
The receptor type-specific attenuation of G protein signaling by RGS8

Short Abstract Talk: Carlo Cifelli, *University of Toronto*
Novel molecular insights into RGS mediated regulation of sinoatrial node function

Abstract Talk: Nikolai Dulin, *University of Chicago*
Regulation of TGF-beta signaling by RGS3

10:00 am — Coffee break: Sponsored by Cell Press

10:30 am — **Session 4 - RGS Action *In Vivo***

Vanna Zachariou, *University of Crete*
A role of RGS proteins in addiction and analgesia

John Traynor, *University of Michigan*
RGS proteins as a potential drug target for depression

John H. Kehrl, *National Institute of Allergy and Infectious Diseases, NIH*
Insights into RGS protein function from the analysis of RGS and Gi alpha knock-out mice

Short Abstract Talk: Caroline Nunn, *University of Western Ontario*
Lean phenotype of RGS2 deficient mice

12:15 pm — Lunch and posters in Marriott Hall 3

2:30 pm — **Session 5 - Novel RGS Interactions/Functions**

Peter Chidiac, *University of Western Ontario*
Novel regulatory properties of RGS2

Abstract Talk: Kirk Druey, *NIAID/NIH*
RGS13 acts as a nuclear repressor of CREB

3:30 pm — Discussion and vote on potential future RGS colloquia

3:45 pm — Coffee break

Final Session

4:15 pm

Marilyn G. Farquhar, *University of California San Diego*
GAIP (RGS-19) functions to terminate Gαi3 Activity during cell migration

Abstract Talk: Thomas Wilkie, *University of Texas Southwestern Medical Ctr*
RGS16 is a glucose-responsive inhibitor of galpha11-stimulated fatty acid oxidation in liver

5:15 pm — Meeting ends

3rd RGS Protein Colloquium
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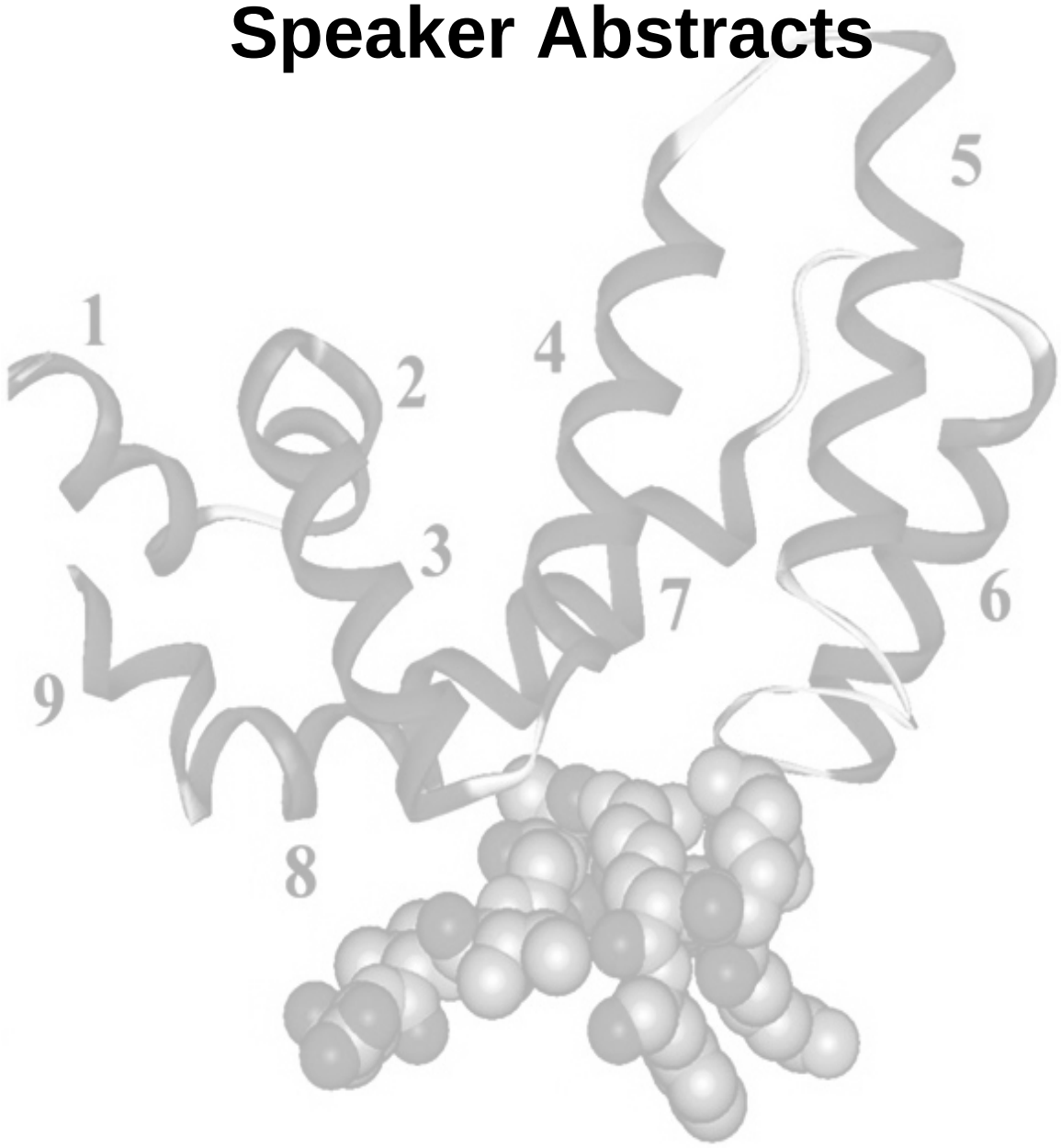
3rd RGS Protein Colloquium

Speaker Abstracts



Roles of RGS proteins and RGS homology domains in signaling scaffolds

John J. G. Tesmer[1,2], Aruna Shankaranarayanan[1,3], David Thal[1], David L Roman[2], Richard R. Neubig[2], Tohru Kozasa[4]

[1]Life Sciences Institute and the [2]Department of Pharmacology, University of Michigan; [3]Institute for Cellular and Molecular Biology, Department of Chemistry and Biochemistry, University of Texas at Austin; [4]Department of Pharmacology and the Department of Anatomy and Cell Biology, University of Illinois, Chicago, IL

G protein-coupled receptor kinase 2 (GRK2) contains a domain homologous to the core domain found in regulator of G protein signaling (RGS) proteins that binds with high affinity to activated $G\alpha_q$. Despite the obvious similarity in structure and function to RGS proteins, the crystal structure of the $G\alpha_q$-GRK2-$G\beta\gamma$ complex [1] revealed that the GRK2 RGS homology (RH) domain binds to the effector site of $G\alpha_q$ and not to the characteristic RGS binding site [2]. This implied that GRK2 could be a previously unrecognized effector target of $G\alpha_q$. If so, then RGS proteins might be expected to regulate the activity of GRK2, and vice versa. To help verify this hypothesis, we have demonstrated that RGS4 can form a ternary complex with $G\alpha_q$ and GRK2 using a flow-cytometry protein interaction assay. A similar ternary complex was demonstrated for another recently identified effector target of $G\alpha_q$, p63RhoGEF [3]. We also evaluated the influence of GRK2 on RGS-mediated GTPase acceleration on $G\alpha_q$ subunits and showed that GRK2 can enhance RGS4 activity on $G\alpha_q$. RGS2, a $G\alpha_q$-selective RGS protein, exhibited remarkable differences from RGS4 in both binding and GAP assays, indicating a different mode of inhibition. Together, our results suggest that RGS proteins and effectors can regulate each other's binding affinity for $G\alpha_q$ subunits, and that this regulation most likely occurs through allosteric modulation of the switch region and not necessarily by direct effector-RGS protein interactions. RGS proteins thus have the potential to modulate the activity of GRK2 just as they would for any other $G\alpha_q$ effector. Our data also support the existence of discrete complexes of activated G proteins, effectors and RGS proteins that are assembled in a specific orientation at the membrane, providing us a glimpse of the molecular framework that underlies transmembrane signaling.

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Gβ5/R7 proteins: the other Gβγ dimer

Matthew L. Cheever[1,3], Jason T. Snyder[2,4], Svetlana Gershburg[1], David P. Siderovski[1,3], T. Kendall Harden[1,3], **John Sondek**[1,2,3]

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Regulator of G protein Signaling (RGS) proteins were originally identified as GTPase-activating proteins (GAPs) for heterotrimeric G protein α subunits. However, many RGS proteins possess highly conserved domains in addition to the signature RGS box responsible for GTPase activation. For example, the R7 subfamily of RGS proteins (RGS6, -7, -9 and -11) contain a distinctive G-γ-like (GGL) domain that mediates specific and obligate heterodimer formation with the atypical G protein subunit, Gβ5. Recent work describing the functional and structural integration of Gβ5/R7 dimers within conventional GPCR machinery will be discussed.

R7BP: A palmitoylation-regulated membrane anchor and shuttling protein for the RGS7 family and Gβ5

Kendall J. Blumer
Washington University School of Medicine
St. Louis, MO 63110

I will present recent work from my lab indicating that the intracellular trafficking and function of complexes containing any member of the RGS7 (R7) family (RGS6, 7, 9 and 11) bound to Gβ5 can be regulated by R7BP (R7 family binding protein), a novel palmitoylated SNARE-like protein. When palmitoylated, R7BP directs R7-Gβ5-R7BP complexes to the plasma membrane. When depalmitoylated, R7BP shuttles R7-Gβ5-R7BP complexes into the nucleus. Complexes containing unpalmitoylated R7BP shuttle in and out of the nucleus, potentially allowing R7-Gβ5-R7BP complexes to be re-targeted to the plasma membrane. Palmitoylation-dependent plasma membrane targeting is required for R7BP to augment function of RGS7-Gβ5-R7BP complexes in Xenopus oocytes. In brain, R7BP is not detected significantly within the nucleus, indicating that at steady state it is efficiently palmitoylated and membrane-targeted. However, depalmitoylation-triggered nuclear import may occur under conditions yet to be identified. Whereas we find that R7 and Gβ5 proteins are obligate binding partners of R7BP, R7BP is not an obligate binding partner of RGS7 and Gβ5. Indeed, RGS7-Gβ5 complexes are likely to function both in concert with and independently of R7BP, because whereas R7BP is exclusively membrane bound in brain extracts, RGS7 and Gβ5 exist in both membrane-bound and soluble pools. In brain, R7BP concentrates in postsynaptic structures (dendrites and spines), but not in nerve terminals, suggesting that R7-Gβ5-R7BP complexes are likely to regulate postsynaptic GPCR signaling. Gβ5 and R7BP protein expression is strikingly upregulated during the second and third weeks of postnatal life, when synapses mature, suggesting that these complexes may play an unsuspected role in postnatal maturation of the nervous system.

Macromolecular Complexes of RGS9 – Master Regulators of G protein Signaling in Retina and Striatum

Kirill A. Martemyanov
University of Minnesota Medical School

A member of RGS family, RGS9, belongs to the R7 family RGS proteins that share similar multi-domain organization and exist as constitutive dimers with type 5 G protein beta subunit, Gβ5. Two splice isoforms of RGS9 exhibit a very specific expression patterns: the short splice isoform, RGS9-1 is present only in retina whereas the long splice variant, RGS9-2 is enriched in the striatum. Studies with transgenic and knockout mice indicate that RGS9-1 essentially determines the duration of photoreceptor responses to light mediated by phototransduction cascade while RGS9-2 sets the sensitivity of signaling in reward and locomotor control circuits mediated by dopamine and opioid receptors in the brain. The goal of our studies is to understand molecular mechanisms that determine the function of RGS9 isoforms in these diverse G protein signaling pathways.

We and others have found that RGS9/Gβ5 dimers form stable complexes with a new class of membrane proteins. RGS9-1 is bound to photoreceptor specific protein R9AP (RGS9 Anchor Protein) and RGS9-2 associates with widely expressed R7BP (R7 family Binding Protein). We studied the roles of these new interactions by both gain-of-function and loss-of-function approaches *in vitro* and in models of genetically modified mice and found that association with R9AP and R7BP control both expression level and subcellular localization of RGS9. Specific degrons on RGS9 isoforms predispose them to rapid proteolytic degradation by cysteine proteases resulting in their short lifetime and low expression levels. Association with either R9AP or R7BP protects RGS9/Gβ5 complexes, and thus serves to set their levels in both photoreceptors and striatal neurons. Furthermore, R9AP and R7BP carry signals that allow targeting RGS9 isoforms to the specific intracellular compartments: ciliary structure outer segment in photoreceptors and dendritic compartment postsynaptic density in striatal neurons. These findings indicate that RGS9 is tightly integrated into a macromolecular complex, the components of which critically modulate its function in the cell.

Structure and function of Gbeta5-R7 complexes: 10th anniversary

Vladin Z. Slepak
University of Miami

Gβ5 is a unique G protein beta subunit that forms obligatory dimers with RGS proteins of R7 subfamily. These novel types of G protein complexes were discovered 10 years ago when it was established that the interaction of Gβ5 with R7 subunits occurs instead of the interaction with Gg subunits. While Gβ5 is capable of binding to Gγ subunits in vitro, up to date, the Gβ5-Gγ complexes were not found in native tissues. Gβ5-R7 complexes are present in cytosol and membranes of neuronal cells. Membrane binding of Gβ5-R7 proteins occurs via the third subunit, R7BP or R9AP. All three subunits are necessary for stability of the complex and degrade in cells if one of the subunit is knocked out. Functional assays implicated Gβ5-R7 proteins in regulation of Gi and Gq-mediated pathways in vertebrates and invertebrates. Recent studies suggested novel roles of in synaptogenesis and other functions. The role of the Gβ5 subunit within the complex remains enigmatic. This talk will summarize the recent data on inter-domain interaction within the Gβ5 complexes, which might be important for regulation of certain functional activities, and discuss the phenotypes of some relevant mouse models.

Is RGS14 a Novel Integrator of G protein and MAPkinase Signaling Cascades Important for Hippocampal-based Learning and Memory?

John R. Hepler, F-J Shu, S.E. Lee, J.P. Schroeder*, C.P. Vellano, J.P. Waters, S. Ramenini, D.P. Cowan, and D. Weinshenker*
*Depts of Pharmacology & *Human Genetics,*
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RGS14 is a highly unusual signaling protein with poorly understood functions. RGS14 contains an RGS domain, which binds *active* Gi/oα-GTP; a GoLoco/GPR (GL) domain, which selectively binds *inactive* Giα-GDP; and a tandem (R1 and R2) Ras/Rap binding domain (RBD), which is reported to bind Rap GTPases. We initiated studies to determine the importance of RGS14 interaction with its binding partners on its cellular functions. In HeLa cells, RGS14 localizes to the cytosol but is recruited to the plasma membrane when co-expressed with inactive Giα1 or Giα3 to form a stable complex; complex formation is mediated by the GL domain of RGS14. By contrast, neither Giα2 nor constitutively active Giα1-3 recruit RGS14 to the plasma membrane. We find that RGS14 binds directly to active Rap2 and H-Ras, but not Rap1 in HeLa cells, and binding is disrupted by mutation of the first (R1) RBD domain of RGS14. In cell imaging studies, RGS14 is recruited by Rap2 or H-Ras to co-localize at the plasma membrane. RGS14 also binds Raf-1 and B-Raf Ser/Thr kinases, effectors of Ras/Rap. RGS14 forms a stable complex in cells with each kinase and also as a heterotrimeric complex with activated Rap2 or H-Ras. B-Raf binding to Rap2 is enhanced in the presence of RGS14, suggesting that RGS14 may promote Rap2/B-Raf association. RGS14/Rap-Ras/Raf complexes co-localize at membranes in cells, indicating a functional complex.

Stimulated GPCRs and growth factor receptors activate MAPkinase signaling and Erk1/2 phosphorylation, and RGS14 regulates each pathway differently. Expression of RGS14 in HeLa cells blocks Erk1/2 activation by PDGF, but not EGF. However, co-expression with inactive Giα1 prevents RGS14 from inhibiting PDGF signaling, suggesting that Giα1 sequesters RGS14 from PDGF signaling components. RGS14 has different effects on 2nd messenger regulated MAPKinase signaling. Cyclic AMP and Ca^{++}/DAG activate guanine nucleotide exchange factors (GEFs) for Rap/Ras-family members to stimulate Erk1/2 phosphorylation. Carbachol activation of Gq/$InsP_3$/Ca^{++} linked muscarinic receptors, and dibutyryl cAMP (dbcAMP) stimulate Erk1/2 phosphorylation. Expression of Rap2 blocks 2nd messenger-stimulated Erk1/2 phosphorylation, but signaling is restored by co-expression with RGS14 thereby suggesting a scaffolding/switch role for RGS14 in G protein and MAPkinase signaling cascades.

RGS14 protein is most highly expressed in brain and hippocampus (HPC). RGS14 partners also are present in the HPC, and Rap2, H-Ras and Gi/o are reported to regulate defining features of postsynaptic plasticity at HPC neurons (neurite retraction, dendritic remodeling, and trafficking of excitatory AMPA-glutamate receptors). To determine physiological roles for RGS14 in brain, we obtained and characterized a novel mouse model that lacks the RGS14 gene/protein (R14-KO). Loss of RGS14 mRNA/protein is confirmed by PCR of genomic DNA; RT-PCR of mRNA; and specific immunoblot of RGS14 protein. R14-KO mice are fully viable, and exhibit no apparent gross phenotype. Wild type (WT) and R14-KO animals were subjected to the Morris Water Maze (MWM), a test of spatial learning capacity linked to hippocampal synaptic plasticity. WT and R14-KO mice show similar latency to platform on Day 1 (WT=43+/-4 sec; R14-KO=41+/-3 sec). WT mice exhibit typical learning behavior that improves each day in their latency to platform, reaching a steady-state minimal value by Day 4-5 (26.1+/-6 sec). In stark contrast, R14-KO mice exhibit a significantly enhanced initial rate of spatial learning and memory that is evident each day relative to WT (Day 5: 13.2 +/- 2 sec; $P < 0.008$ two-tailed paired t-test).

Together, these findings suggest that RGS14 is a multifunctional signal switch/scaffolding protein that integrates Gi/o and Rap/Ras-Raf kinase signaling pathways to modulate biochemical mechanisms of learning and memory in brain and hippocampal neurons.

The molecular basis of the pleiotropic effects of RGSs in the regulation of G-protein gated K+ channels

Andrew Tinker
UCL, London

G-protein gated K+ channels are responsible for vagally mediated heart rate slowing. A canonical pathway exists in which acetylcholine binds to M2 muscarinic receptors and leads to activation of inhibitory heterotrimeric G-proteins. The direct binding of the Gβγ subunit to the channel follows this, which results in current activation and membrane hyperpolarisation. I will discuss the current views of the "assembly" of this signalling complex and how RGSs might integrate into that picture. In addition, I will discuss the importance of G_{i2} in dictating the normal physiological response in the heart. I will compare these results with the published studies on mice with a knockin mutation of a RGS insensitive G-protein.

A role of RGS proteins in addiction and analgesia

Vanna Zachariou
Department of Pharmacology, University of Crete, and Department of Psychiatry, UT Southwestern Medical Center

The RGS family of GTPase accelerating proteins shows abundant expression in brain, with distinct patterns of regional and cellular distribution. Our group aims to elucidate the role of RGS9-2, RGSz and RGS4 in neuropsychiatric disorders. The striatal-enriched RGS9-2 is a potent determinant of sensitivity to drugs of abuse. RGS4 and RGSz are smaller proteins, with simpler structure, but wider distribution than RGS9-2. RGS4 is highly abundant in the cortex, with lower levels in several brain regions mediating addiction and analgesia, including the striatum, the locus coeruleus and the dorsal horn of the spinal cord. The Galphaz associated RGSz protein, shows moderate expression levels throughout the brain. To assess the roles of these proteins in analgesia, addiction and addiction-related depression, we varied their activities in particular brain regions using genetic mouse models. Overexpression of RGS9-2, RGS4 or RGSz in particular brain regions was achieved using adeno associated virus (AAV) constructs. Local knockout of RGS4 or RGSz was achieved following bilateral infection of the nucleus accumbens (or other brain regions) of floxed RGS4 (or floxed RGSz) mice with an AAV-Cre recombinase expressing virus. We complemented the behavioural studies, with biochemical, electrophysiological and cell culture assays, to improve our understanding of the mechanisms via which RGS9-2, RGS4 and RGSz modulate neuronal function. Our data indicate that signalling complexes in striatum containing RGS9-2 play a critical role in the development of drug addiction and addiction associated depression. In addition our findings support a role of RGS4 in locus coeruleus as modulator of opiate dependence. Finally, our results demonstrate that RGSz is a robust modulator of analgesic responsiveness but has no role in mood disorders. Taken together, our findings position RGS9-2, RGSz and RGS4 as novel pharmacological targets for analgesia and addiction.

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RGS proteins as a potential drug target for depression

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Regulators of G proteins signaling proteins by their GTPase accelerating protein (GAP) activity control the potency and efficacy of agonists acting at Gαi/o-coupled receptors (Clark et al, 2003). However, assigning physiology to individual members of the RGS family is a daunting task due to the large number of proteins with GAP activity, many of which are co-expressed in the same cells and tissues (Gold et al, 1997) and the potential for functional redundancy As an alternative we have used a knock-in mouse expressing RGS-insensitive Gαi2. In this mouse the GAP activity of all endogenous RGS protein is silenced by a point mutation in the switch I region of Gαi2 without altering its intrinsic functions (Huang et al, 2006). Serotonin-1A (5HT1A) receptors preferentially couple to Gαi2, are negatively regulated by RGS proteins in overexpression systems (Ghavami et al., 2004) and are implicated in mood disorders. Therefore, we hypothesized that mice expressing RGS-insensitive Gαi2 would show increased responsiveness to anti-depressant drugs targeting serotonin systems. Using the tail-suspension test as a model these mice exhibit an anti-depressive-like phenotype that is reversed by the 5HT1A antagonist WAY 100635, suggesting an enhanced response to endogenous serotonin. In addition, the 5HT1A agonist 8-hydroxy-DPAT and the serotonin selective re-uptake inhibitor (SSRI) fluvoxamine have increased potency in mice expressing RGS-insensitive Gαi2 compared to their wild-type littermates. This is a highly selective effect since there is no change in the activity of desipramine, a norepinephrine uptake inhibitor, or in 8-hydroxy-DPAT-mediated hypothermia. Taken together the findings suggest that inhibitors of RGS proteins may provide novel therapeutics for the treatment of depression by selectively enhancing the action of endogenous serotonin or as adjuncts to currently available SSRI's.

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Insights into RGS protein function from the analysis of RGS and Gi alpha knock-out mice

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Regulator of G protein signaling (RGS) proteins play a crucial role in the adaptation of cells to stimulation by G protein-coupled receptors. In addition, RGS proteins likely regulate G-proteins at sites distinct from their traditional one at the cell membrane. Many of the genes that encode RGS proteins have now been disrupted in mice. In addition, a Gnai2 allele that encodes Giα2 resistant to RGS proteins has been knocked-in to the wild type locus to generate mice that express Giα2 insensitive to RGS protein regulation. Very significant phenotypes have been described for Rgs2, Rgs3, Rgs9, Rgs10, and Rgs14 knock-out mouse strains. In addition, the Gnai2 RGS resistant mice exhibit a complex phenotype affecting heart, myeloid cells, skeletal, and central nervous system. Milder phenotypes have been found for Rgs1, Rgs5, Rgs7 and Rgs13 disrupted mice. No or very minimal phenotype has been reported for Rgs4 and Rgs11. While Rgs6, Rgs8, Rgs12, Rgs16, Rgs18, and Rgs19 have been targeted no report of the analysis of these mice is yet available. The status of targeting of Rgs8 and Rgs20-22 is not publicly available. Several of the Rgs genes have been targeted more than once including Rgs2, Rgs4, Rgs5, Rgs10, and Rgs14. The reported phenotypes have varied significantly indicating that the particular targeting approach and/or mouse background can profoundly alter the observed phenotypes. Analysis of mice lacking individual Gα subunits in conjunction with individual RGS proteins can also provide some insight into RGS protein function. To date mice lacking two or more RGS proteins have not been reported. The analysis of the mouse strains indicated above has highlighted the functional importance of RGS proteins yet our knowledge of the roles of specific family members still remains limited. The continued analysis of these mice especially on different genetic background should help identify them.

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Novel regulatory properties of RGS2

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RGS2 is a highly inducible protein, and its upregulation in response to G protein signaling can attenuate signals triggered by GPCR agonists(Roy et al., 2006). However, RGS2 is also upregulated in a number of cells and tissues by other factors which have no obvious link to G protein signaling. In particular, the induction of RGS2 by various forms of cellular stress suggests that it has additional actions(Song and Jope, 2006). One well established consequence of stress is decreased global protein synthesis(Proud, 2005). The rate at which mRNA is translated into new protein is limited by the initiation process, wherein an mRNA, a ribosome, and the first tRNA molecule are brought together, and our findings point to RGS2 as a previously unrecognized inhibitor of this process. Using a yeast two-hybrid approach, we identified the ε-subunit of the eukaryotic initiation factor eIF2B (eIF2Bε) as a novel binding partner for RGS2, and confirmed this interaction through co-immunoprecipitation experiments with both transfected and non-transfected cells. At the functional level, RGS2 inhibits translation and impedes the ability of eIF2B to act as a guanine nucleotide exchange factor and thus promote the activation of its binding partner eIF2. This novel function of RGS2 is independent of its effects on G protein signaling and maps to a subdomain within the RGS box. Notably, the region of RGS2 identified bears a moderate homology to the region of eIF2 that interacts with eIF2B. This suggests that RGS2 can inhibit protein synthesis by competing or otherwise interfering with the binding of eIF2 to eIF2B and thus blocking initiation. This novel mechanism is distinct from established inhibitory effects on translation mediated via kinases that target eIF2 and eIF2B, and moreover it provides a plausible role for the upregulation of RGS2 in response to cellular stress.

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GAIP (RGS-19) functions to terminate Gαi3 activity during cell migration

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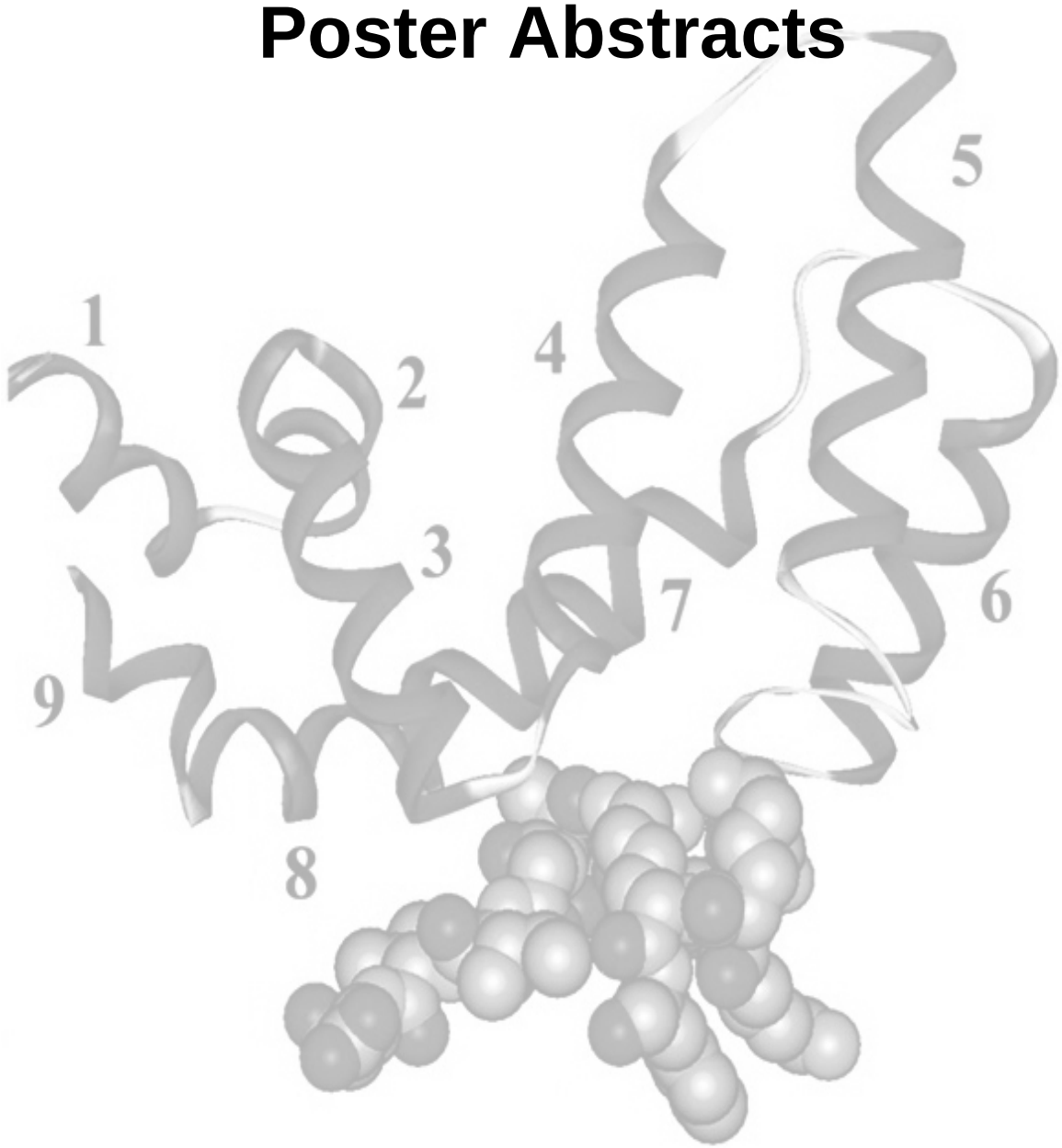
GAIP was discovered by us in a 2-hybrid screen with Gαi3 as bait. GAIP is composed largely of an RGS domain and has a cysteine string motif at the N-terminus that serves as a palmitoylation site and a PDZ binding motif at its extreme N-terminus through which it interacts with GIPC. We established earlier that GAIP is membrane anchored through palmitoylation and, like Gαi3, is found on both the Golgi and at the cell membrane (PM) where it is concentrated in clathrin-coated pits. We further obtained evidence that upon activation of delta opioid receptors Gαi3 moves into clathrin coated pits where it encounters GAIP and is inactivated. What remains to be established is in which cellular processes GAIP functions to terminate G protein activity. We have recently obtained evidence that Gαi3 translocates from the Golgi to the PM and is found preferentially at the leading edge of migrating cells where it serves as a molecular switch that enhances activation of Akt and actin remodeling cooperatively with GIV/Girdin. Mechanistically, activation of the G protein was a key event in functioning of the switch. Recently, we have used the RGS domain of GAIP fused to CFP (RGS-CFP) as a biosensor to detect active Gαi3, and using live cell imaging we have demonstrated that activation of Gαi3 indeed occurs at the leading edge during epithelial cell migration induced by scratch wounding. Furthermore, overexpression of GAIP impaired cell migration in scratch-wound assays. Our results thus indicate that GAIP functions to terminate Gαi3 activity at the leading edge during cell migration.

3rd RGS Protein Colloquium

Poster Abstracts



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RGS13 restricts G-protein-coupled receptor-induced responses in mast cells

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Mast cell degranulation and release of vasoactive mediators and cytokines promotes allergic reactions and innate immunity. Regulator of G protein signaling (RGS) proteins attenuate G-protein-coupled receptor (GPCR)-evoked signaling pathways by binding heterotrimeric G protein alpha subunits and acting as GTPase accelerating proteins (GAPs). Several RGS proteins were expressed in mast cells including RGS13. We found previously that RGS13 suppressed IgE-mediated degranulation of mouse bone marrow-derived mast cells (BMMCs) and anaphylaxis (Bansal et al Nat Immunol in press). To determine how RGS13 affected GPCR-mediated signal transduction in human mast cells, we examine responses of the human mastocytoma cell line (HMC-1) overexpressing RGS13 or expressing an RGS13-specific short, hairpin siRNA (shRNA) to GPCR stimulation. Compared to control cells, HMC-1 cells with reduced RGS13 amounts exhibited heightened Ca2+ influx in response to adenosine, C5a, sphingosine-1-phosphate (S-1P), and CXCL12. Chemotaxis and cytokine (interleukin 8, IL-8) secretion were increased in shRGS13-HMC-1 cells while overexpression of RGS13 in HMC-1 cells inhibited CXCL12-induced Ca2+ mobilization and chemotaxis. These results suggest that RGS13 may exert control over GPCR-evoked biological responses in human mast cells.

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Suppression of IgE-mediated allergic responses by Rgs13

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Mast cells provoke allergic responses through degranulation and release of proinflammatory mediators after antigen crosslinking of the high affinity immunoglobulin E (IgE) receptor (Fc.ÕRI). Regulator of G protein Signaling (RGS) proteins negatively control G-protein-coupled receptor-mediated signaling through GTPase accelerating protein (GAP) activity. Here, we show that Rgs13 inhibits allergic responses by physically interacting with the regulatory p85 alpha subunit of PI3K in mast cells and disrupting its association with an FceRI-activated scaffold complex. Rgs13¡V/¡V mice exhibited increased IgE-mediated mast cell degranulation and anaphylaxis. Thus, apart from its regulation of GPCRs, Rgs13 inhibits immune receptor-induced signalosome assembly in MCs. Abnormal Rgs13 expression or function may underlie some cases of idiopathic anaphylaxis or disorders of amplified MC activity.

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An RGS-domain FRET sensor demonstrates Gαi3 activity at the leading edge of migrating cells

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Gαi3 activity plays a vital role in cell migration. To study the localization of Gαi3 activity during cell migration we developed a biosensor based on the RGS domain of GAIP (RGS19) fused at its C-terminus to CFP (RGS-CFP). In resting HeLa cells RGS-CFP is largely cytosolic; addition of AlF_4^- to activate G-proteins redistributed RGS-CFP to Golgi and plasma membranes and dramatically increased FRET with Gαi3-YFP. RGS-CFP was next used to study Gαi3-YFP activity during cell migration. It is thought that definition of the leading edge arises from a gradient of external ligand that is detected by an array of GPCRs and G-proteins distributed around the plasma membrane; these in turn activate downstream factors in a similar gradient and additional feedback hones the leading edge. In HeLa cells migrating to close a wound Gαi3-YFP was concentrated in psuedopods at the leading edge rather than distributed homogeneously around the PM. RGS-CFP was also enriched at these sites. FRET measurements showed increased Gαi3-YFP activity at the leading edge and were confirmed with an alternative FRET sensor, β1-CFP. These results demonstrate that Gαi3 activity is indeed localized to the leading edge of a migrating cell and extend the model of leading edge formation by suggesting that additional feedback acts on Gαi3 itself to enhance its localization and signaling at the leading edge.

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Crystal Structures of a RhoGEF-RGS:Galpha-13 Complex at Various GTP Hydrolysis States

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RGS domain-containing RhoGEFs function as direct links between G12 class heterotrimeric G proteins and small GTPase Rho. These RhoGEFs possess a RhoGEF-RGS domain (rgRGS) that interacts with activated Galpha-12/13. Some rgRGS domains function as GAPs for Galpha-13 by stabilizing its transition state (GDP-AlF-bound) conformation. The rgRGS domain of PDZRhoGEF however, is not a GAP for Galpha-13. Isothermal titration calorimetry experiments indicate that it has a higher binding affinity towards Galpha-13 in the ground state (GTP-gamma-S-bound) than the transition state conformation. We have determined crystal structures of PRG-rgRGS in complexes with Galpha-13 in the inactive state (GDP-bound), the transition state (GDP-AlF-bound) and the ground state (GTP-gamma-S-bound) for GTP hydrolysis. PRG-rgRGS interacts extensively with the helical domain and the effector binding site on G13 in all three complexes. PRG-rgRGS forms stable, high affinity interactions with G13 in the ground state rather than in the transition state, causing slight rearrangement in switch regions of G13. GAP activity can be restored in PRG-rgRGS by replacing key residues at its N-terminus with those from p115RhoGEF. Our study suggests that rgRGS domains, though structurally similar, form interactions with Galpha subunits that are unique to each state in the hydrolytic pathway.

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Novel molecular insights into RGS mediated regulation of sinoatrial node function

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Heart rate is tightly controlled by the opposing activities of sympathetic and parasympathetic inputs to the sinoatrial node (SAN). Parasympathetic signalling in SAN myocytes is mediated by acetylcholine-dependent stimulation of M_2 muscarinic receptors coupled to Gi/o □ subunits. RGS proteins are potent inhibitors of Gi/o signalling in multiple tissues. The RGS protein(s) involved in parasympathetic regulation of pacemaker cells remain to be identified. Our results demonstrate that RGS4 mRNA levels are higher in the SAN compared to right atrium and that LacZ staining in hearts from mice expressing LacZ under the control of the RGS4 promoter show high RGS4 expression in the SAN and atrioventricular node. Moreover, mice lacking RGS4 activity showed lower basal heart rates than wild-type controls and greater heart rate increases following systemic atropine administration. Also, retrograde perfused hearts from RGS4-deficient mice showed enhanced negative chronotropic responses to carbachol, and increased susceptibility to conduction defects. Consistent with a role for RGS4 in the modulation of GIRK channels, myocytes from the SAN of RGS4-deficient mice showed altered modulation of GIRK channel desensitization, as well as on and off rates following carbachol stimulation. Taken together, our studies establish that RGS4 is a key regulator of sinus rhythm in mouse hearts by inhibiting parasympathetic signalling and I_{KACh} activity.

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RGS13 acts as a nuclear repressor of CREB

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Cyclic AMP (cAMP)-induced phosphorylation of the transcription factor CREB elicits expression of genes involved in diverse biological functions. In lymphoid organs, the neurotransmitter norepinephrine stimulates b_2-adrenergic receptors on B lymphocytes to promote CREB-dependent expression of genes like the B-cell specific Oct 2 coactivator (OCA-B). Although CREB phosphorylation recruits co-activators to stimulate transcription, protein inhibitors of CREB-DNA interactions have not been defined. Here, we identified RGS13, a member of the Regulator of G protein Signaling (RGS) protein family enriched in B lymphocytes, as a nuclear factor that suppresses CREB-mediated gene expression. cAMP or Ca^{2+} signaling induced RGS13 translocation to the nucleus and direct binding to phosphorylated CREB. RGS13-CREB complex formation required the kinase-inducible domain of CREB and the RGS13 amino terminus. shRNA-mediated knockdown of RGS13 enhanced CREB DNA binding activity, occupancy of CRE promoter sites in vivo, and expression of CREB target genes. B lymphocytes from *Rgs13*$^{-/-}$ mice had augmented phosphoCREB-DNA binding and OCA-B expression. Thus, RGS13 mediates a heretofore unknown mechanism regulating CREB-dependent transcription through disruption of CREB-mediated activation of gene promoters.

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Regulation of TGF-beta signaling by RGS3

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Regulator of G protein signaling (RGS) proteins bind G-alpha subunits of heterotrimeric G proteins and accelerate their GTPase activity. Through this mechanism, RGS3 (a member of the RGS family) regulates the signaling mediated by Gq and Gi proteins. In this study, we show that RGS3 interacts with the novel partners, Smad2, Smad3 and Smad4 - the transcription factors that are activated by transforming growth factor-beta (TGF-beta), who's signaling is not mediated by G proteins. The interaction of RGS3 with Smads can be detected by co-immunoprecipitation of overexpressed, as well as of endogenous RGS3 and Smads. Smad3 binds RGS3 at the region outside of the RGS domain; and Smad3 does not affect the ability of RGS3 to regulate G protein signaling. In contrast, RGS3, interacting with Smad's Mad homology 2 (MH2) domain, inhibits Smad-mediated gene transcription. The RGS3 mutant, defective in GTPase activation of G proteins, inhibits Smad-mediated gene transcription as well as the wild type RGS3, suggesting that regulation of Smad signaling by RGS3 is not related to regulation of G protein signaling by RGS3. Regarding the TGF-beta signaling, RGS3 does not affect TGF-beta - induced Smad phosphorylation, but it prevents heteromerization of Smads, which is mediated by their MH2 domains, and which is required for transcriptional activity of Smads. Functionally, RGS3-Smad interaction translates to inhibition of TGF-beta – induced myofibroblast differentiation (smooth muscle alpha-actin expression in fibroblasts) by RGS3. In conclusion, this study identifies a novel, non-canonical function of RGS3 as a regulator of TGF-beta signaling through RGS3-Smad interaction.

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Expression of Multiple Splice Variant Forms of RGS6 in Mouse Ventricular Myocytes

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GPCR signaling in cardiac myocytes is essential for normal cardiovascular function. RGS proteins are key regulators of GPCR signaling due to their GAP activity toward G proteins. Recent studies have shown an essential role of RGS proteins in regulating chronotropic responses in mice, although the identity of the RGS proteins involved is unknown. RGS6 is one of three RGS genes expressed highly in atrial and ventricular cardiac myocytes (VCM). We undertook studies to determine whether RGS6 exists in multiple splice forms in VCM, in view of our evidence for the existence of thirty-six human RGS6 splice forms. RGS6 immunoreactivity in VCM lysates co-migrates with the DEP domain-containing RGS6L splice forms. Nine C-terminal forms of RGS6L exist in humans and each contains a complete or incomplete GGL domain. We amplified and cloned RGS6Lα2, a major RGS6L form in most tissues, from VCM and found it to be 98.7% identical to the human form. Using primers based upon mouse genomic sequence data, PCR was performed to determine which RGS6L C-terminal splice forms are expressed in VCM. VCM express transcripts encoding each of the RGS6L C-terminal splice forms, designated α1, α2, β1, β2, γ, δ, ε η and ζ. Notably, PCR products corresponding to the –GGL forms of these transcripts are also present in VCM. Thus, VCM express multiple splice forms of RGS6L which may possess distinct roles in GPCR signaling in heart (AHA 0750057Z, NIH GM075033).

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Effect of pentoxifylline treatment on protein degradation in skeletal muscle from diabetic rats

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We investigated the in vivo effect of pentoxifylline (PTX; cAMP-phosphodiesterase inhibitor) on the rate of overall proteolysis and on the activity of proteolytic systems in extensor digitorum longus (EDL) muscles from normal and acutely diabetic rats by measuring the rate of tyrosine release. Methods and Results: Male Wistar rats (70-80g) were injected with streptozotocin (135mg/STZ/kg, i.v.). Diabetic and normal rats treated with PTX (100mg PTX/kg, s.c. one day before and during three days after STZ administration or citrate buffer) or with saline were killed and EDL muscles were incubated in appropriate medium. PTX treatment induced significant decreases in total rates of protein breakdown (17% in diabetic group), in Ca2+-dependent (47%) and ATP-proteasome-dependent (23%) proteolytic pathways. Western blotting analysis showed that increased m-calpain content in muscles from diabetic rats (115% as compared to normal group) was reduced by 70% with PTX treatment, without effect on the calpastatin content. In addition, the higher protein levels of 20S proteasome C2 subunit in the diabetic group were reduced by 24% after PTX treatment. RT-PCR analysis showed that atrogin-1 transcripts levels in EDL from diabetic rats were higher than controls and were 65% reduced with PTX. PTX treatment induced an increase in the cAMP content, assessed by an enzyme immunoassay system, in EDL muscles from diabetic rats. Conclusions: The present data suggest that PTX exerts an inhibitory effect on protein degradative systems in muscles from acutely diabetic rats, probably involving the participation of cAMP intracellular dependent cascades.
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RGS9–2 Negatively Modulates L-dopa-Induced Dyskinesia

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L-dopa treatment of Parkinson's disease (PD) often leads to debilitating involuntary movements, termed L-dopa-induced dyskinesia (LID), mediated by dopamine (DA) receptors. RGS9–2 is a GTPase-accelerating protein that inhibits DA D2 receptor-activated G proteins. Herein, we assess the role of RGS9–2 in LID. In monkeys, RGS9–2 levels are not altered by MPTP-induced DA denervation and/or chronic L-dopa. In MPTP monkeys with LID, striatal RGS9–2 overexpression – achieved by intra-striatal viral injection – diminishes the LID intensity without lessening the anti-parkinsonian effects of the D1/D2/D3 receptor agonist L-dopa. In contrast, striatal RGS9–2 overexpression diminishes both LID intensity and anti-parkinsonian effects of the D2/D3 receptor agonist ropinirole. In unilaterally 6-OHDA-lesioned rats with LID, we show that the time course of viral-mediated RGS9–2 expression parallels the time course of amelioration of LID. We also find that unilateral 6-OHDA-lesioned RGS9-/- mice are more susceptible to L-dopa-induced involuntary movements than unilateral 6-OHDA-lesioned wt mice, albeit the rotational behavior – taken as an index of the anti-parkinsonian response – is similar between the 2 groups of mice. Together, these findings suggest that RGS9–2 plays a pivotal role in LID pathophysiology. However, the findings also suggest that increasing RGS9–2 function in PD patients may only be a suitable therapeutic strategy when nonselective DA agonists such as L-dopa are used. (MJFF, AHA, NARSAD, FDF).

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RGS Protein Suppression of $G\alpha_o$ protein-mediated α_{2A}-Adrenergic Inhibition of Mouse Hippocampal CA3 Epileptiform Activity

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G-protein coupled α_2 adrenergic receptor (AR) activation by epinephrine (EPI) inhibits epileptiform activity in the mouse hippocampal CA3 region. The mechanism underlying this action is unclear. This study investigated which subtypes of α_2ARs and G-proteins ($G\alpha_o$ or $G\alpha_i$) are involved in this response using recordings of CA3 epileptiform bursts in mouse brain slices. First, we determined that this effect was mediated by the α_{2A}AR subtype as the inhibitory action of EPI on burst frequency was abolished in α_{2A}AR, but not α_{2C}AR knockout mice. Next, using mice with Gα subunit (G184S) knock-ins that prevent inhibition by regulators of G-protein signaling (RGS), we found enhanced α_{2A}AR effects with mutant $G\alpha_o$ but not $G\alpha_{i2}$. These results indicate the EPI-mediated inhibition of mouse hippocampal CA3 epileptiform activity is through an α_{2A}AR $G\alpha_o$ mediated pathway under inhibitory control by RGS proteins. This suggests a role for RGS inhibitors as a novel antiepileptic drug therapy.

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The RGS2 gene product from a candidate hypertension allele shows decreased plasma membrane association and inhibition of Gq

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Hypertension is a leading risk factor for the development of cardiovascular disease. Data from human and animal studies suggest that RGS2, a potent inhibitor of Gq signaling, is important for blood pressure regulation. Recently, several *RGS2* mutations in the Japanese population were found to be associated with hypertension. The product of one of these alleles, R44H, is mutated within the amino terminal amphipathic alpha helix domain, the region responsible for plasma membrane-targeting. The functional consequence of this mutation and its potential link to the development of hypertension, however, are not known. Here, we show that R44H is a weaker inhibitor of receptor-mediated Gq signaling than wild type RGS2. Confocal microscopy reveals that YFP-tagged R44H binds to the plasma membrane less efficiently than wild type RGS2. R44 is one of the basic residues positioned to stabilize lipid bilayer interaction of the RGS2 amphipathic helix domain. Tryptophan fluorescence and circular dichroism studies of this domain show that the R44H mutation prevents proper entrenchment of hydrophobic residues into the lipid bilayer without disrupting helix-forming capacity. Together, these data suggest that decreasing the side chain length and flexibility at R44 prevents proper lipid bilayer association and function of RGS2. Lastly, the R44H protein does not behave as a dominant negative interfering mutant. Thus, our data are consistent with the notion that a R44H missense mutation in human RGS2 produces a hypomorphic allele that may lead to altered receptor mediated Gq-inhibition and contribute to the development of hypertension in affected individuals.

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Regulation of p115RhoGEF by the C-terminus of Gα13

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The monomeric GTPase RhoA regulates a variety of cellular processes, such as actin cytoskeletal rearrangement and gene expression. Two members of the heterotrimeric G-protein family, Gα12 and Gα13, have been shown to mediate signals from GPCRs leading to RhoA activation. In this signaling pathway, one of the GEFs for RhoA, p115RhoGEF, serves as a direct link between Gα13 and RhoA. The goal of the present study is to isolate and characterize the region of Gα13 responsible for stimulating p115's RhoGEF activity. To do this, two sets of Gα12/13 and Gα13/12 chimeras were constructed, in which the fusion point between the two proteins is located after the switch regions or in the α4 helix. The results of cell-based and *in vitro* experiments suggest that the region of Gα13 responsible for stimulation of p115's RhoGEF activity is located between residues 322-377. Further analysis of this region using site-directed mutagenesis revealed that mutating residue R331 in Gα13 to the corresponding residue in Gα12, aspartic acid (D), decreased the ability of Gα13 to activate p115. Interestingly, introducing the R331D mutation and the K204A mutation into Gα13, which has been shown to inhibit Gα13's interaction with p115's RGS domain and impair RhoA activation, failed to further decrease RhoA activity below the level seen with the R331D mutation only. These data suggest a mechanism of p115RhoGEF activation in which Gα13's interaction with both the RGS domain and DH/PH domains of p115RhoGEF are necessary for efficient activation. Furthermore, as R331 lies outside 'classical' effector binding regions, these data suggest that Gα13 regulates its effector p115RhoGEF via a unique biochemical mechanism.

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Septic shock: a role for RGS proteins?

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The profound hypotension in septic shock patients is difficult to treat as they display depressed vascular responses to α-adrenergic agonists. Bacterial lipopolysaccharide (LPS) is the main trigger for most of the cardiovascular alterations occurring in septic shock. Recently, LPS-induced cardiac failure was found to be associated with upregulation of the Regulator of G protein Signalling (RGS) proteins RGS4 and RGS16 (Cardiovasc Res 53:156, 2002). In this study we investigate the effects of LPS exposure on vascular contractility in general and the role of RGS proteins in the LPS-induced vascular alterations. Exposure of rat aortic rings to various LPS concentrations (1, 3, 10, 30 μg ml^{-1}) for 22 hours had differential effects on the contractile responses to agonists at four distinct G-protein coupled receptors. Phenylephrine- and angiotensin II-induced contraction was reduced whereas serotonin-induced contraction was enhanced. The endothelin-1-induced contraction was unaffected. Concomitantly, LPS treatment increased the RGS16 mRNA expression level both in aortic rings and cultured vascular smooth muscle cells (VSMCs) but RGS2, RGS3, RGS4 and RGS5 was unaffected. Regulation of RGS16 mRNA in VSMCs was also time- and concentration-dependent. The changes in RGS16 mRNA might contribute to the differential regulation of the contractile responses in an LPS model of septic shock.

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Control of short-term synaptic plasticity and anxiety circuits by RGS2

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RGS2, one of the small members of the regulator of G protein signaling (RGS) protein family, is highly expressed in brain and regulates Gi/o as well as Gq coupled receptor pathways. RGS2 modulates anxiety, aggression and blood pressure in mice suggesting that RGS2 regulates synaptic circuits underlying animal physiology and behavior. How RGS2 in brain influences synaptic activity is unknown. We therefore analyzed the synaptic function of RGS2 in hippocampal neurons by comparing electrophysiological recordings from RGS2 knock out and wild type mice and show that RGS2 regulates synaptic strength. RGS2 deficient neurons have increased paired-pulse facilitation, which is caused by a shift in the Ca^{2+} dependence of transmitter release to higher Ca^{2+} concentrations leading to a reduced vesicle release probability during action potential stimulations. The decreased Ca^{2+} influx in the absence of RGS2 can be explained by an increase in the membrane delimited, G protein mediated Ca^{2+} channel inhibition involving Gi/o but not Gq coupled receptor pathways. To test the possibility that RGS2 regulates synaptic strength and neuronal activity in neuronal circuits involved in anxiety, we next characterized the expression and function of RGS2 in the serotonergic transmitter system of mice. Our data suggest that RGS2 is expressed within serotonergic neurons early in development and may regulate the spontaneous firing of the serotonergic neurons in the dorsal raphe nuclei.

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Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) may phosphorylate an R7 RGS protein in *C. elegans* to regulate neurotransmission

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CaMKII is a Ser/Thr kinase that undergoes autophosphorylation when Ca^{2+} enters a neuron. This activated CaMKII serves as a molecular memory that a Ca^{2+} spike has occurred and functions to. control neurotransmitter release. Genetic interactions in *C. elegans* suggest CaMKII acts by inhibiting the R7 RGS protein EGL-10. I am investigating whether CaMKII directly phosphorylates EGL-10 to control neurotransmission and behavior. We found that EGL-10 protein from CaMKII mutants undergoes a shift in both one- and two-dimensional gels, suggesting CaMKII modifies the EGL-10 protein. Purified EGL-10 can be phosphorylated *in vitro* by purified rat brain CaMKII. Further, mutating the putative CaMKII phosphorylation sites in EGL-10 leads to increased EGL-10 function in transgenic animals, suggesting CaMKII phosphorylation may inhibit EGL-10. However, *in vitro* phosphorylation of purified EGL-10 does not shift the mobility of the protein. These results suggest that CaMKII may directly phosphorylate EGL-10, but that second phosphorylation-dependent modification may be responsible for the observed gel shift. I plan to use mass spectrometry to identify the CaMKII-dependent modifications in EGL-10, and to measure the *in vitro* GTPase activation activities of modified and unmodified EGL-10. Finally, I will use a FRET based system to monitor CaMKII activity in live animals to determine how phosphorylation of EGL-10 regulates specific behaviors.

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Lysophosphatidic Acid-stimulated cellular migration and activation of p44/42 MAP kinases is modulated by endogenous RGS proteins in Ovarian Cancer Cells

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Lysophosphatidic acid (LPA) is the predominant growth factor driving the progression of ovarian cancer by promoting proliferation, migration, invasion, and survival of ovarian cancer cells. The effects of LPA are mediated through at least five G-protein coupled receptors (GPCRs), all of which are capable of activating multiple G-proteins. We hypothesized that RGS proteins may regulate G-protein signaling pathways initiated by LPA in ovarian cancer cells. To determine the effect of endogenous RGS proteins on LPA signaling in ovarian cancer cells, we compared the activity of LPA in ovarian cancer cells expressing G_i subunit constructs that are either insensitive to RGS protein regulation (RGSi) or their wild-type counterparts (RGSwt) which are sensitive to RGS regulation. LPA-mediated inhibition of forskolin-stimulated adenylyl cyclase activity was enhanced in cells expressing RGSi G_i proteins as compared to RGSwt G_i, suggesting that endogenous RGS proteins in ovarian cancer cells normally attenuate signaling by these G-proteins. Further, LPA-cell migration and p44/42 MAP kinases phosphorylation was enhanced in ovarian cancer cells expressing $G\alpha_{i2}$ RGSi as compared to cells expressing $G\alpha_{i2}$ RGSwt. These data establish RGS proteins as important regulators of LPA signaling in ovarian cancer cells.

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Structural diversity in the RGS domain and its interaction with G-alpha subunits

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RGS proteins accelerate G-alpha GTP hydrolysis and facilitate termination of signaling initiated by G protein-coupled receptors. RGS proteins hold great promise as disease intervention points [Neubig R. & Siderovski D. 2002 Nature Rev Drug Disc. 1, 187-197], given their signature role as negative regulators of GPCRs to which the largest fraction of approved medications are currently directed. The human genome encodes three dozen RGS proteins with varied G-alpha specificities. We have taken a systematic structural biology approach in cataloging the structural diversity present among RGS domains and identifying molecular determinants of their differential G-alpha selectivities. Fourteen new structures were derived from NMR and x-ray crystallography of members of the R4, R7, R12, and RZ subfamilies: ten uncomplexed RGS domains and four RGS domain/G□ complexes. Heterogeneity observed in the structural architecture of the RGS domain, as well as in engagement of switch III and the all-helical domain of the G-alpha substrate, suggests that unique structural determinants specific to RGS protein/G-alpha pairings exist and could be utilized to achieve selective inhibition.

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Deciphering RGS - G protein specificity and selectivity by combining computational and experimental methods

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It has been generally assumed that the majority of "classical" RGS proteins (and in particular isolated RGS domains) interact promiscuously with most Gα subunits. This promiscuity is surprising, given the substantial RGS-domain sequence divergence. More generally, RGS sequence diversity makes it difficult to pinpoint which residues underlie the similar interactions with Gα subunits, and which residues contribute to those cases where selectivity is observed. To understand the structural basis of these interactions, we combined experimental and computational approaches to compare the GTPase activation of $G\alpha_o$ by a large array of RGS domains. As expected, a group of RGS domains (e.g. RGS1, RGS7, RGS16) were found to significantly accelerate $G\alpha_o$ GTPase, and to a similar rate. However, another group (including previously well-characterized RGS2, but also RGS14, RGS17 and RGS18) exhibited significantly lower and more varied GAP activities. Interestingly, the subfamily classification of the RGS domains did not correlate with their activity. Using structure comparison, structure prediction and electrostatic/energetic calculations, we identified key residues that are likely to determine the efficiency of the RGS-$G\alpha_o$ interactions. These residues include, but are not limited to, several residues identified in published studies. This approach provides a framework for understanding Gα-RGS interactions quantitatively at the level of the whole families, and can be generalized to study interaction specificity across other large protein families.

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p115RhoGEF Self-assembles into Homomultimer That Undergo Dynamic Conformational Changes by Interacting with Gα13 and RhoA

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Rho GTPases control fundamental cellular processes, including cytoskeletal reorganization transcription and transformation. Rho guanine nucleotide exchange factors (RhoGEFs) compose a large and diverse family of related proteins that activate Rho GTPases. RGS-RhoGEFs, p115RhoGEF, LARG, and PDZ-RhoGEF, are members of RhoGEF family, exhibiting a unique structural feature containing a homologous region of RGS domain. Gα12 and Gα13 interact with this RGS-like (RGL) domain and regulate the activity of these RhoGEFs. Thus, RGS-RhoGEF serves as a direct link from these heterotrimeric G proteins to RhoA. Interestingly, while characterizing a purified recombinant p115RhoGEF protein by using gel filtration column chromatography, we found that the protein eluted from the column with an apparent molecular weight of approximately 630 kDa, suggesting that p115RhoGEF may form a hexamer in solution. We also observed that p115RhoGEF expressed in HEK293 cells forms oligomer with similar size. The point mutations in a putative coiled-coil domain at the C-terminal region of p115RhoGEF reduced the size of the oligomer to about 400 kDa, indicating that p115RhoGEF contains multiple self-assembling domains. The gel filtration and dynamic light scattering analyses also demonstrated that oligomeric p115RhoGEF interacts with Gα13 and RhoA accompanied with significant conformational changes. These results indicate that p115RhoGEF homooligomer is the functionally active state to interact with Gα13 and RhoA.

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Discovering Alternate Mechanisms of G-protein Regulation through a Coarse-Graining Approach

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Biochemical systems are nonlinear and complex due to the presence of multiple species, reactions and their complexes. Computational analysis of these systems in full detail is impractical due to the unavailability of parameter values. Thus, there is a need to develop methods to reduce the size and complexity of computational models of biochemical networks while retaining mechanistic and functional features and predictive accuracy. Such coarse-graining, in addition to resulting in a reduced-order model, can provide insights into the core mechanisms responsible for eliciting the desired functional response of the biological system and can suggest alternate mechanisms operating in the network. Here we present a mixed integer nonlinear programming-based method for coarse-graining which we tested on a detailed model of the GTPase-cycle module of M1 muscarinic acetylcholine receptor, Gq, and regulator of G-protein signaling 4 (RGS4). When applied to the case study of the GTPase cycle module, the coarse-grained models obtained are better than those obtained using our previous multiparametric variability analysis approach. Additionally, by automatically searching the model-structure space, an alternative mechanism of the GTPase cycle module was found which predicts that, in the presence of RGS, increasing the density of active receptor (analogous to increasing ligand) can result in a biphasic (bell-shaped) response in G-protein activity, a result that has been observed in pharmacology but not explained by RGS. This highlights the hypotheses generation capability of our approach.

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A novel RGS regulates multiple G-alpha subunits during mechano-transduction and fungal pathogenesis

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Conidial germ tubes of rice-blast fungus Magnaporthe grisea must differentiate into an infection structure called the appressorium in order to penetrate its host. Apart from hydrophobicity, the other host-surface characteristics responsible for appressorium initiation are poorly understood. We identified Regulator of G-protein Signaling 1 (Rgs1) based on its essential function in proper appressorium formation and in coupling surface dependency with pathogenesis. Rgs1 is novel and physically interacts with active forms of all three G-alpha subunits (s, i and class II) in Magnaporthe and controls important developmental events during asexual and pathogenic development (Liu et al., 2007). Chemical genetic studies and global transcriptome analyses related to surface signaling indicated that thigmo-morphogenesis is initiated within two hours after conidia germination and uses calcium signaling mediated by ion channels. Biophysical analyses allowed us to estimate the critical hardness necessary for efficient initiation of infection in Magnaporthe. Our preliminary results suggest a possible role for stretch-activated ion channels and a non-canonical GPCR in hardness sensing and host infection. These will be discussed along with a possible function for RGS1 in elaborating the extra-cellular matrix during the pathogenic development in Magnaporthe. [Collaborators: David Siderovski, Francis Willard, Shen Lu]

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A Novel Mechanism of Translational Control by RGS2

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We have identified a novel role for RGS2 in the control of protein synthesis that is independent of its established RGS domain function. The objective of this research was to characterize further the molecular determinants for this new aspect of RGS2 signaling. RGS2 was found to bind to the eukaryotic initiation factor 2 ε-subunit (eIF2Bε) in a yeast two-hybrid screen, and this interaction was confirmed at the protein level by co-immunoprecipitation. RGS2 was able to significantly decrease *de novo* protein synthesis *in vitro* and also in a cell-based assay, whereas RGS1 and RGS4 did not interact with eIF2Bε or have an effect on protein synthesis. The ability of RGS2 to interact with eIF2Bε and inhibit protein synthesis was dependent on a subdomain within its RGS domain, spanning residues 79-116. An RGS2 peptide based on this eIF2Bε-interacting region was capable of inhibiting protein synthesis in the *in vitro* translation assay.

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Lean Phenotype of RGS2 Deficient Mice

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RGS proteins negatively regulate G protein signaling via their actions as GTPase activating proteins (GAPs) and effector antagonists. RGS2 is selective for Gq over Gi/o proteins in GTPase activity assays, and is also able to inhibit signals through Gs-coupled receptors. RGS2 knockout mice are fertile and observed phenotypes include decreased male aggression, increased anxiety, reduced T cell proliferation and hypertension. We have recently discovered a previously unreported phenotype of the *rgs2*$^{-/-}$ mice in our laboratory in which they are resistant to age-induced obesity. Thus, 21-24 month old animals weigh significantly less than age-matched wild-type mice, apparently due to decreased fat storage since *rgs2*$^{-/-}$ mice had significantly decreased epididymal white adipose tissue stores. Serum lipids and adiposity markers were also altered in aged *rgs2*$^{-/-}$ mice. In young mice a difference in body weight was significant from 4 weeks of age. At 3 months old there was no difference in basal body temperature or food intake, however liver weight was significantly lower in *rgs2*$^{-/-}$ mice and gene expression of UCP-1 and SREBP-1c was reduced, suggesting a possible impairment in adipocyte differentiation. In conclusion we report a novel role of RGS2 in body weight regulation which may be due to altered energy balance and/or adipocyte function.

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Using *C. elegans* to investigate R7 protein complex formation, function and localization

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R7 family RGS proteins exist in complexes with a $G\beta_5$ subunit and a membrane-anchoring protein. The function of $G\beta_5$ is unknown, and whether reversible lipid modifications on the anchoring protein dynamically regulate these complexes is unclear. To resolve these issues, we are using the powerful combination of genetic and biochemical techniques possible in *C. elegans* to investigate how R7 protein complexes form, function and localize in live animals. In the *C. elegans* nervous system, the G proteins $G\alpha_o$ and $G\alpha_q$ act antagonistically to regulate neurotransmitter release. $G\alpha_o$ and $G\alpha_q$ are regulated by the R7 proteins, EGL-10 and EAT-16, respectively, which each require $G\beta_5$. Null mutations in $G\beta_5$ disrupt EGL-10 and EAT-16 activity equally, resulting in no net effect on neurotransmitter release. By analyzing an unusual set of point mutations in $G\beta_5$ that unexpectedly increase neurotransmitter release, apparently by differentially affecting EGL-10 and EAT-16 function, we hope to obtain new insights into the function of $G\beta_5$ and the specificity of the R7•$G\beta_5$ interaction. To date no membrane anchoring protein has been described in *C. elegans*. However we have identified a candidate, generated a knockout strain and begun characterization. Preliminary data unexpectedly suggests that this protein is required for EAT-16 but not EGL-10 function. We have also generated transgenic animals containing a functional fluorescently-tagged version of each R7 protein. By genetically altering signaling and observing the effects on R7 protein localization and function, this powerful system will allow us to further investigate the mechanisms by which the anchor protein may dynamically regulate the localization and activity of R7 protein complexes.

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High Throughtput Screening Using A Polyplexed Flow Cytometry Protein Interaction Assay (FCPIA) for Small Molecule Inhibitors of Regulator of G protein Signaling (RGS) Proteins

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Signaling networks are comprised of many combinations of protein-protein interactions and present a number of targets for potential drug discovery. Here, we focus on the interaction of Regulators or G protein signaling (RGS) proteins with the G protein Gαo, using a flow cytometery protein interaction assay (FCPIA). FCPIA is capable of accurately measuring this protein-protein interaction and has been used in high throughput screening, resulting in the discovery of a small molecule RGS4 inhibitor (Roman, Talbot et al. 2007). We have expanded on this method by performing a high throughput screen in a polyplexed format. In this method, we attach 5 different RGS proteins (4,6,7,8 and 16) to 5 uniquely dyed bead sets and co-incubate them with fluorescently labeled Gαo to measure the protein-protein interaction. We established Z' factor values for individual RGS proteins in multiplex of 0.73 to 0.92, indicating a suitable HTS method. For the screen, 5 RGS proteins on beads were combined and aliquoted into 96 well plates that contained 4 distinct compounds per well, incubated with fluorescently labeled Gαo, and then measured using FCPIA. This approach was used to screen 8,000 compounds. After individual well deconvolution, we have preliminarily identified a number of RGS and RGS-subfamily specific novel RGS inhibitors, on which further mechanistic evaluation is underway.

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Inhibitors of RGS4 Identified in a One-Bead, One-Compound Peptide Library

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RGS proteins accelerate the hydrolysis of GTP by the Gα subunit of the heterotrimeric G-protein and therefore quicken the inactivation of G-protein signaling. The distinct expression patterns and pathophysiologic regulation of RGS proteins suggest that inhibitors of their activity may have therapeutic potential. The objective of this study is to identify peptide inhibitors of RGS4. We synthesized a focused One-Bead, One-Compound peptide library that retains structural features known to be necessary for the activity of a previously identified peptide. The library of 2.5 million peptides was screened and peptide-beads with increased binding to a fluorescently labelled RGS4 were isolated. One hit peptide, (**2**, Ac-Gly-Thr-[Cys-Phe-Gly-Thr-Cys]-Trp-NH_2, S-S) inhibited RGS4 activity in a GTPase assay with some RGS selectivity. Another hit peptide (**9**, Tyr-Trp-[Cys-Lys-Leu-Cys]-Lys-NH_2, S-S) blocked the interaction between biotin-RGS4 on avidin beads and a fluorescent Gαo with an IC50 of 28 μM in a Flow Cytometry Protein-Protein Interaction Assay (FCPIA). It also had RGS selectivity. Surprisingly, inhibition of RGS4 by **9** was irreversible. Current efforts include determining how the RGS is modified using mutagenesis and mass spectrometry. In conclusion, although the library was designed based on the structure of a lead compound, the hit peptides do not necessarily have similar mechanisms of action as the lead. This study provides new peptide inhibitors of RGS proteins with novel mechanisms.

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The receptor type-specific attenuation of G protein signaling by RGS8

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We previously identified RGS8, which belongs to the B/R4 subfamily and specifically binds to Gαo and Gαi3. We also identified a short splice varient, RGS8S, which has unique N-terminal 7 amino acids instead of 1-9 amino acids of RGS8. It was revealed that RGS8 decreased the response upon activation of M1 muscarinic receptor (mAChR), but did not remarkably inhibit the signaling from M3 receptor. In contrast, RGS8S showed much less inhibition of the response of either of the Gq-coupled receptors. Thus, RGS8 suppressed Gq signaling depending on the receptor types in spite of its weak binding to the Gαq protein, but the inhibitory activity of RGS8S was weak. To elucidate molecular mechanisms underlying receptor-specific attenuation by RGS8, we first examined whether or not RGS8 interacts with certain G-protein-coupled receptors (GPCRs). By pull-down assay, bioluminescence resonance energy transfer (BRET) experiments, and electrophysiological analyses, it was demonstrated that RGS8 directly interacts with M1-mAChR via its N-terminus (6-9aa, MPRR) at the third intracellular loop (i3L) of the receptor, and this binding was shown to play important roles in receptor type-specific suppression by RGS8. To further explain the receptor type-specific regulation by RGS8, we next carried out a two-hybrid screening for RGS8-interacting proteins and identified Spinophilin (SPL). SPL has been reported as a scaffolding protein binding to i3L of several GPCRs. By *in vitro* binding assay using a series of deletion mutants of RGS8, the SPL binding site was found to be localized at the MPRR, which was previously characterized as the M1 receptor binding site. Then, we observed that the M1 receptor and SPL compete for the same binding site of RGS8 by BRET and pull-down experiments using recombinant proteins. We next examined how SPL modifies the regulatory function of RGS8 on the M1 signaling. Interestingly, we observed that the expression of SPL significantly enhances the inhibitory function of RGS8. These results suggest that SPL removes directly bound RGS8 from the M1 receptor, but that the M1 receptor is regulated by RGS8 indirectly bound to the M1 through SPL. This signaling complex was considered to function more efficiently. In summary, it was shown by the present study that RGS8 selectively regulates receptor system by using the direct and indirect receptor-recognition, that SPL is involved in the indirect receptor-recognition and that the presence of SPL may change the selectivity and efficiency of regulatory function of receptor signalings by RGS8.

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Oxidative Stress Increases RGS2 Expression which Suppresses Increased cAMP Accumulation in Response to CRF Activation in CATH.a Cells

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Regulator of G-protein Signaling Protein (RGS)-2 has been suggested as a modulator of anxiety and dysregulation of oxidative stress is implicated in the development of anxiety. In the present study, we report that hydrogen peroxide-induced oxidative stress increases RGS2 expression and increases nuclear translocation of RGS2 in CATH.a cells. CATH.a cells express CRF_1 receptors and RGS2 and are a model of locus coeruleus neurons. It is well known that stress causes excessive CRF_1 receptor activation, increased cAMP production and increased locus coeruleus firing. We have observed that the cAMP response to CRF_1 receptor activation is reduced by chronic hydrogen peroxide treatment in CATH.a cells. This reduced cAMP response may be the result of direct inhibition of adenylyl cyclase by RGS2 in response to oxidative stress. In addition, expression of antioxidant proteins including superoxide dismutase and heme oxygenase are also increased in response to increased oxidative stress. In conclusion, we hypothesize that the hydrogen peroxide treatment increases RGS2 expression as part of a compensatory mechanism to protect against oxidative stress. This protection is via two mechanisms; 1) increasing the expression of antioxidant proteins, and 2) direct inhibition of adenylyl cyclase to reduce cAMP accumulation caused by excessive CRF_1 receptor activation.

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RGS2 is repressed by Flt3-ITD and inhibits Flt3-ITD mediated transformation of myeloid progenitor cells

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Activating mutations of the receptor tyrosine kinase Flt3 are one of the most common genetic aberrations in Acute Myeloid Leukemia (AML) and cause malignant transformation of myeloid progenitors in vitro and in vivo. We identified RGS2 as a gene repressed by Flt3-ITD mutations in 32D cells. RGS2 belongs to the R4 family RGS-Proteins and is an effective inhibitor of G-alpha-q. Further Gene expression analyses revealed a significant repression of RGS2 mRNA in primary bone marrow of AML patients compared to healthy bone marrow, with very low expression in Flt3-ITD positive samples. Coexpression of RGS2 with Flt3-ITD in 32D antagonized Flt3-ITD induced transformation and signaling events. Furthermore, RGS2 coexpression could overcome the block differentiation caused by Flt3-ITD. We also found strong induction of RGS2 in myeloid cell lines during granulocytic differentiation. Studies on the mechanism of action of RGS2 in myeloid cells revealed impact of the RGS2 target G-alpha-q in Flt3-ITD induced transformation. Further, we found nuclear localization and DNA-binding of RGS2 in these cells. This study shows a close interaction between an activated receptor tyrosine kinase and heterotrimeric G-protein signaling in transformation and differentiation of myeloid cells. Furthermore, it gives hints to so far unknown cellular effects or RGS2.

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Mice lacking RGS protein activity at Gαi2 exhibit a 5HT1A receptor-mediated antidepressant-like phenotype

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Serotonin (5HT) acting through 5HT1 receptors plays an integral role in regulating mood. Multiple lines of evidence suggest 5HT1 receptors preferentially couple to Gαi2 relative to the more abundant Gαo. Therefore, transgenic "knock-in" mice that express RGS-insensitive Gαi2 (G184S; RGS-i) were evaluated in the tail suspension test (TST), a classical assay of antidepressant-related behavior in rodent models. In the TST, RGS-i animals exhibited basal antidepressant-like behavior and were 5-10 times more sensitive to the antidepressant-like effects of the SSRI fluvoxamine and the 5HT1A-selective agonist 8-OH-DPAT. In contrast, the antidepressant potency of the norepinephrine reuptake inhibitor desipramine was unchanged. Yet, 8-OH-DPAT-mediated hypothermia was unaltered in animals of either genotype. Furthermore, the antidepressant-like phenotype of RGS-i animals was completely reversed by treatment with the 5HT1A-selective antagonist WAY-100635 without altering spontaneous motility. These data point to a critical role for endogenous RGS protein regulation at Gαi2 in mediating the antidepressant-like effects of 5HT1A receptor activation. By selectively enhancing the antidepressant effects of serotonin, RGS proteins represent a novel therapeutic target for the treatment of depression. Supported by GM39561 and the Bower, Bennet, & Bennet Endowed Chair Research Award.

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Genetic deletion of RGS protein activity enhances buprenorphine antinociception while limiting withdrawal behaviors associated with chronic administration

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Understanding the mechanisms that govern opioid signaling is important to improve therapy for pain while limiting abuse potential. Opioid signaling is controlled by regulators of G protein signaling (RGS) proteins. In vitro evidence suggests that partial efficacy mu-opioid agonists such as buprenorphine are more sensitive to RGS protein regulation than those that are fully efficacious. To determine the relevance of this in vivo, transgenic "knock-in" mice were developed that express RGS-insensitive Gαi2 (G184S; RGS-i). Buprenorphine was twice as efficacious in RGS-i animals compared to wild-type littermates in antinociceptive tests. However, morphine activity was identical in animals of either genotype. In contrast, when administered chronically (5-day treatment), both buprenorphine and morphine induced the same degree of tolerance in RGS-i and wild-type animals, indicating that mechanisms underlying opioid tolerance are insensitive to RGS regulation of Gαi2. Importantly, withdrawal behaviors, such as naltrexone-precipitated jumping, were substantially reduced in chronically-treated RGS-i mice relative to wild-type littermates. These data suggest that inhibition of RGS protein activity at Gαi2 leads to enhanced antinociception but reduced physical dependence to buprenorphine.

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RGS10 Promotes Dopaminergic Neuron Survival Via Regulation of the Microglial Inflammatory Response

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Epidemiological studies suggest that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) lowers the incidence of Parkinson's disease (PD) in humans and implicate neuroinflammatory processes in the death of dopamine (DA) neurons. We tested the hypothesis that Regulator of G protein signaling-10 (RGS10), a microglia-enriched GTPase accelerating protein (GAP) for Gα subunits modulates microglial activation in the brain. RGS10-deficient mice displayed increased microglial burden in the CNS and exposure to chronic systemic inflammation induced nigrostriatal pathway degeneration, suggesting a protective role for RGS10. To investigate how RGS10 regulates microglial phenotype and impacts DA neuron survival, we established a functional assay utilizing BV2 cells and MN9D dopaminergic cells as effector and target cells, respectively. Knockdown of RGS10 in the BV2 microglia cell line resulted in dysregulated inflammatory gene expression, overproduction of Tumor Necrosis Factor (TNF), and enhanced neurotoxicity of BV2 microglia on MN9D dopaminergic cells which was abolished by addition of the TNF decoy receptor etanercept. Given the neuroprotective effects of RGS10 against inflammation-induced death of DA neurons, RGS10 emerges as a novel drug target for the treatment of neurodegenerative conditions like PD. *[NIH-NINDS R01RNS049433-01]*

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Identification of Gαq mutants specifically uncoupled with PLCβ and GRK2

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The activation of PLCβ isozymes by Gαq produces DAG and IP_3 from PIP_2 and plays critical functions in a variety of physiological processes. Recently, we established a novel method to purify soluble Gαq in large scale with Sf9-baculovirus expression system using Gαi/q chimera which consists of the amino-terminus of Gαi1 followed by Gαq. Using this soluble Gαq, the atomic structure of Gαq-GRK2-Gβγ complex was determined. The structure of the complex demonstrated that RGS domain of GRK2 binds with the interface of Gαq which is similar to the PLCβ-interacting surface (effector interface) but different from RGS3/4-interacting surface (GAP interface). Here, in order to further investigate the molecular mechanism of the interaction of Gαq with PLCβ and GRK2, we isolated Gαq mutants specifically uncoupled with each target; GRK2-insensitive mutant (Y261L), PLCβ-insensitive mutant (A253K), and GRK2/PLCβ-insensitive mutant (T257E). The results indicate that Gα subunit interacts with multiple effectors with specific footprint on effector interface. Together with RGS3/4-insentitive mutant (R214A), our new effector selective Gαq mutants will become useful tools to elucidate the molecular mechanism of the regulation of GPCR-Gq signaling.

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Molecular Studies of Regulators of G-Protein Signaling (RGS) Proteins in Prostate Cancer

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Prostate cancer is the most commonly diagnosed noncutaneous cancer in American men and the second leading cause of cancer mortality. Acquisition of androgen independence and metastasis by prostate cancer are the key problems during prostate cancer treatment. The precise mechanisms underlying progression of prostate cancer to a state of androgen-independence remain an active area of research. Several lines of evidence suggest that androgen-independent activation of androgen receptor (AR) is one underlying mechanism of androgen independence. More recently, it has been shown that AR can be indirectly activated by factors other than androgens. These factors often activate cell-surface receptors such as G-protein coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are major regulators of important cellular responses. Aberrant GPCR signaling contributes to many human diseases including prostate cancer. Regulators of G-protein Signaling (RGS) proteins inhibit GPCR signaling, thus playing a key role in the regulation of GPCR function *in vivo* (Fig.1).

Because RGS proteins regulate cellular proliferation and differentiation, they have been suggested as potential tumor suppressors. We recently reported in *Oncogene* that RGS2, a member of the RGS protein superfamily, is specifically down-regulated in androgen-independent prostate cancer cells. Re-expression of RGS2, but not other RGS proteins, significantly inhibited androgen-independent AR activity in androgen-independent prostate cancer cells. RGS2 inhibits Gq-coupled GPCR signaling. In prostate cancer cells, expression of RGS2 blocked androgen-independent activation of AR by constitutively activated Gq or Gq-coupled α1A-adrenergic receptors. Furthermore, extracellular signal-regulated kinases 1/2 (ERK1/2) were required for RGS2-mediated regulation of androgen-independent AR activity.

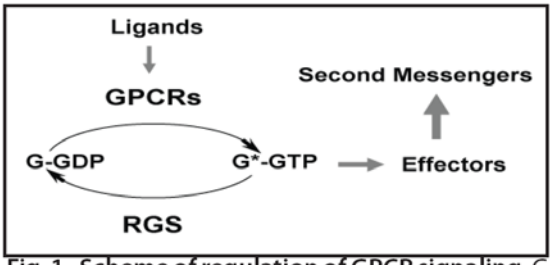

Fig. 1. Scheme of regulation of GPCR signaling. G proteins stimulate intracellular signaling proteins (effectors) when they bind GTP in response to ligand bound GPCRs; signaling ends when bound GTP is hydrolyzed. RGS proteins stimulate GTP hydrolysis of G proteins by as much as 1000-fold.

We also observed that altering RGS2 expression levels inversely affected androgen-independent proliferation of prostate cancer cells, and ectopic overexpression of RGS2 in androgen independent prostate cancer cells by RGS2-adenovirus infection suppressed tumorigenicity of xenografts in castrated nude mice (Fig. 2). In addition, the weight of prostate glands significantly increased in RGS2-knockout mice, suggesting that RGS2 plays a role in regulating prostate cell growth.

Interestingly, we found that RGS2 repression in androgen-independent prostate cancer cells is not simply due to loss of RGS2 gene. Instead, the RGS2 gene promoter is hypermethylated and the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine is able to induce re-expression of RGS2 in androgen-independent prostate cancer cells, which provides a mechanism for the RGS2 repression in human prostate tumors.

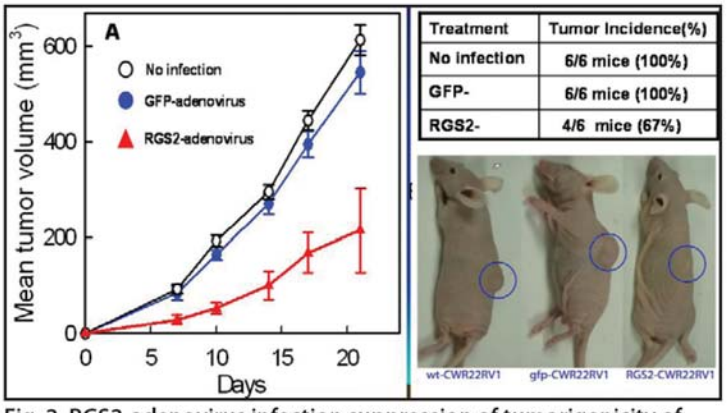

Fig. 2. RGS2-adenovirus infection suppression of tumorigenicity of xenografts in castrated nude mice.

Based on these results, we put forward a novel concept that repression of a specific member of the RGS family such as RGS2 contributes to androgen-independent prostate cancer progression. Moreover, our research also has several important translational implications: first, our studies may provide potential novel diagnostic markers for early tumor detection and prognosis prediction for prostate cancer, and, second, it may provide additional therapeutic targets and treatment strategies.

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Rgs16 is a Glucose-responsive Inhibitor of G□11-stimulated Fatty Acid Oxidation in Liver

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G protein coupled receptor (GPCR) pathways regulate glucose and fatty acid metabolism and feeding behaviors that influence the onset of obesity and metabolic syndrome. Regulators of G protein Signaling (RGS) proteins are negative regulators that control the intensity and duration of G protein signaling. One RGS gene, *Rgs16*, is up regulated 20-fold in liver during fasting and rapidly down regulated by feeding. Rgs16 is specifically expressed in periportal hepatocytes that preferentially oxidize fatty acids and produce glucose during fasting. Interestingly, the bHLH transcription factor carbohydrate response element binding protein (ChREBP) is required for glucose-dependent induction of *Rgs16* transcription. Transgenic mice that express Rgs16 protein specifically in liver have reduced expression of genes promoting fatty acid oxidation, lower rates of fatty acid oxidation in liver extracts, and lower plasma □-ketone levels. By contrast, fasted Rgs16 knockout mice exhibit the reciprocal phenotypes, increased fatty acid oxidation gene expression, increased fatty acid oxidation in liver and elevated plasma b-ketone levels. Rgs16 is a glucose-responsive inhibitor of G□11-stimulated fatty acid oxidation in liver. We are currently investigating broader roles of Rgs16 in glucose homeostasis in healthy, obese and diabetic conditions.

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RGS13 Suppresses CREB-dependent Gene Transcription

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cAMP response element binding protein (CREB) promotes transcription of genes involved in neuronal development, metabolism, and immunity. Norepinephrine (NE) secreted into lymphoid organs stimulates Gαs-coupled β2-adrenergic receptors on B lymphocytes, leading to CREB-dependent transcription of the Oct 2 coactivator (OCA-B). Regulator of G protein Signalling (RGS) proteins attenuate GPCR signaling by inhibiting Gαi and Gαq but not Gαs. We show that B-cell-enriched RGS13 is a direct transcriptional repressor of CREB. Phosphorylation of CREB induced RGS13 nuclear translocation, where it bound pCREB and inhibited CREB-DNA contact. RGS13 abundance affected pCREB promoter occupancy and CREB target gene transcription in HEK293T cells. Two discontinuous sites on RGS13 (aa15-33 and aa93-117) were required for CREB binding and inhibition of CREB activity, which depended on Arg13, Glu15, and Ser104 within these two regions. RGS13 interacted with the kinaseinducible domain (KID) of CREB, which required the α-helical conformation of KID induced by CBP binding. *Rgs13*./. B cells exhibited increased β2AR-stimulated pCREB-DNA binding and OCA-B expression. Thus, RGS13 may impact humoral immune responses under sympathetic neural control and other physiological responses mediated by CREB.

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RGS2 is a negative regulator of $G_{q/11}$-mediated signaling and function in adult rat ventricular fibroblasts

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Cardiac fibroblasts (CF) contribute to cardiac remodeling by assuming an "activated" myofibroblast (MyoFb) phenotype. Many G protein-coupled receptors are involved in CF activation. G proteins are controlled by Regulators of G protein Signaling (RGS). RGS expression and function in CF and MyoFb are unknown. We set out to identify which RGS are expressed in isolated adult rat ventricular CF (passage P0-P2) and to begin to delineate their function. Morphologic changes and expression of MyoFb markers (e.g., α-smooth muscle actin) indicated gradual conversion from CF (P0) to MyoFb (P2). We detected mRNA of 14 RGS by RT-PCR that were differentially regulated during CF to MyoFb conversion. Only RGS2 mRNA was markedly up-regulated in response to short-term stimulation of $G_{q/11}$-coupled Angiotensin II (Ang II) and G_s-coupled β-adrenergic receptors. Adenoviral RGS2 expression blunted Ang II-induced phospholipase C β activation by 65±2% (P0), 66±2% (P1) and 78±3% (P2) (n=3, P<0.05), CF proliferation (BrdU incorporation) by 37±5% (n=10, P<0.05) and collagen synthesis by 49±11% (n=3, P<0.05). RGS2 had no effect on G_s-coupled cAMP accumulation. Thus, while RGS2 is selectively up-regulated in response to $G_{q/11}$ and G_s activation, it negatively regulates only $G_{q/11}$-mediated signaling, proliferation and collagen synthesis in CF and MyoFb.

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Regulation of RGS4 Gap Activity by Goloco Motif Containing Protein G18

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Heterotrimeric G proteins play an important role in cellular signaling, which occurs via a complex network of signaling proteins and other molecules. Factors that can regulate G protein signaling include G protein coupled receptors, RGS proteins and proteins that contain GoLoco motifs. The latter bind to inactive Gαi/o and impede GDP dissociation. G18 (also known as AGS4 or GPSM3) is an 18kDa protein that contains three tandem GoLoco motifs and a short proline-rich N terminus. We examined the binding of G18 to Gαi1 and Gαo as well as its effects on the GTPase activities of these two G proteins under steady-state conditions in the presence and absence of RGS4. Both full length G18 (wtG18) and an N terminal truncated form of G18 (Δ N-G18) bound to the inactive form of Gαi1 but not Gαo. Surprisingly, wtG18 also bound to the AlF_4^- activated forms of Gαi1 and Gαo. Activated Gαo, but not Gαi1, bound to a full length G18 mutant in which the three GoLoco motifs were nonfunctional (G18mGL), but neither of the activated G protein bound to Δ N-G18. An N terminal deletion mutant of G18 with non-functional GoLoco domains (Δ N-G18mGL) did not bind to inactive or activated Gαi1 or Gαo. In functional assays, wtG18, G18mGL and Δ N-G18 all inhibited receptor- and RGS4-promoted Gαi1 and Gαo GTPase activities, whereas Δ N-G18mGL had little or no effect. Taken together, these results implicate the N terminal domain of G18 as a previously unrecognized G protein binding domain which may regulate G protein signaling in a novel manner.