The Division for Molecular Pharmacology of
The American Society for Pharmacology and Experimental Therapeutics
Presents:

The Second RGS Protein Colloquium

Organized by:

Vadim Y. Arshavsky
Harvard Medical School and Massachusetts Eye and Ear Infirmary
and
David P. Siderovski
University of North Carolina at Chapel Hill

April 17, 2004
Congressional Hall B
Renaissance Washington, D.C. Hotel
999 Ninth Street, N.W., Washington D.C.

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999 Ninth Street, N.W., Washington, D.C.
9:00 am – 5:30 pm

Chairs: Vadim Y. Arshavsky and David P. Siderovski

Time	Speaker
9:00 AM	**Introduction**
9:10 AM	***RGS proteins: Past, present, future*** David P. Siderovski, *University of North Carolina at Chapel Hill*
9:40 AM	**Regulator of G-protein signaling 2 mediates vascular smooth muscle relaxation and blood pressure** Michael Mendelsohn, *Tufts University*
10:10 AM	***RGS protein control of centrosome movement* during mitosis in *C. elegans* embryos** Michael Koelle, *Yale University*
10:40 AM	**COFFEE BREAK**
11:00 AM	**Role of the RGS domain in G protein-coupled receptor kinase function** Jeffrey L. Benovic, *Thomas Jefferson University*
11:30 AM	**Short talk: Crystal structure of the p115RhoGEF rgRGS domain in a complex with Galpha(13):Galpha(i1) Chimera: A novel G protein GAP mechanism** Zhe Chen, *University of Texas Southwestern Medical Center*
11:45 AM	**RGS insensitive G proteins as probes of physiological RGS function** Richard R. Neubig, *University of Michigan*
12:15 PM	**LUNCH & POSTERS**

2:15 PM **Short talk: Targeted disruption of *Rgs1* leads to excessive B-lymphocyte response to chemokines, disturbed plasma cell localization, and distorted immune tissue architecture**
Chantal Moratz, *NIAID, NIH*

2:30 PM **Short talk: RGS9-2 is a negative modulator of morphine actions**
Venetia Zachariou, *University of Texas Southwestern Medical Center*

2:45 PM **Mechanisms of feedback inhibition by RGS protein induction and turnover**
Henrik G. Dohlman, *University of North Carolina at Chapel Hill*

3:15 PM **Investigation of RGS proteins toward modulation of neurobiological disorders**
Kathleen H. Young, *Wyeth Research*

3:45 PM **COFFEE BREAK**

4:15 PM **Functional analysis of RGS proteins in intact cells: Lessons from photoreceptors**
Marie E. Burns, *UC-Davis*

4:45 PM **Building RGS protein specificity through its domain composition**
Vadim Y. Arshavsky, *Harvard Medical School and Massachusetts Eye and Ear Infirmary, Boston*

Presented by:
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Speaker Abstracts

RGS Proteins: Past, Present, Future

David P. Siderovski
University of North Carolina at Chapel Hill

To open the Second RGS Protein Colloquium, Dr. Siderovski will present a brief historical overview of the discovery of the "regulators of G-protein signaling" (RGS) protein superfamily and survey the current "state-of-the-art". Interspersed will be vignettes from Dr. Siderovski's own research, including recent work in developing real-time, fluorescence-based approaches to measuring RGS protein action (*e.g.*, Kimple *et al.*, 2003) and mining the genomes of diverse organisms to uncover novel members of the RGS protein superfamily (*e.g.*, Chen *et al.*, 2003)

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A seven-transmembrane RGS protein that modulates plant cell proliferation
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Regulator of G-protein Signaling 2 Mediates Vascular Smooth Muscle Relaxation and Blood Pressure

Michael E. Mendelsohn, M.D, Mary Tang, Ph.D.[1], Guang Wang [1], M.D.,
Ping Lu [1*], M.D., Richard H. Karas[1], M.D., Ph.D., Mark Aronovitz[1],
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Vascular contraction by many agonists is mediated by G_q-coupled receptor activation, calcium mobilization and myosin light chain (MLC) phosphorylation. Nitric oxide (NO) inhibits vascular contraction by activating cGMP-dependent protein kinase I α (PKGI-α), which causes MLC dephosphorylation and vascular smooth muscle relaxation. In recent studies, we find support for a model in which PKGI-α attenuates signaling by the G_q-coupled thrombin receptor PAR-1 by directly activating the regulator of G-protein signaling, RGS2. NO donors and cGMP cause cGMP-mediated inhibition of PAR-1 and membrane localization of RGS2. PKGI-α binds directly to and phosphorylates RGS-2, significantly increasing the GTPase activity of G_q. Disruption of the RGS2-PKGIα interaction reverses inhibition of PAR-1 signaling by nitrovasodilators and cGMP. In addition, Rgs2 $^{-/-}$ mice develop marked hypertension and their blood vessels have enhanced contraction and decreased cGMP-mediated relaxation. Thus, PKGIα binds to, phosphorylates and activates RGS-2, attenuating G_q-coupled receptor-mediated vascular contraction. The presence of vasomotor dysfunction and hypertension in the RGS-2 knockout mice raises the possibility that vasoconstriction alone is sufficient to cause hypertension, a hypothesis that is being tested at present. Vasoconstriction alone could produce hypertension in at least two different ways. Hypertension could arise de novo from alterations in the 'set point' of resting vascular tone of the resistance vessels that can modify blood pressure. Alternatively, intrinsic abnormalities in the vascular smooth muscle cells of renal vessels might produce altered renal perfusion, secondarily creating the recognized effects on the kidney that promote and/or sustain blood pressure elevations. In summary, the data to be discussed support that vasodilatators facilitate a decrease in vascular smooth muscle tone by PKGI-α-mediated activation of RGS-2 and a consequent reduction of signaling by G_q-coupled receptors. Thus, RGS-2 is required for normal vascular function and blood pressure and may be a useful new drug target for therapies to treat abnormalities of blood pressure.

RGS Protein Control of Centrosome Movement During Mitosis in *C. elegans* Embryos

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To identify the biological functions of RGS proteins, we produced knockout mutations for all 12 *C. elegans* RGS genes. Only one of these genes, *rgs-7*, is required for viability. *rgs-7* mutants die as embryos, and time-lapse video microscopy shows defects in mitosis starting with the first cell division of the zygote. This division is asymmetric, resulting in daughter cells of different sizes that take on different developmental fates. For this division to occur correctly, microtubule-based forces must act on the two centrosomes so that they generate a mitotic spindle in the proper orientation and position within the mother cell. In wild-type embryos, the posterior centrosome is subjected to greater pulling forces than is the anterior centrosome, and the posterior centrosome thus undergoes faster and larger movements, resulting in an asymmetrically positioned mitotic spindle and an asymmetric cell division. In *rgs-7* mutant embryos, the posterior centrosome undergoes movements that are even faster and larger than in the wild type, resulting in a hyper-asymmetric first cell division. Centrosome movement defects continue to occur in subsequent cell divisions, eventually leading to death of the embryo.

RGS-7 functions by regulating two redundant $G\alpha_o$ proteins, GOA-1 and GPA-16. Double RNAi inactivation of these G proteins results in slower and smaller movements of the posterior centrosome, a defect precisely opposite that seen in *rgs-7* mutants[1]. The purified RGS domain of RGS-7 can act as a GTPase activator on purified GOA-1 protein, and GOA-1/GPA-16 double RNAi is genetically epistatic to the *rgs-7* knockout mutation. These results suggest that the GOA-1/GPA-16, in their GTP-bound forms, somehow increase forces on the posterior centrosome, and that RGS-7 inactivates the G proteins by driving them to hydrolyze GTP.

RGS-7 acts as part of an unconventional G protein cycle in which a set of soluble proteins substitute for the role usually played by a seven-transmembrane receptor. Genetic experiments suggest that two redundant GoLoco domain proteins, GPR-1 and GPR-2, as well as another soluble protein, RIC-8, function together to activate the GOA-1/GPA-16 G proteins[2,3]. RNAi inactivation of GPR-1/2 or RIC-8 results in a phenotype similar to that seen in GOA-/1GPA-16 RNAi, and all of these RNAi treatments are epistatic to the *rgs-7* knockout mutation. We have also shown that purified RIC-8 protein has guanine nucleotide exchange activity on purified GOA-1 G protein. These results are consistent with a model in which GPR-1/2 and RIC-8 function to generate active Gα-GTP protein, while RGS-7 functions as a GTPase activator to eliminate Gα-GTP. Genetic studies show that a similar unconventional G protein cycle controls asymmetric cell divisions in *Drosophila*[4], and the proteins involved all have mammalian homologs that are likely to control centrosome forces in mammalian cell divisions.

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Role of the RGS Domain in G Protein-coupled Receptor Kinase Function

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G protein-coupled receptors (GPCRs) transduce cellular signals from hormones, neurotransmitters, light and odorants by activating heterotrimeric guanine-nucleotide binding (G) proteins. For many GPCRs, short-term regulation is initiated by agonist-dependent phosphorylation by GPCR kinases (GRKs) resulting in G protein/receptor uncoupling. GRKs are serine/threonine protein kinases and consist of three primary domains, an N-terminal RGS homology (RH) domain, a central kinase catalytic domain, and a C-terminal lipid-binding domain. Interestingly, the recent crystal structure of GRK2 reveals that the RH domain consists of two discontinuous regions with the characteristic nine-helix bundle in the N-terminal region and two additional helices following the kinase domain. The RH domains of GRK2 and GRK3 have been shown to specifically interact with $G\alpha_q$ family members including $G\alpha_q$, α_{11}, and α_{14} but not $G\alpha_{16}$, $G\alpha_s$, $G\alpha_i$, or $G\alpha_{12/13}$ (1, 2). In contrast, the RH domains of GRK5 and GRK6 do not appear to bind $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$, or $G\alpha_{12/13}$ (1). GRK2 binds effectively to both the GDP/AlF_4^- and GTPγS forms of $G\alpha_q$ and possesses weak GAP activity toward $G\alpha_q$. Nevertheless, GRK2 serves as an effective inhibitor of $G\alpha_q$-mediated activation of phospholipase C-β both *in vitro* and in intact cells, most likely via sequestration of activated $G\alpha_q$. Recent studies have demonstrated that the primary binding site for $G\alpha_q$ on GRK2 is the α5 helix of the RH domain with Arg-106, Asp-110, Met-114 and Leu-118 in GRK2 being particularly important (3). This is in contrast to RGS4 and RGS9 where Gα binding is localized to the loops between helices α3 and α4, α5 and α6, and α7 and α8 and the RH domain of axin where APC binding involves residues on α helices 3, 4, and 5. Mutation of residues within the GRK2 RH domain that effectively disrupt $G\alpha_q$ interaction appear to have no effect on the ability of the kinase to phosphorylate receptor substrates. In addition, GRK2 binds equally well to wild type and an RGS-resistant mutant (G188S) of $G\alpha_q$ suggesting that the residues of $G\alpha_q$ that form the interface for binding GRK2 are distinct from those used for binding the RH domain of RGS proteins. In summary, GRKs are bi-functional regulators of GPCR signaling operating directly on both receptors and heterotrimeric G proteins.

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Crystal Structure of the p115RhoGEF rgRGS Domain in a Complex with Galpha(13):Galpha(i1) Chimera: A Novel G Protein GAP Mechanism

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As a Guanine nucleotide Exchange Factor (GEF) for Rho, p115RhoGEF serves as a direct regulatory link between G protein Coupled Receptors that activate G12 class Ga subunits and Rho-responsive cellular pathways. p115RhoGEF is both a negative regulator and a downstream effector of Ga13. It contains an N-terminal RhoGEF RGS (rgRGS) domain with low sequence identity to classic RGS domains. The rgRGS domain has specific GTPase Activating Protein (GAP) activity toward Ga12 and Ga13, and requires elements outside of the RGS domain to function as a GAP. We have engineered a chimeric Ga13:Gai1 protein (Chi_5) that is over-expressed in bacteria and serves as a substrate for the GAP activity of rgRGS. We have determined the crystal structure of GDP•Mg^{2+}•AlF_4^- •Chi_5 in complex with the rgRGS domain of p115RhoGEF. Although the core of the rgRGS domain is similar in fold to classic RGS domains, the rgRGS domain of p115RhoGEF employs a completely different mechanism to regulate the GTPase activity of Ga13. Two surfaces of rgRGS interact, cooperatively, with Chi_5: one of these is composed of the N-terminal subdomain that precedes the RGS-box; the other comprises loops from the RGS domain. Both the helical domain and the switch regions of Chi_5 are involved in the interaction with rgRGS. These interactions appear to stabilize the GDP•Mg^{2+}•AlF_4^- -bound state of the Ga subunit through contacts that are analogous to those observed in the structures of RGS4 with Gai1 and RGS9 with Gat, but involve different structural elements of rgRGS. The interface between the RGS subdomain of rgRGS and Chi_5 also shares striking similarity to those observed in adenylyl cyclase:Gas and PDEg:Gat complex structures. Thus, rgRGS shares the structural properties of both GAP and effector. Because the functional surfaces that mediate GAP activity in rgRGS and the conventional RGS domains are non-homologous, it is probable that GAP function is an evolutionarily convergent property of the RGS family of protein domains.

RGS Insensitive G Proteins as Probes of Physiological RGS Function

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A major question in the field of regulators of G protein signaling (RGS) proteins is the physiological role of RGS proteins (1). Knockouts are beginning to reveal some of these roles but their full contribution to G protein function may be obscured by redundancy of the function of different RGS proteins. An alternative approach to understand how RGS proteins affect G protein signaling is to use mutant G proteins that aren't inhibited by RGS proteins. Genetic studies in S. cervesiae revealed a point mutation in the yeast G protein Gpa1 that prevents the action of the yeast RGS (2). The analogous mutation in mammalian Gi and Gq family proteins blocks RGS-mediated GTPase acceleration and RGS-Gα binding interactions (3). In this presentation we describe the use of mutant $G\alpha_o$ and $G\alpha_{i2}$ proteins in expression and knock-in studies to assess the role of RGS proteins in signal transduction by these two Gi family G proteins. Opioid signaling is greatly enhanced by transfection of these mutants. Inhibitory regulation of cardiac function is also enhanced by means of genomic knock-ins. These genetic tools should prove useful to assess the full range of actions of RGS proteins in G protein signaling. (supported by GM39561-RRN, T32HL007853-XH, DA004087- JRT and an AHA Predoctoral fellowship -YF)

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Targeted Disruption of *Rgs1* Leads to Excessive B-Lymphocyte Response to Chemokines, Disturbed Plasma Cell Localization, and Distorted Immune Tissue Architecture

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Normal lymphoid tissue development and function depends upon chemokine directed cell migration. Since chemokines signal through heterotrimeric G-protein coupled receptors, RGS proteins, which act as GTPase activating proteins for Gα subunits, likely fine-tune the cellular responses to chemokines. RGS1 was initially characterized by its expression predominantly in human germinal center B cells. Further studies determined that in a RGS1 transfected B cell line; diminished migrational responses to SDF-1 were detected, as well as muted Ca^{++} responses to PAF. To further decipher the physiologic role of RGS1, RGS1 deficient mice were made by targeted gene disruption. Here we show that *Rgs1* -/- mice possess B-cells that respond excessively and desensitize improperly to the chemokines CXCL12 and CXCL13. Many of the B cell follicles in the spleens of *Rgs1* -/- mice have germinal centers even in the absence of immune stimulation. Furthermore, immunization of these mice leads to exaggerated germinal center formation; partial disruption of the normal architecture of the spleen and Peyer's patches; and abnormal trafficking of Ig secreting cells. These results reveal the importance of a regulatory mechanism that limits and desensitizes chemokine receptor signaling.

RGS9-2 Is a Negative Modulator of Morphine Actions

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Opioid alkaloids exert their analgesic and reinforcing effects by activating the mu opioid receptor. Upon activation this receptor couples via the Gi/o family of G proteins to various effectors, including the adenylyl cyclase signaling pathway, a system highly involved in the molecular adaptations following chronic drug use. Regulators of G- protein signaling (RGS) proteins are GTPase activating proteins that inhibit G protein function by reducing the duration of the activated GTP bound state of the G protein subunit. They may also have additional functions related to the scaffolding or trafficking of receptor signaling components. Previous studies have shown that RGS proteins are present in the brain and several subtypes exhibit striking regional specificity (Gold et al., 1997). An example is RGS9-2, which is very abundant in the striatum and also expressed at moderate levels in other areas mediating responses to opiates, such as the periaqueductal gray (PAG) and the superficial dorsal horn of the spinal cord. Each of these regions is rich in mu opioid receptors, raising the possibility that RGS9-2 may modulate opioid receptor function. In this study, we are showing that activation of the mu opioid receptor by acute or chronic morphine treatments regulates RGS9-2 protein expression in each of these three CNS regions. Acute morphine administration results in upregulation of RGS9-2 protein levels, whereas chronic morphine causes a dramatic downregulation. To further investigate the influence of RGS9-2 on morphine responses, we used mice with a functional deletion of the RGS9 gene and monitored their responses to morphine in several behavioral paradigms. Mice lacking RGS9 show a tenfold greater sensitivity to morphine's rewarding effects compared to their wild type littermates, an effect that can be reversed upon expression of RGS9 to the nucleus accumbens of the knockout animals using virally mediated gene transfer. While deletion of the RGS9 gene does not affect pain thresholds, it potentiates morphine analgesia and slows the development of morphine tolerance. Morphine dependence is also affected by the absence of RGS9, since RGS9-/- mice experience more severe opiate withdrawal. These data provide in vivo evidence for a physiological role of RGS9-2 as a negative regulator of morphine's actions.

Mechanisms of Feedback Inhibition by RGS Protein Induction and Turnover

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Cellular responses to hormones and neurotransmitters are necessarily transient. The mating pheromone signal in yeast is typical. Signal initiation requires a cell surface receptor (Ste2), a G protein heterotrimer (Gpa1, Ste4/Ste18) and downstream effector kinases (e.g. the MAPKK Ste7). Signal inactivation requires an RGS protein (Sst2) that accelerates G protein GTPase activity. We conducted a quantitative analysis of RGS and G protein expression, and devised computational models that describe their activity in vivo. This analysis indicates that pheromone-dependent transcriptional induction of the RGS protein constitutes a negative feedback loop that leads to desensitization. We have also found evidence for additional feedback loops that regulate the pathway. In particular, we find that the RGS protein Sst2, as well as the downstream kinase Ste7, is ubiquitinated and degraded in response to pheromone stimulation. Identification of multiple positive and negative feedback loops accounts for the transient response to external signals observed in vivo. Successful modeling of the pheromone pathway in yeast (with experimental validation of those models) should lead to improved models of signaling events in more complex organisms, and promises to improve our understanding of how cellular changes in disease states can be predicted and managed.

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Investigation of RGS Proteins Toward Modulation of Neurobiological Disorders

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G-protein signaling is a fundamental pathway contributing to function and dysfunction of the central nervous system. Many therapeutics for the treatment of neurobiological disorders target neurotransmitter systems that function by activating G-protein coupled receptors (GPCRs). Modulation of the G-protein signaling system is of paramount importance for optimal neurobiological function. Regulator of G-protein signaling (RGS) proteins function as GTPase accelerating proteins (GAPs) for Gα subunits to negatively regulate and 'turn-off' G-protein signaling. Modulation of RGS proteins, directly, would provide an alternative approach in the modulation of the GPCR/G-protein system.

To further understand the impact of this protein family, and identify modulators, we have undertaken several experimental approaches. Functional, and protein-interaction, automated high-throughput screening platforms were designed and implemented for the identification of small molecule modulators for specific RGS proteins. These screens enabled the identification of small molecules capable of inhibiting *in vitro* RGS GAP activity; provide potential biological tools, as well as serving as a basis for SAR evaluation.

To further understand the impact of specific RGS proteins on cell biology and neuronal function, RGS effects on G-protein signaling via 5HT1A function were investigated. Both RGS4, and RGSz, modulated $5HT1_A$ receptor function. RGS4 also appeared to have a direct effect on affect adenylyl cyclase. To determine if RGS4 could modulate $5HT1_A$ receptor function *in vivo*, RGS4 was expressed in the somatodendrictic neurons in the dorsal raphe nucleus, determine subsequent effects on extracellular 5-HT in the striatum were determined. To further investigate the impact of RGS proteins we have generated RGS knockout animals, and initial behavioral assessments are underway. These animals may be of particular interest given previous studies on RGS modulation of GPCR signaling associated with in various mood disorders, pain, and addiction.

RGS proteins provide intriguing potential to further understand the underlying mechanisms in neurobiology. Further understanding of this intriguing protein family, and their biological function and utility, will provide potential new opportunities in therapeutic design.

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Functional Analysis of RGS Proteins in Intact Cells: Lessons from Photoreceptors

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Few G protein cascades are as amenable to quantification as the cascade that mediates vision in dim light. In response to photon absorption, the phototransduction cascade of retinal rod photoreceptors produces a decrease in cyclic GMP (cGMP) concentration and a corresponding decrease in the conductance of cGMP-gated cation channels in the plasma membrane. The cGMP-dependent current of the intact rod can be readily recorded with a suction electrode and provides a real-time assay for signal transduction kinetics under physiological conditions. In this talk, I will summarize results from a series of experiments in which perturbations in the photoreceptor-specific RGS protein, RGS9-2, its binding partners Gβ5-L and R9AP, and their target, the transducin/phosphodiesterase complex, has led to changes in the time course of the rod's light response. I will also discuss recent data that suggests that deactivation of the transducin/phosphodiesterase complex normally rate-limits the time course of the light response, which may have important implications for the fine tuning of signal transduction kinetics through the regulation of RGS expression in other systems.

Building RGS Protein Specificity Through Its Domain Composition

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The abundance of signaling cascades where RGS and G proteins are involved raises the question of how the specificity of their mutual recognition is achieved on the molecular and cellular levels. In general, this specificity can be attained either by precise patterns of protein-protein recognition or by intracellular compartmentalization of individual RGS and G proteins. I will use the example of two splice isoforms of RGS9, one from the retina and another from the brain, to illustrate how both of these principles could be realized through the function of multiple non-catalytic domains of RGS proteins and/or by the incorporation of RGS proteins into larger signaling complexes. I will first introduce the concept of "affinity adapters" which are domains or subunits primarily specializing in providing high affinity interactions between an RGS protein and its specific G protein target. Dependent on the timing needs of different signaling pathways, these adapters can be positioned at various pathway components, such as the effectors or RGS proteins themselves. I will then show how the precise intracellular targeting of the photoreceptor-specific isoform of RGS9, as well as its stability in the cell, is achieved via the interaction of its DEP domain with a novel SNARE-like protein R9AP.

POSTER ABSTRACTS

Exploring the Role of Protein Folding on RGS Domain Activity

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Much is known about RGS protein binding partners, localization and function. There is a high degree of sequence and structural homology between various RGS domains. Currently little is known about the thermodynamics and kinetics of RGS protein folding. We are interested in studying how the protein folding properties of RGS domains contribute to their function in cells. One of our goals is to introduce stabilizing and destabilizing mutations in these RGS domains without perturbing the overall structure or binding sites. Equilibrium and kinetic studies of the folding of these variants can give insights into the effects of RGS proteins' energetics on their biological roles and regulation. We plan to express these variants in cells and observe the differences in their function.

We have expressed and purified a collection of RGS domains from RGS4, RGSz, Axin, and Sst2. We express the proteins in E. Coli Rosetta DE3 cells and purify the proteins without a tag for biophysical characterization. We are using circular dichroism and fluorescence spectroscopy to determine the thermodynamic stability and folding kinetics of wild-type and variant RGS domains.

Identification of a Novel Sequence in PDZ-RhoGEF That Mediates Interaction with the Actin Cytoskeleton

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Small GTPases of the Rho family are crucial regulators of actin cytoskeleton rearrangements. Rho is activated by members of the Rho guanine-nucleotide exchange factor (GEF) family; however, mechanisms that regulate RhoGEFs are not well understood. This report demonstrates that PDZ-RhoGEF, a member of a sub-family of RhoGEFs that contain regulator of G protein signaling (RGS) domains, is partially localized at or near the plasma membranes in 293T or COS-7 cells, and this localization is coincident with cortical actin. Disruption of the cortical actin cytoskeleton in 293T cells using latrunculin B prevents the peri-PM localization of PDZ-RhoGEF. Co-immunoprecipitation and F-actin co-sedimentation assays demonstrate that PDZ-RhoGEF binds to actin. Extensive deletion mutagenesis revealed the presence of a novel 25 amino acid sequence in PDZ-RhoGEF, located at amino acids 561-585, that is necessary and sufficient for localization to the actin cytoskeleton and interaction with actin. Lastly, PDZ-RhoGEF mutants that fail to interact with the actin cytoskeleton display enhanced Rho-dependent signaling compared to wild type PDZ-RhoGEF. These results identify interaction with the actin cytoskeleton as a novel function for PDZ-RhoGEF, thus implicating actin interaction in organizing PDZ-RhoGEF signaling.

Mast Cells Express Multiple Regulator of G Protein Signaling (RGS) Proteins

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Regulators of G Protein Signaling (RGS) proteins inhibit G-protein-coupled receptor (GPCR) responses and are thus potential drug targets for treatment of asthma and other allergic diseases. We evaluated expression of RGS proteins in mast cells (MCs), an initiator of allergic reactions in asthma, and their potential role in MC function. *rgs1* and *rgs18* were identified from murine MC and cell line (RBL, MC/9, P815) RNA by RT-PCR using degenerate primers encompassing a highly conserved region (RGS box). cDNA microarray analysis of human MCs from peripheral blood (PBMCs) suggested 1000-fold higher *rgs13* expression in PBMCs than any other human tissue. *rgs13* expression correlated with MC maturation. FceRI aggregation increased *rgs13* mRNA levels, while IL-4 treatment was associated with decreased *rgs13* expression. RT-PCR of mouse bone marrow-derived MCs (BMMCs) revealed abundant *rgs13* mRNA. Immunocytochemistry using RGS13-specific antisera demonstrated cytoplasmic RGS13 localization in BMMCs. Mice deficient in RGS13 with *LacZ* knocked-into the *rgs13* genomic locus were generated by homologous recombination. Immunoblotting confirmed RGS13 expression in BMMCs from wild type but not knockout mice. BMMCs and toluidine-blue^{+} MCs from *rgs13*$^{-/-}$ mice in skin, conjunctiva, and gastrointestinal tract exhibited cytoplasmic b-galactosidase staining suggestive of physiological RGS13 expression. Studies of the activation and migration of *rgs13*$^{-/-}$MCs are underway.

This work was supported by the Division of Intramural Research, NIAID/NIH.

Polymorphisms in Regulators of G Protein Signaling (RGS) Genes Associated with a Decreased Risk of Bladder Cancer

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RGS proteins negatively regulate heterotrimeric G proteins signaling. Recent reports have shown that RGS proteins modulate neuronal, cardiovascular and lymphocytic activity; yet their role in carcinogenesis has not been explored in any depth. In a hospital-based case-control study of 477 bladder cancer patients and 446 matched controls, we explored the association between 11 non-coding single nucleotide polymorphisms (SNPs) in five RGS genes and risk of bladder cancer, a cause of over 12,000 deaths in the United States annually. Overall, the RGS6- rs2074647 variant genotypes were associated with a statistically significant 35% reduction in bladder cancer risk (OR: 0.65; 95% CI: 0.46-0.93). On stratified analysis, the protective effects were more evident in ever smokers (OR: 0.62; 95% CI: 0.40-0.95), individuals who started smoking before age 17 (OR: 0.39; 95% CI: 0.19-0.79) and younger patients (OR: 0.47; 95% CI: 0.24-0.91). Similar findings were noted for the RGS2-rs4606 and RGS6-rs3784058 polymorphisms. The presence of a variant genotype at all three sites reduced the odds ratio to 0.22 (95% CI: 0.08-0.29). This risk was further reduced to 0.15 (95% CI: 0.05-0.49) in ever smokers. In the multivariate model, we found that variant RGS6-rs2074647, RGS2-rs4606, and RGS6-rs3784058 genotypes were independently associated with reduced bladder cancer risk and there was a strong significant negative interaction among these three polymorphisms. In patient-derived lymphoblastoid cell lines, the variant genotypes did not influence the levels of RGS2 or RGS6 transcripts nor did they alter RGS6 mRNA splicing patterns. However, we are currently investigating whether changes in protein translation rates provide a mechanistic explanation for the significantly reduced risk of bladder cancer we observed. These data provide the first evidence that RGS proteins may be important modulators of cancer risk.

RGS Proteins Bind Directly and Selectively to the Third Intracellular Loops of GPCRs to Modulate Gq/11α Signaling

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RGS proteins serve as GTPase activating proteins (GAPs) and/or effector antagonists to modulate Gα signaling events. In live cells, members of the B/R4 subfamily of RGS proteins selectively modulate G protein signaling depending on the associated receptor (GPCR). Here we examine whether GPCRs selectively recruit RGS proteins to modulate linked G protein signaling. We report the novel finding that RGS2 binds directly to the third intracellular (i3) loop of the Gq/11-coupled M1 muscarinic cholinergic receptor (M1mAChR; M1i3). This interaction is selective since closely related RGS16 does not bind M1i3, and neither RGS2 nor RGS16 binds to the Gi/o-coupled M2i3 loop. When expressed in cells, RGS2 and M1 mAChR co-localize to the plasma membrane whereas RGS16 does not. The N-terminal region of RGS2 is both necessary and sufficient for binding to M1i3, and RGS2 forms a stable heterotrimeric complex with both activated Gqα and M1i3. RGS2 potently inhibits M1 mAChR-mediated phosphoinositide hydrolysis in cell membranes by acting as an effector antagonist. Deletion of the N-terminus abolishes this effector antagonist activity of RGS2 but not its GAP activity towards G11α in membranes. These findings predict a model where the i3 loops of GPCRs selectively recruit specific RGS protein(s) via their N-termini to regulate linked G protein. Consistent with this model, we find that the i3 loops of the mAChR subtypes (M1-M5) exhibit differential profiles for binding distinct B/R4 RGS family members, indicating that selectivity for RGS proteins exists among receptor subtypes. To test whether this novel mechanism for GPCR modulation of RGS signaling may extend to other receptors, we also examine the selective interactions of RGS proteins with the ic3 loops of Gq/11-coupled α1 adrenergic receptor (α_1-AR). We find that RGS2 binds to α_{1A}-AR but not α_{1B} or α_{1D}-AR, and that RGS2 can be recruited from the nucleus to the plasma membrane of HEK293 cells by co-expression of the α_{1A}-AR. Together these data suggest that selective recruitment of RGS proteins by GPCRs may be a general mechanism for controlling downstream signaling in cells.

Computational Modeling Reveals How Interplay between Components of the GTPase Cycle Regulates Signal Transduction

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In this study, we predict how the concentrations of components of the GTPase cycle—which are known to be spatially and temporally regulated in cells—regulate G protein activity. We do this using a computational model based on a general kinetic framework of the GTPase cycle and parameters of a single GTPase cycle: m1 muscarinic acetylcholine receptor, Gq, and Regulator of G protein signaling 4 (RGS4). Our simulations predict, quantitatively, that (a) G protein activity occurs within four distinct limits determined by receptor and RGS concentrations; but that (b) the effect of receptor or RGS is partly controlled by G protein concentration, which determines whether mass action or stoichiometric interactions occur; and (c) ~10-fold variation in average whole cell GTP and GDP concentrations alters G protein activity. These predictions result from the kinetics of our GTPase cycle model, which permits mechanisms of collision coupling, kinetic scaffolding, and others. Our simulations also provide a quantitative explanation for data that has appeared paradoxical, namely that RGS proteins do not necessarily attenuate G protein activity, but do accelerate the onset and desensitization of activity. Overall, our model shows how interplay between concentrations of molecular players in the GTPase cycle results in a dynamic range of G protein activity that quantitatively explains cellular signaling scenarios.

Crystal Structure of the p115RhoGEF rgRGS Domain in a Complex with Galpha(13):Galpha(i1) Chimera: A Novel G Protein GAP Mechanism

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See Speaker Abstracts

RGS14 Expression Interferes with Normal Exit from Cytokinesis

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The Regulator of G protein Signaling (RGS) family of highly diverse proteins down-regulates heterotrimeric G protein-coupled receptor signaling pathways mainly by enhancing GTPase activities of G□ subunits. RGS14, a member of the RGS family contains RGS, Rap-interacting, and GPR/GoLoco domains likely participating in multiple cellular processes. In this study, we have identified a centrosome protein, ninein as RGS14-interacting protein in a yeast 2-hybrid screen. Coimmunoprecipitation and confocal microscopy studies demonstrate that RGS14 associates with ninein in the centrosomes in HeLa cells. Many different cell lines such as HeLa, MCF-7 and HEK293 express a lower level of RGS14 mRNA compared to that previously found in immune cells. In these cell lines, endogenous RGS14 co-localizes with various centrosome markers such as centrin, pericentrin, and □-tubulin, and appears to mainly associate with mother centriole. Mutation in the nuclear export signal or treatment with leptomycin B results in nuclear accumulation of RGS14. The result indicates that RGS14 is also a nuclear-cytoplasmic shuttling protein as shown with certain centrosome proteins such as centrin and pericentrin. Prolonged expression of RGS14 results in formation of multinucleated cells containing supernumerary centrosomes as well as formation of micronuclei, a hallmark of unequal chromosome segregation. Time-lapse imaging shows that cells expressing RGS14 are defective in completion of cytokinesis. We have generated mutants defective in various activities of RGS14 and an shRNA construct that reduces the expression of RGS14. We are currently examining cells expressing the RGS14 mutants or with reduced amount of RGS14 to further understand the role of RGS14 in cell cycle progression.

Role of Gα Subtypes and RGS Proteins in a Denylyl Cyclase Supersensitization

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In C6 glioma cells expressing the μ opioid receptor (C6μ) we have shown that chronic μ opioid activation of exogenous Gαo leads to adenylyl cyclase supersensitization and this effect is decreased by endogenous regulators of G protein signaling (RGS) proteins. Here we examine the hypothesis that opioid induced adenylyl cyclase supersensitization can be mediated by a variety of inhibitory Gα subtypes and all are modulated by endogenous RGS proteins. C6μ cells were transfected with pertussis toxin (PTX) insensitive inhibitory Gα subunits, in which the PTX sensitive cysteine was replaced with either isoleucine or glycine. Following PTX treatment to inactivate endogenous Gα, the isoleucine substituted PTX insensitive mutants provided a much better coupling between G protein and the μ opioid receptor than the corresponding glycine mutants (>10 fold increase in maximal stimulation of [^{35}S]GTPγS binding by the μ agonist, DAMGO). Treatment of either Gαi1C351I, Gαi2C352I or Gαi3C351I expressing cells for 18 h with 1μM DAMGO, followed by challenge with naloxone gave an increase in cAMP levels, indicating adenylyl cyclase supersensitization mediated by each Gαi subtype. The degree of supersensitization was increased when Gαi also contained a RGS-insensitive mutation, confirming that endogenous RGS proteins decrease supersensitization mediated by different inhibitory Gα subunits. Suppported by DA04087.

Modulation of Ion Channels by RGS-Insensitive $G\alpha_q$ Chimeras

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Rapid termination of ion channel modulation mediated by heterotrimeric $G\alpha_{i/o}$ proteins depends on the GTPase accelerating activity of RGS proteins. Activation of $G\alpha_{q/11}$ proteins also modulates ion channels, often by activating phospholipase C-β (PLCβ). However, it is not known whether RGS proteins regulate the kinetics of $G\alpha_{q/11}$-mediated responses. To address this question we examined inhibition of endogenous 2P domain potassium (K2P) channels in primary cerebellar granule neurons by RGS-sensitive and RGS-insensitive (RGSi) variants of $G\alpha_q$. Activation of endogenous $G\alpha_{q/11}$ proteins by native m3 muscarinic receptors inhibits K2P channels in these cells. We reconstituted similar inhibition by transfecting chimeric Gα subunits comprised largely of $G\alpha_q$ with a $G\alpha_i$-derived carboxy terminus ($G\alpha_{qi9}$) and cotransfecting $\alpha 2_A$ adrenoreceptors (α2ARs). The α2AR agonist norepinephrine inhibited K2P channels in neurons that were cotransfected with α2ARs and $G\alpha_{qi9}$ chimeras, but not in neurons that were transfected with receptor alone. This result suggests that α2ARs do not inhibit K2P channels in these neurons by coupling to endogenous $G\alpha_{q/11}$ subunits. K2P inhibition mediated by an RGSi mutant chimera ($G\alpha_{qi9}$ G188S) recovered more slowly than that mediated by $G\alpha_{qi9}$. The time to half recovery ($T_{1/2}$) from inhibition mediated by $G\alpha_{qi9}$ G188S was 25 ± 2 seconds (n=11), whereas that mediated by $G\alpha_{qi9}$ was 11 ± 1 seconds (n=9). This change reflected the appearance of a lag period before the onset of recovery, rather than a change in the rate of recovery. In addition, the onset of K2P inhibition mediated by $G\alpha_{qi9}$ G188S was markedly slower than that mediated by $G\alpha_{qi9}$. Interestingly, another mutation shown previously to render Gα subunits RGSi (S211D) had a much more modest effect on K2P inhibition ($T_{1/2}$ = 14 ± 1 seconds; n=7). Neither RGSi mutation markedly altered the concentration-response sensitivity of K2P inhibition. These results suggest that the kinetics of ion channel modulation by $G\alpha_{q/11}$ proteins are not regulated by RGS proteins to the same extent as modulation mediated by $G\alpha_{i/o}$ proteins. It is likely that other GTPase accelerating proteins (e.g. PLCβ) modulate the kinetics of responses mediated by $G\alpha_{q/11}$ proteins.
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Characterization of the GRK2 Binding Site of $G\alpha_q$

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Heterotrimeric guanine nucleotide-binding proteins (G proteins) transmit signals from membrane bound G protein-coupled receptors (GPCRs) to intracellular effector proteins. The G_q subfamily of Gα subunits couples GPCR activation to the enzymatic activity of phospholipase C-β (PLC-β). Regulators of G protein signaling (RGS) proteins bind to activated Gα subunits, including $G\alpha_q$, and regulate Gα signaling by acting as GTPase activating proteins (GAPs), increasing the rate of the intrinsic GTPase activity, or by acting as effector antagonists for Gα subunits. GPCR kinases (GRKs) phosphorylate agonist-bound receptors in the first step of receptor desensitization. The amino-termini of all GRKs contain an RGS homology (RH) domain (Siderovski et al., Curr. Bio., 1996) and binding of the GRK2 RH domain to $G\alpha_q$ attenuates PLC-β activity (Carman et al., J. Biol. Chem. 1999). The RH domain of GRK2 interacts with $G\alpha_{q/11}$ through a novel Gα binding surface termed the "C" site (Sterne-Marr et al., J. Biol. Chem. 2003). Here, molecular modeling of the $G\alpha_q$-GRK2 complex and site-directed mutagenesis of $G\alpha_q$ were used to identify residues in $G\alpha_q$ that interact with GRK2. The model identifies Pro^{185} in Switch I of $G\alpha_q$ as being at the crux of the interface, and mutation of this residue to lysine disrupts $G\alpha_q$ binding to the GRK2-RH domain. Switch III also appears to play a role in GRK2 binding because the mutations $G\alpha_q$-V240A, $G\alpha_q$-D243A, both residues within switch III, and $G\alpha_q$-Q152A, a residue that structurally supports switch III, are defective in binding GRK2. Furthermore, GRK2-mediated inhibition of $G\alpha_q$-Q152A-R183C-stimulated inositol phosphate release is reduced in comparison to $G\alpha_q$-R183C. Interestingly, the model also predicts that residues in the helical domain of $G\alpha_q$ interact with GRK2. In fact, the mutants $G\alpha_q$-K77A, $G\alpha_q$-L78D, $G\alpha_q$-Q81A and $G\alpha_q$-R92A have reduced binding to the GRK2-RH domain. Finally, while the mutant $G\alpha_q$-T187K has greatly reduced binding to RGS2 and RGS4 it has little to no effect on binding to GRK2. Thus the RH domain A and C sites for $G\alpha_q$ interaction rely on contacts with distinct regions and different switch I residues in $G\alpha_q$.

RGS16 Inhibits Signaling Through the Gα13-Rho Axis

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The heterotrimeric G protein Gα13 directly stimulates guanine nucleotide exchange factors (GEFs) for the monomeric G protein Rho, such as p115RhoGEF. The resultant activation of Rho induces a variety of cellular responses including actin polymerization, serum response element (SRE)-dependent gene transcription, and transformation. p115RhoGEF also contains a domain with homology to the Regulators of G protein signaling (RGS proteins), which confers GTPase activating protein (GAP) activity specifically toward Gα12 and 13. Classical RGS proteins (such as RGS16 and RGS4) exhibit RGS domain-dependent GAP activity on Gαi and Gαq, but not Gα12/13. Here we show that RGS16 inhibits Gα13-mediated, RhoA-dependent cellular processes including reversal of stellation and SRE activation. The RGS16 amino- terminus binds Gα13 directly, resulting in Gα13 translocation to detergent-resistant membranes and impaired interaction with p115RhoGEF. In contrast, RGS4 does not bind Gα13 or attenuate Gα13-dependent responses, and neither RGS16 nor RGS4 interacts with Gα12 or affects Gα12-coupled signaling. These results elucidate a novel means by which a classical RGS protein regulates Gα13-mediated signal transduction through its amino-terminus, independently of the RGS box.

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RGS 2 is Upregulated in the PVN of Restrained Male Sprague-Dawley Rats

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The regulator of G-protein signaling (RGS) proteins are involved in G-protein coupled receptor (GPCR) signaling and show gene expression changes after various stimuli. For example, RGS 4 mRNA has been reported to be downregulated in the paraventricular nucleus of the hypothalamus, but upregulated in the locus coeruleus after exposure to unpredictable stress (Ni et al., 1999). More recently, it has been suggested that RGS 2 and RGS 4 mRNA expression may undergo opposite regulation following manipulation of the dopaminergic system (Taymans et al., 2003). In the present study, we investigated whether these two RGS are also oppositely regulated following acute stress exposure. RGS 2 and RGS 4 mRNA expressions were evaluated at different time points (0-24h) after a 1-hour restraint stress in male adult Sprague-Dawley rats. As expected, restraint stress induced a marked activation of the hypothalamic-pituitary axis, as indicated by an increase in plasma corticosterone (6-fold) and ACTH (9-fold) immediately after stress exposure. Main observation was that *in situ* hybridization on frozen brain sections demonstrated a rapid upregulation of RGS 2 mRNA after restraint stress in the paraventricular nucleus of the hypothalamus. RGS 2 peaked 2 hours after stress, reaching a maximum of 240 ± 30 % of control values. These new data on expression changes in mRNA suggest that RGS 2 and RGS 4 exert different regulatory functions on G-proteins in the mediation of the cellular response to stress.

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Application of RGS-Box Proteins to Evaluate G Protein Selectivity in Receptor-Promoted Signaling

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Regulator of G protein signaling (RGS) domains bind directly to GTP-bound Gα subunits and accelerate their intrinsic GTPase activity. The rate of intrinsic GTPase activity of Gα subunits is stimulated up to several thousand-fold *in vitro*, and selectivity of RGS proteins for individual Gα subunits has been illustrated. Thus, expression of RGS proteins can be used to inhibit signaling pathways activated by specific G protein-coupled receptors (GPCRs). Here, we describe the use of specific RGS domain constructs to discriminate between $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$-mediated activation of phospholipase C (PLC) isozymes in Cos-7 cells. Constructs derived from G protein-coupled receptor kinase 2 (GRK2), RGS2, RGS4, and p115 RhoGEF were used to delineate signaling by a variety of GPCRs. These analyses revealed that some RGS domain constructs can be universally applied to inhibit signaling, whereas others exhibit receptor-selective effects and therefore require appropriate controls to support their usage as discriminators of GPCR/heterotrimer couplings.

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RGS2 Protein Inhibits G_q-mediated Signaling and Hypertrophy in the Heart

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RGS2 protein belongs to a family of Regulators of G protein Signaling. It negatively regulates G_q signaling, enhancement of which is known to cause hypertrophy and heart failure in the myocardium. The goal of this study was to determine whether RGS2 exerts inhibitory restraint on G_q-mediated signaling and hypertrophy in cardiomyocytes and to test the hypothesis that changes in the amount or function of RGS2 play a critical role in cardiac remodeling in response to enhanced G_q signaling. We examined the effect of RGS2 on phospholipase C β activation and hypertrophy in response to G_q-coupled receptor stimulation in neonatal and adult rat ventricular myocytes using adenoviral gene transfer to overexpress RGS2. RGS2 dose-dependently inhibited endothelin-1- and phenylephrine-induced generation of [^{3}H]labeled inositol phosphates with almost complete inhibition at 10 MOI in both neonatal and adult myocytes compared to empty adenovirus controls. RGS2 also inhibited the characteristic doubling in cell size and protein synthesis and increase in ANF expression in response to α_1-adrenoceptor-mediated G_q stimulation in neonatal cardiac myocytes, as assessed by immunostaining and radiolabeled protein content. Northern blots and RT-PCR analysis were used to determine mRNA expression levels of RGS2 and three other major cardiac RGS proteins (RGS3-RGS5) in ventricular tissue from two different hypertrophic mouse models due to enhanced G_q signaling: (i) cardiac-specific transgenic expression of constitutively active $G\alpha_q$ and (ii) pressure overload by ascending aortic constriction. Only RGS2 mRNA was reduced by 60-80 % compared to age-matched wild-type or sham-operated controls, respectively, both *prior* to and *after* cardiac hypertrophy developed. RGS2 mRNA down-regulation occurred in ventricular myocytes and translated into a reduction in RGS2 protein, as shown in $G\alpha_q$ transgenic hearts. We conclude that RGS2 is a potent inhibitor of G_q-mediated PLCβ activation and hypertrophy in cardiomyocytes. The selective, early and sustained reduction of RGS2 in hearts from two different models of hypertrophy with enhanced G_q signaling suggests that down-regulation of RGS2 plays an important role in G_q-mediated cardiac remodeling via insufficient inhibitory restraint on G_q signaling.

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The Role of RGS4 in Ligand-directed Signaling of the Dopamine D_2 Receptor

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RGS4 is a member of a recently identified large family of proteins, called Regulators of G protein signaling. RGS4 is known to enhance the GTPase activity of specific members of the $G_{i\alpha}$ and $G_{q\alpha}$ subfamily of G proteins, thereby inhibiting the G protein-mediated signaling [Berman et al., JBC 271(44):27209-27212, 1996]. Interestingly, gene expression profiling of the prefrontal cortex of schizophrenic patients revealed a down-regulation of RGS4 [Mirnics et al., Mol Psychiatry 6:293-301, 2001]. Therefore, it was hypothesized that a dysregulation of RGS4 contributes in part to schizophrenic symptoms.

Current treatment of schizophrenia involves dopamine D_2 antagonists, based on the notion that the dopaminergic system may be overactive in schizophrenia. To determine the role of RGS4 in D_2 signaling, CHO cells stably expressing the human dopamine D_{2L} receptor were used. This receptor is known to attenuate the accumulation of cAMP through the $G_{i\alpha}$ subfamily. cAMP activity was measured using the CREB-sensitive reporter gene secretable alkaline phosphatase which was stably expressed in the CHO-D_{2L} cells.

We stably overexpressed RGS4 in the CHO-D_{2L} cells and determined the effects on the D_2 receptor-mediated efficacy and potency of agonists and antagonists. We selected two cell lines with unique characteristics and evaluated them pharmacologically. Surprisingly, it was found that the potency and efficacy of agonists and antagonists were differentially affected by RGS4. These results indicate that RGS4 plays an important role in D_2 receptor signaling and is considered a useful tool in approaching ligand-directed signaling for compound selection. Moreover, regulation of RGS4 expression itself may yield novel therapeutic opportunities for future drug development.

Role of a Seven-transmembrane RGS Protein in Sugar Signaling in Arabidopsis

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Arabidopsis contains a single RGS1 protein. This RGS (AtRGS1) contains a seven-transmembrane domain at its amino-terminal half and a functional RGS box at its cytosolic-facing, C-terminal half. D-glucose serves as both a metabolite and a hormone-like signal that regulates plant cell proliferation. High D-glucose arrests cell proliferation, but Arabidopsis mutants lacking AtrRGS1 are insensitive to high sugars. Conversely, plants ectopically expressing *AtRGS1* are hypersensitive to sugars. D-glucose, but not L-glucose, causes rapid internalization of a AtRGS1:GFP fusion protein. Loss of AtRGS1 is dependent on a protein designated GIP1 which was identified as an interactor to the single canonical heterotrimeric G protein alpha subunit. GIP1 serves to desensitize cells to glucose by controlling the steady-state levels of AtRGS1 protein. These results raise the possibility that AtRGS1 is a glucose receptor that controls cell proliferation through regulation of the active state of its cognate G-protein complex.

Reciprocal Control of RGS Protein Function by a Phoshoinositide and Calmodulin

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Regulators of G-protein-signaling (RGS) proteins are a family of proteins, which accelerate intrinsic GTP-hydrolysis on □subunit of trimeric G-proteins and play crucial roles in the physiological regulation of G-protein mediated cell signaling. If RGS proteins were active unrestrictedly, it would completely suppress various G protein-mediated signalings as has been seen in the overexpression experiments of RGS proteins. Therefore, it is quite important to understand how the actions of RGS proteins are regulated in various physiological conditions. The modulatory mechanisms of RGS-action *per se* have, however, been poorly clarified. We have been shown a physiological mode of action of a RGS protein (Ishii *et al*., *Circ Res* 2001; Ishii *et al*., *PNAS* 2002). The voltage-dependent formation of Ca^{2+}/calmodulin (CaM) facilitated the GTPase-activity of RGS protein via removing intrinsic inhibition mediated by a kind of phospholipid, phosphatidylinositol-3,4,5,-trisphosphate (PIP_3). This modulation of RGS-action underlies a characteristic property, named 'relaxation', of G-protein-gated K^+ (K_G) channels in native cardiac myocytes. These studies unexpectedly provide us with a novel principle that the cell excitation can dynamically regulate G protein signaling via apparent voltage-dependent behavior of cytosolic RGS proteins. Further examination using protein-lipid co-sedimentation assay detected the specific interaction between RGS4 and PIP_3 (but not other PIPs), which was abolished by Ca^{2+}/CaM. Interestingly, this reciprocal modulation is exclusively performed within RGS domain, which is also responsible for GTPase-accelerating activity. We identified the clusters of positively charged residues in helix 4 of RGS domain as a candidate of the molecular switch of PIP_3/CaM-modulation. Because the residues are conserved in almost all RGS protein subtypes, the allosteric modulation of RGS proteins should be important in the physiological regulation of G-protein signalling by various RGS proteins in different cell types.

Association and Linkage Analyses of RGS4 Polymorphisms in Schizophrenia

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Gene expression analyses of postmortem cerebral cortex suggest that transcription of the Regulator of G-protein Signaling 4 (RGS4) is decreased in a diagnosis specific manner in subjects with schizophrenia (Mirnics K et al, 2001). We have shown significant transmission distortion at this locus in three samples ascertained independently in Pittsburgh, New Delhi and by the NIMH Collaborative Genetics Initiative (Chowdari K et al 2002). Among 13 SNPs spanning approximately 300 kb, significant associations involved four SNPs localized to a 10 kb region at RGS4, but the associated haplotypes differed amongst populations. Consistent with the significant transmission distortions, samples with affected siblings (NIMH, India) showed higher levels of allele sharing, identical by descent, at RGS4. To further probe the disparate allele and haplotype associations, we are conducting meta-analyses and clinical sub-group analyses in an additional 1,500 family based case-parent trios and 6,500 case/control samples independently ascertained by 9 different investigators. In addition, we have identified several novel SNPs localized in a 30 kb region upstream to the RGS4 locus for further analysis in our sample to elucidate the putative association of the RGS4 gene in the pathogenesis of schizophrenia.

Alternative Splicing of RGS9 Sets the Specificity of its Target Recognition

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RGS proteins regulate the duration of cell signalling by stimulating the hydrolysis of GTP on G protein a subunits. RGS9 exists as two splice isoforms that differ in their C-termini. The short isoform, RGS9-1, regulates the lifetime of activated transducin (G_t) in the phototransduction pathway. Critical to this pathway is the ability of RGS9-1 to interact selectively with G_t bound to the γ-subunit of its effector, cGMP phosphodiesterase (PDEγ). This specificity is achieved through the ability of PDEγ to increase the affinity between RGS9-1 and G_t. The long isoform, RGS9-2, is thought to regulate the activity of $G_{o/i}$ in the brain. We have shown that G_o is the preferred target of RGS9-2 *in vitro*. We further found that the C-terminal domain of RGS9-2 acts as a functional analogue of PDEγ by inducing high affinity binding of RGS9-2 to Ga_o. This functional analogy is underlied by significant structural similarities between PDEγ and the C-terminus of RGS9-2 that indicate a possible evolutionary relationship. Our results suggest that RGS proteins employ affinity adapter proteins or domains as a general strategy in target recognition.

The DEP Domain Determines Subcellular Targeting of the GTPase Activating Protein RGS9 *in vivo*

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DEP (Disheveled, EGL-10, Pleckstrin) homology domains are present in numerous signaling proteins, including many in the nervous system, but their function remains mostly elusive. We report that the DEP domain of a photoreceptor-specific signaling protein, RGS9, plays an essential role in RGS9 delivery to the intracellular compartment of its functioning, the rod outer segment. We generated a transgenic mouse where RGS9 was replaced by its mutant lacking the DEP domain. We then utilized a combination of the quantitative technique of serial tangential sectioning/Western blotting with electrophysiological recordings to demonstrate that mutant RGS9 is expressed in rods in the normal amount but is completely excluded from the outer segments. The delivery of RGS9 to rod outer segments is likely to be mediated by the DEP domain interaction with a transmembrane protein, R9AP, known to anchor RGS9 on the surface of photoreceptor membranes and to potentiate RGS9 catalytic activity. We show that both of these functions are also abolished upon the DEP domain deletion. These findings indicate that a novel function of the DEP domain is to target a signaling protein to a specific compartment of a highly polarized neuron. Interestingly, sequence analysis of R9AP reveals the presence of a conserved R-SNARE motif and a predicted overall structural homology with SNARE proteins involved in vesicular trafficking and fusion. This presents the possibility that DEP domains might serve to target various DEP-containing proteins to the sites of their intracellular action via interactions with the members of extended SNARE protein family.

Reduced Models of Biochemical Networks: GTPase Cycle as an Exemplar

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Complex signaling pathways can be viewed as being composed of smaller modules that can be subject to precise quantitative measurements and computations. The ubiquitous GTPase cycle is one such module that gates signal transduction in G protein signaling networks. Despite its apparent simplicity this module involves a number of kinetic events that parse the extracellular signal into activation of the G-protein. Once the parameters of these kinetic events are accurately measured the module can be modeled mathematically in order to obtain quantitative estimates of the modular response. However, the lack of sufficient experimental data and the complexity of the module warrant exploration of more coarse-grained models that will have the ability to capture the important features of the underlying biochemistry. In an accompanying poster, we constructed a computational model of the GTPase cycle of m1 muscarinic acetylcholine receptor, Gq, and RGS4, that is based on experimental data including a detailed biochemical reaction scheme of the GTPase cycle. Here we demonstrate, how beginning with a detailed model, we can systematically construct reduced models that capture important biochemical steps, yet provide simplicity to the biochemical picture of the cycle. These reduced models provide knowledge about the dependence of various parameters as well as their range under which G protein cycles operate in cells. We use multi-dimensional sensitivity analysis (MDSA) to analyze the effect of simultaneous perturbations of all model parameters on the model's ability to simulate experimental data. Parameters with little influence on simulation output are removed from the model. Whenever the model is reduced, all parameters are re-estimated within the constraints of experimental data with a genetic-algorithm (GA)-based optimizer. Model reduction is iterative. The minimal models we construct provide interesting insights into the GTPase cycles. Further, the approach outlined here is extensible to other networks thus providing a framework for simplifying mathematical modeling approaches for biochemical networks.

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Targeted Disruption of *Rgs1* Leads to Excessive B-Lymphocyte Response to Chemokines, Disturbed Plasma Cell Localization, and Distorted Immune Tissue Architecture

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Critical Role of a Switch I Residue of G alpha 13 for Rho GEF Activation‡

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Regulator of G protein signaling domain-containing Rho guanine nucleotide exchange factors (RGS-RhoGEFs) were recently shown to constitute a direct signaling link from the G alpha 13 subunit to the Rho GTPase. Identification of residues within G alpha 13 and the RGS domain which mediate this interaction is currently under investigation. Based on information derived from structures of the RGS4-G alpha i1 complex and the RGS domain of p115-RhoGEF, we decided to mutate lysine 204 within switch region I of G alpha 13 to alanine (G alpha 13 KA) and to characterize the effect of this mutation. We found this lysine residue to be critically involved in G alpha 13/RGS-RhoGEF interaction. In HeLa cells, expression of G alpha 13 KA was much less effective than wild-type G alpha 13 for Rho activation, either in the absence or presence of co-expressed LARG. Lysine 204 may be directly involved in the mechanism of RhoGEF activation through the GDP-GTP cycle of G alpha 13, as the GTPase-deficient (Q226L) mutation rescued the activity of G alpha 13 KA. Co-immunoprecipitation studies demonstrated the defective interaction of G alpha 13 KA with RGS domains of p115 or LARG, and GTPase-activating protein (GAP) activity of these RGS domains toward G alpha 13 KA was severely impaired. Stimulation of the GEF activity of p115 or LARG by G alpha 13 was drastically reduced in the background of the KA mutation. These data suggest a critical role of lysine 204 of G alpha 13 in mediating interaction with the RGS domain and in the mechanism of RhoGEF activation through the GDP-GTP cycle.

* These authors contributed equally to this work.
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RGS 12 Binds to the SNARE-binding Region of Neuronal Calcium Channels

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$GABA_B$ receptor-mediated inhibition of calcium channel requires the activation of Src kinase and downstream activation of the MAP kinase pathway. Upon activation of the tyrosine kinase the pore-forming subunit of the calcium channel is tyrosine-phosphorylated and the thereby recruits the binding of the phosphotyrosine-binding (PTB) domain of RGS12. Using a combination of biochemical and electrophysiological approached we have determined that RGS12 PTB domain binds to the SNARE-binding (synprint) region of the calcium channel. Overlay assays show that a recombinant protein containing the N-terminal PDZ and PTB domains of RGSG12 binds to the synprint region. This region contains two tyrosine residues that are phosphorylated by Src kinase, Y804 and Y815. Affinity chromatography and electrophysiological experiments show that Y804 is required for the binding of RGS12 to the channel. Our preliminary experiments how that RGS12 competes with syntaxin for the same binding region. As the binding of SNARE protein is crucial for the modulation of calcium channels and exocytosis, RGS12 binding to the synprint region might have profound physiological consequences.

RGS2 is an Effector of the Nitric Oxide-cGMP Signaling Pathway That Relaxes Blood Vessels and Regulates Blood Pressure

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Vascular tone and blood pressure are regulated by the opposing actions of vasoconstrictor and vasodilatory agonists that signal via G protein coupled receptors. Vasoconstrictor receptors in smooth muscle cells use Gq to trigger Ca2+ release from intracellular stores and Ca2+ entry across the plasma membrane, resulting in myosin light chain phosphorylation and contraction. Vasoconstrictor signaling and contraction are antagonized by vasodilatory receptors that trigger production of nitric oxide (NO). NO relaxes smooth muscle cells by incompletely understood mechanisms that attenuate Ca2+ transients and decrease Ca2+ sensitivity of the contractile apparatus.

We hypothesized that NO-mediated activation of cGMP-dependent protein kinase (cGK) targets RGS2, thereby attenuating Gq-coupled vasoconstrictor receptor signaling and promoting relaxation. Indeed, we previously showed that RGS2 -/- mice exhibit elevated blood pressure and impaired cGKI-mediated vascular relaxation *ex vivo* [1, 2].

To determine the mechanism by which RGS2 promotes relaxation, we analyzed the ability of cGKI to inhibit vasoconstrictor-induced Ca2+ transients in vascular smooth muscle cells from wild type and RGS2 -/- mice. In the absence of cGKI activation, RGS2 -/- cells displayed augmented vasoconstrictor-triggered Ca2+ responses. Strikingly, whereas activation of cGKI in wild type cells strongly inhibited vasoconstrictor-induced Ca2+ transients, cGKI activation had little inhibitory effect in RGS2 -/- cells even though cGKI□ and cGK1□ expression and activation was normal. Activated cGK phosphorylated RGS2 in cells. Therefore RGS2 is a novel effector of the NO-cGMP pathway that attenuates vasoconstrictor-induced Ca2+ signaling, promotes vascular relaxation and regulates blood pressure. Because RGS2 and cGKI are co-expressed in several tissues and cell types, this crosstalk mechanism may be used widely to attenuate Gq-stimulated Ca2+ signaling. Impairing this mechanism may contribute to the development of hypertension, and augmenting it may provide a novel means of treating hypertension.

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Hepatic Rgs16 Regulation by Dietary Carbohydrate and Fats

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G protein signaling in liver helps maintain carbohydrate and lipid homeostasis. Regulators of G protein Signaling (RGS) proteins are GTPase-activating proteins (GAPs) of G_i and G_q class of alpha subunits and function as inhibitors of G_i/G_q signaling. RGS mRNA and protein expression can be induced by G protein coupled agonists to feedback inhibit these pathways. To characterize the control of liver G_i/G_q signaling during fasting and refeeding we screened the expression of all 20 mouse RGS genes. Only Rgs16 mRNA and protein are regulated by fasting. QPCR and Western blot analysis show that liver Rgs16 mRNA and protein are diurnally regulated in mice fed *ad libitum*, and the expression pattern changes rapidly in response to a restricted feeding schedule. By contrast, the circadian regulation of Rgs16 in the suprachiasmatic nucleus (SCN) is entrained by light and does not respond to feeding. In mice maintained on normal chow, Rgs16 mRNA is upregulated (40-fold) in periportal hepatocytes in response to fasting and down regulated by refeeding in 30 minutes. When mice were given free access to sucrose(5%)-water for 3 days Rgs16 mRNA is hyper-induced (160 fold) when denied food on day 3, but quickly declined either by refeeding or i.p. injection of the satiety hormone Cholecystokinin-8 (CCK). Male transgenic mice that express Rgs16 protein specifically in liver under the control of a doxycyclin-inducible promoter are being characterized for defects in glycogen storage and utilization. We propose Rgs16 feedback inhibits a G_i/G_q pathway that controls glucose and fatty acids metabolism during transitions between fasted and fed states in liver.

Work was supported by NIH and Welch Foundation grants to TMW.

Toward Small Molecule Inhibitors of RGS Proteins: Development of Computational and *in vitro* Fluorescence-based Approaches.

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Ligand-bound seven transmembrane receptors cause G-protein alpha subunits to bind guanosine 5'-triphosphate (GTP) and activate effector pathways. Signal termination is facilitated by the intrinsic GTPase activity of G-protein alpha subunits. Regulator of G-protein signaling (RGS) proteins accelerate the GTPase activity of the G-protein alpha subunit, and thus negatively regulate G-protein-mediated signal transduction. Given their multiplicity, dynamic spatio-temporal regulation, receptor-selectivity, and pleiotropic signaling capacity, RGS proteins are promising drug targets (Neubig, R.R. and Siderovski, D.P.; Nat. Rev. Drug. Discov. 2002 1:187-197).

We have performed structure-based drug discovery at the G-alpha-i1/RGS4 interface to identify possible RGS inhibitors. Using DOCK we have screened 200,000 compounds *in silico* for their ability to bind and inhibit RGS proteins. Computationally high-scoring compounds were then tested, *in vitro*, using novel fluorescence based assays of RGS protein function. The standard *in vitro* assay for RGS-protein activity is the measurement of G-protein alpha subunit GTPase acceleration (GAP) activity. The conventional paradigm makes use of radiolabeled GTP and scintillation counting. Here, we describe fluorescence-based methodologies to study heterotrimeric G-protein alpha subunit regulation by RGS proteins *in vitro*. We utilize fluorophore-conjugated GTP analogues in kinetic assays for RGS protein GAP activity. Similarly, we utilize fluorescence resonance energy transfer (FRET)-based assays for RGS protein/G-alpha interaction to identify compounds that inhibit RGS protein/G alpha interaction.

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RGS9-2 Is a Negative Modulator of Morphine Actions

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Genetic Analysis of RGS-PX1 Functions in Mice

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We have shown that RGS-PX1, a member of the regulator of G protein signaling (RGS) protein family, is able to attenuate Gαs-mediated signaling through its GAP activity and to regulate the endocytic trafficking and degradation of the EGF receptor through its sorting nexin activity in biochemical and cell culture studies (*Science 2001, 294:1939*). Northern Blot analysis indicated that RGS-PX1 is ubiquitously expressed with the highest levels in heart and skeletal muscle. To understand the physiologic roles of RGS-PX1, we have employed both knock out and transgenic strategies in mice. Using the Cre/loxP system to generate mice carrying targeted mutations of RGS-PX1, we have found that systemic RGS-PX1-null mice were embryonic lethal around E11.5, similar to what is observed in Gαs knockout mice *(PNAS 1998, 95:8715).* RGS-PX1-null embryos exhibit significant overall growth retardation by E8.5 and striking neural tube closure defects at E10.5. Whole-mount staining of embryos for the endothelial cell marker CD31 (PECAM) revealed dramatic vascularization defects in RGS-PX1 null embryos, especially in the cephalic and intersomatic regions. Analysis of the visceral embryonic yolk sac epithelium revealed morphologic changes in the endocytic system and defective uptake of Texas Red-transferrin, supporting an important role of RGS-PX1 in the regulation of endocytic trafficking. These results demonstrate an essential role of RGS-PX1 in mouse development. Conditional RGS-PX1-null mice are currently being generated.

To investigate the role of RGS-PX1 in cardiac function, we overexpressed the RGS domain of RGS-PX1 in the hearts of transgenic mice using a mouse alpha-myosin heavy chain promoter. Adenylyl cyclase activity assays using myocyte membranes prepared from adult hearts showed that overexpression of RGS-PX1 in transgenic mice blunted isoproterenol-stimulated adenylyl cyclase activation, consistent with the notion that RGS-PX1 inhibits β-adrenergic receptor mediated Gαs signaling in the heart. Taken together, these data suggest that each of the two key functional domains of RGS-PX1 contribute to cellular regulation *in vivo.*

Regulation of EGF Receptor Degradation by RGS-PX1 and Gαs

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Recently we identified and characterized RGS-PX1 and showed that it is localized in early endosomes and acts as a GAP for Gαs through its RGS domain and as a sorting nexin that prolongs epidermal growth factor (EGF) signaling by slowing EGF receptor (EGFR) down-regulation through its phoX (PX) domain (*Science 2001, 294:1939*). These dual activities and its localization suggested that RGS-PX1 serves as a bridge between Gαs and EGFR sorting at endosomes and that Gαs may regulate EGF degradation. To determine whether Gαs can bind to RGS-PX1 *in vivo* we analyzed transfected HEK293 cells and found that Gαs and GFP-RGS-PX1 coprecipitate and colocalize in punctate structures, presumably endosomes. To find out if Gαs can affect EGFR down-regulation we transfected wild-type or a constitutively active mutant Gαs (Q227L) and EGFR into HEK293 cells and found that both promote ligand-dependent EGFR degradation as assessed by immunoblotting. Moreover, overexpression of GFP-tagged Gαs in Cos-7 cells significantly enhances degradation of Texas Red-EGF after 30 or 60 min uptake but does not impair its internalization. Furthermore, inhibition of Gαs expression by RNAi delayed the degradation of EGFR in Cos-7 cells, suggesting that Gαs plays an important role in EGFR degradation. To define the mechanisms whereby Gαs influences EGFR trafficking, we tested whether Gαs interacts with known components of the endocytic sorting machinery. We found that Gαs and RGS-PX1 interact with Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), a critical component of the endosomal sorting machinery *(Curr Opin Cell Biol. 2003, 15:446),* in immunoprecipitation and pull-down assays. By immunofluorescence exogenously expressed Gαs colocalizes with myc-Hrs and GFP-RGS-PX1 in early endosomes in Cos-7 cells and expression of Hrs or RGS-PX1 recruits Gαs to endosomes. These observations define important roles of RGS-PX1 and Gαs in degradation of EGFR and provide mechanistic insights into the functions of RGS-PX1 and Gαs in endocytic trafficking.

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