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# Whither Goest the RGS Proteins?

*David P. Siderovski, Bentley Strockbine, and Cynthia I. Behe*

Department of Pharmacology, School of Medicine, The University of North Carolina at Chapel Hill, Campus Box #7365, Mary Ellen Jones Building, Room 906B, Chapel Hill, NC 27599-7365

**Referee: Dr. Richard Neubig, Department of Pharmacology, University of Michigan at Ann Arbor, 1150 West Medical Center Drive, Ann Arbor, MI 48109–0632**

**ABSTRACT:** Studies of the desensitization of G protein-coupled signal transduction have led to the discovery of a family of guanosine triphosphatase-activating proteins (GAPs) for heterotrimeric G protein alpha subunits — the "regulator of G protein signaling" or RGS proteins. In considering both documented and potential functions of several RGS protein family members with demonstrable multidomain compositions (p115RhoGEF, PDZRhoGEF, Axin, Axil/Conductin, D-AKAP2, the G protein-coupled receptor kinases [GRKs], the DEP/GGL/RGS subfamily [RGS6, RGS7, RGS9, RGS11], and RGS12), this review explores the shift in our appreciation of the RGS proteins from unidimensional desensitizing agents to multifocal signal transduction regulators.

**KEY WORDS:** desensitization, effector proteins, GGL, GoLoco, PDZ, PTB, regulators of G protein signaling, RGS, scaffold proteins, signal transduction

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## I. INTRODUCTION

Signal transduction is the general process cells use to respond to cues from their external environment — packets of information embodied in various forms: hormones, neurotransmitters, growth factors, differentiation factors, photons of light. These diverse stimuli each start a cascade of biochemical reactions, beginning with interaction of the signaling molecule with a cellular receptor and ending with activation of intracellular molecular machinery. This fundamental process of signal transduction controls a wide variety of cellular activities, ranging from release of hormones and neurotransmitters, modulation of transmembrane ion flux, and activation or repression of gene transcription, to integrated responses of cellular survival, proliferation, and differentiation.

One important pathway in this process is the guanine nucleotide-binding protein ("G protein") signaling pathway (Gilman, 1987; Hamm, 1998). In this pathway, serpentine receptors that span the plasma membrane seven times (Figure 1) are coupled to membrane-associated heterotrimeric G proteins composed of α, β, and γ subunits. A variety of these subunits have been identified (20 α, 6 β, and 12 γ to date) that associate to comprise at least four general classes of G proteins (Simon et al., 1991): $G_s$, activators of adenylyl cyclase; $G_i$, inhibitors of adenylyl cyclase; $G_q$, activators of phospholipase C; and $G_{12}$, for which effectors are now being identified (Fukuhara et al., 1999; Hart et al., 1998b; Jiang et al., 1998; Mao et al., 1998). After binding extracellular ligand, the *G protein-coupled receptor* (GPCR) changes the conformation of its intracellular loops to promote the exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit and the subsequent dissociation of Gα and Gβγ. Whereas GTP-bound Gα and free Gβγ subunits are both capable of propagating the signal through their interactions with downstream effector proteins, deactivation of the

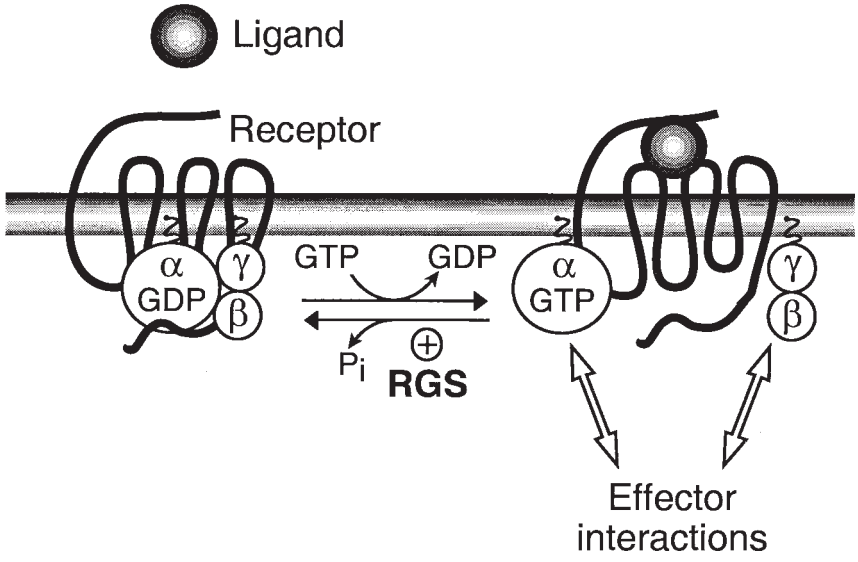


**FIGURE 1**. General scheme of signal transduction by serpentine G protein-coupled receptors and facilitation of signal termination by RGS proteins that serve as *g*uanosine triphosphatase-*a*ctivating *p*roteins (GAPs) for heterotrimeric G protein alpha subunits. See text for details.

signal is transacted solely by the intrinsic guanosine triphosphatase activity of the Gα subunit (Figure 1). Reassociation of Gβγ with GDP-bound Gα obscures critical effector contact sites, (Ford et al., 1998: Li et al., 1998b), thereby terminating effector activations. Hence, the duration of G protein-coupled signaling is subject to the rate of Gα GTP hydrolysis.

However elegant, this scheme is incomplete. Discrepanies are known to exist, for example, in photoreception (Arshavsky and Pugh, 1998), between the rate at which physiological responses are terminated (~milliseconds) and the rate at which isolated Gα subunits hydrolyze GTP (~seconds). A partial answer to this timing paradox came from the discovery that certain effectors can also act as guanosine triphosphatase-*a*ctivating ₱roteins (GAPs). For example, phospholipase Cβ–1 can enhance the rate of GTP hydrolysis by $G\alpha_{q/11}$ (Berstein et al., 1992; Chidiac and Ross, 1999). Adenylyl cyclase may do the same for $G_{\alpha s}$ (Scholich et al., 1999). Recently, a new family of GAPs for Gα subunits, dubbed "regulators of G-protein signaling" or "RGS" proteins, have been identified by my laboratory and others. This review addresses a paradigm shift currently occurring in our consideration of these proteins: rather than being a group of monolithic Gα GAPs, the RGS family embraces a panoply of diverse signal transduction regulators, all sharing a modular protein-protein interaction domain — the "RGS box".

## II. DISCOVERY

Our discovery of the RGS family had its genesis in the study of immediate-early genes involved in T-cell activation. Initially, we described a set of human lymphocyte $G_0/G_1$-switch regulatory genes ("*G0S* genes"; Siderovski et al., 1990) isolated by differential screening of cDNA prepared from peripheral blood mononuclear cells stimulated with a T-cell mitogen (concanavalin-A) in the presence of the protein synthesis inhibitor cycloheximide. Several of these *G0S* genes were shown to be human orthologues of rodent fibroblast immediate-early genes or members of the c-Fos family of transcription factors (Siderovski et al., 1990; Heximer and Forsdyke, 1993). One member of the *G0S* set, *G0S8*, had no obvious relationship with known immediate-early genes; however, we did report a small region of sequence similarity but unknown function shared between the G0S8 gene product and two known regulators of G protein signaling: the family of G protein-coupled receptor kinases (GRKs) and the Sst2p protein of the budding yeast *Saccharomyces cerevisiae* (Siderovski et al., 1994). This finding was reaffirmed in our later report on the chromosomal localization of *G0S8* (Wu et al., 1995), with the polypeptide sequence similarity between G0S8 and Sst2p extended to a ~100-residue tripartite domain also found in an early regulator of *Aspergillus nidulans* conidiophore development (flbA; Lee and Adams, 1994), an anonymous open-reading frame from the *Caenorhabditis elegans* genome project (C05B5.7; Wilson et al., 1994), and the human B-cell-specific immediate-early gene product 1R20/BL34 (Murphy and Norton, 1990; Hong et al., 1993).

*sst2* was originally isolated by genetic analysis of *S. cerevisiae* mutants supersensitive to the cell-cycle-arresting effects of mating pheromone (Chan and Otte, 1982a,b). Identification of Sst2p as a negative regulator of pheromone signaling, a well-characterized G protein-mediated pathway (Sprague and Thorner, 1992; Kurjan, 1993), afforded us an opportunity to test the functional significance of the G0S8/Sst2p sequence similarity. Indeed, expression of

G0S8 in haploid *S. cerevisiae* strains bearing either wild-type *SST2* or mutant *sst2* alleles dramatically decreased sensitivity to pheromone-induced cell-cycle arrest (Siderovski et al., 1996). Previous genetic studies established that Sst2p functioned either upstream of, or directly on, the Gα subunit (Gpa1p) coupled to the pheromone receptor (Dohlman et al., 1995); and references therein). Hence, we proposed that the growing collection of protein sequences sharing homology with G0S8 and Sst2p represented a new family of G protein-coupled receptor desensitizing factors, with a common function most likely to be direct association with activated Gα subunits (Siderovski et al., 1996). Our hypothesis was strengthened by the identification of a human *G-a*lpha *i*nteracting *p*rotein (GAIP), within which a core 125 amino acid domain bearing homology with G0S8 and Sst2p was found to be critical for Gα binding (De Vries et al., 1995).

Other groups came to similar conclusions. For example, Koelle and Horvitz (1996), in reporting the cloning of the *C. elegans* gene *egl-10*, established the current moniker for these proteins as "regulators of G-protein signaling", given genetic evidence for regulation by EGL-10 of the Gα subunit GOA-1 involved in *C. elegans* egg-laying and locomotion behaviors. This same group isolated nine partial RGS gene sequences by polymerase chain-reaction (PCR) amplifications of rat brain cDNA using degenerate oligonucleotide primers designed to bind to conserved "RGS domain" sequence elements; in this way, a number-based nomenclature for the first 14 mammalian RGS genes was established (the expressed-sequence tag described by Koelle and Horvitz as "RGS15" was in fact the human orthologue of rat RGS3). In addition, a subsequent report confirmed the ability of G0S8 (now RGS2) and its B-cell homologue, 1R20/BL34 (now RGS1), to decrease pheromone sensitivity in yeast (Druey et al., 1996). An independent account detailing this simultaneous discovery of the RGS family by several groups was also published around this time (Roush, 1996).

Very soon after identification of the mammalian RGS proteins came empirical evidence of their effects on Gα subunits. Berman, Wilkie, and Gilman (1996) purified recombinant GAIP and RGS4 proteins and tested each in biochemical assays with isolated Gα subunits *in vitro*. Neither GAIP nor RGS4 had any effect on steady-state GTP hydrolysis that, in the absence of activated receptor, is a measure of the rate-limiting step of product release (i.e., GDP dissociation). Both GAIP and RGS4 did, however, dramatically accelerate GTP hydrolysis by $G_i$ subfamily alpha subunits ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$, $G_{\alpha z}$) as measured in single-turnover assay conditions where GDP dissociation was not rate limiting (Berman et al., 1996). Subsequent demonstration by Dohlman and colleagues of *in vitro* Gα GAP activity by Sst2p brought the RGS discovery story full circle (Apanovitch et al., 1998; reviewed in Dohlman et al., 1998) (Figure 2).

## III. ORIGINAL VIEW

The role of Sst2p induction in yeast adaptation to pheromone signaling established the original viewpoint of the role of the RGS proteins in G protein signaling attenuation, as exemplified in the higher eukaryote by RGS1 (Figure 3). *RGS1* was originally described by Murphy and Norton (1990) as *1R20*, isolated by virtue of its rapid transcriptional up-regulation after treatment of human B-cell chronic lymphocytic leukemia cells with phorbol ester (PMA), an activator of protein kinase C (PKC). Independently, Kehrl and co-workers identi-

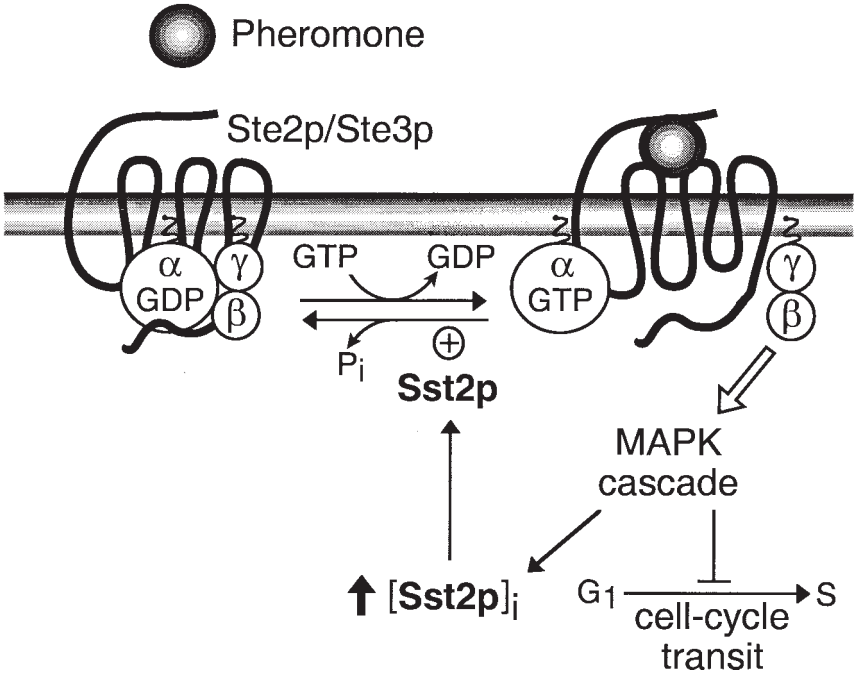


**FIGURE 2.** Scheme of homologous desensitization of pheromone signaling by yeast RGS protein Sst2p. Haploid yeast sense extracellular mating pheromone using the G protein-coupled receptor Ste2p or Ste3p (depending on haploid type). Bound receptor catalyzes guanine nucleotide exchange on the G protein α subunit (Gpa1p), leading to release of the Gβγ subunit (Ste4p/Ste18p) and the activation of a mitogen-activated protein kinase (MAPK) cascade. Extracellular pheromone thus stimulates integrated mating responses such as cell-cycle arrest and changes in cell polarity and morphology. This signal also stimulates *SST2* gene transcription and accumulation of Sst2p protein levels, leading to acceleration of GTP hydrolysis by Gpa1p and recovery from pheromone-induced mating responses in the absence of mating "consummation".

fied *BL34*, a human B-cell-specific cDNA trapped by subtraction of mRNA from mitogen/PMA-treated B-cells with that of mitogen/PMA-treated T-cells (Hong et al., 1993). Sequence data from *1R20* and *BL34* cDNAs revealed them both to be derived from the same gene, encoding a 196 amino acid protein with a predicted alpha-helical character and sequence similarity to the G0S8/RGS2 gene product (Hong et al., 1993; Newton et al., 1993). Unpublished data from Kang and Scheschonka, cited obliquely by Kehrl (Druey et al., 1998), suggests that *RGS1* expression is confined largely to a subset of B-cells that localize to the germinal center region of lymphoid tissue (a site of antigen-stimulated activation and somatic hypermutation). Druey and colleagues (1996) observed increased RGS1 expression within a human B-cell lymphoma line, HS-Sultan, after treatment with platelet-activating factor (PAF). Moreover, ectopic overexpression of RGS1 in HS-Sultan cells diminished PAF-induced mitogen-activated protein kinase (MAPK) activation. The PAF receptor couples to both the Gq and Gi families of G proteins (e.g., Honda et al., 1994), and both of these G protein subclasses can activate MAP kinase signaling by the release of Gβγ heterodimers (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994). RGS1 accelerates the GTPase activity of $G_i$class Gα subunits in *in vitro* single-turnover assays (Watson et al., 1996b). In trans-

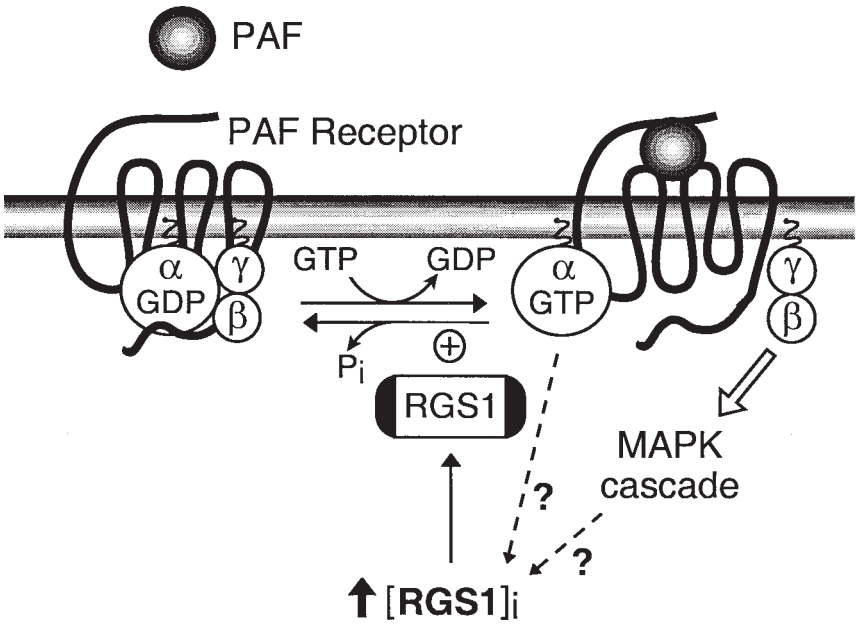


**FIGURE 3.** Homologous desensitization of platelet-activating factor (PAF) signaling in B-cells by RGS1 as a model for the original view of RGS protein-mediated signal attenuation. While PAF treatment has been observed to increase RGS1 protein levels in B-cells (Druey et al., 1996), the particular signal transduction cascade leading to this increase remains uncharacterized, hence the dotted lines and question marks.

fected cells, RGS1 inhibits PAF-activated p38 and extracellular signal-regulated kinase (ERK) arms of MAPK signaling (Zhang et al., 1999), and is also a potent inhibitor of Gq-dependent calcium signaling in pancreatic acinar cells (Xu et al., 1999), suggesting that RGS1 can accelerate GTP hydrolysis by $G_{\alpha q/11}$ subunits as well. Hence, the hypothesis proposed by Druey et al. that RGS1 participates in a negative feedback loop to decrease PAF receptor signaling could be realized in the following manner: an accumulation of RGS1 stimulates GTP hydrolysis by PAF-activated $G_{\alpha i}$ and $G_{\alpha q/11}$ subunits, thus causing a reassociation of free $G\beta\gamma$ heterodimers with GDP-bound $G\alpha$ and a termination of signaling (Figure 3). PAF-stimulated accumulation of RGS1 might also play a role in heterologous desensitization of other G protein-coupled receptor systems, as Bowman and colleagues (1998) have subsequently demonstrated that ectopic RGS1 expression potently inhibits B-cell chemotaxis stimulated through various G-protein-coupled chemoattractant receptors, including those for formyl-Met-Leu-Phe (fMLP), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1).

## IV. HARBINGERS OF THE PARADIGM SHIFT

Many different types of mammalian proteins have now been reported to possess the conserved ~125 amino acid "RGS box" (Siderovski et al., 1996; Koelle, 1997; Dohlman and Thorner, 1997; Berman and Gilman, 1998; De Vries and Farquhar, 1999), and thus it has become apparent how misleading it is to consider all of them as simple, unidimensional desensitizing agents. Some RGS box-containing proteins not only

harbor Gα GAP activity, but also appear to exploit their interaction with active GTP-bound Gα to activate a coincident effector function. Others contain the RGS box, but, to this point, have not demonstrated Gα GAP activity. RGS box-containing proteins have also been found bearing complex, multidomain structures that suggest an active orchestration of interdependent signaling events. Examples of all of these scenarios are detailed below.

## A. p115RhoGEF and PDZRhoGEF: Effector Proteins for $G_{\alpha 12/13}$

The majority of *bona fide* RGS proteins (i.e., those with demonstrable Gα GAP activity) have exhibited selective activity toward Gα subunits of the Gi and/or Gq classes (reviewed in DeVries and Farquhar, 1999); in this regard, it is interesting to note that no RGS box-containing protein has yet been shown to GAP $G_{\alpha s}$, although reports exist that RGS2 (Tseng and Zhang, 1998) or RGS3 (Chatterjee et al., 1997) might directly affect $G_{\alpha s}$-coupled signaling pathways. Two related RGS box-containing proteins have now been discovered to act as GAPs for $G_{\alpha 12}$ and $G_{\alpha 13}$, sole members of $G_{12}$ — the fourth distinct class of Gα subunits.

Kozasa and colleagues (1998) first reported detecting a weak RGS box signature (Figure 4) within the N-terminus of p115RhoGEF, a ubiquitously expressed guanine nucleotide exchange factor (GEF) (Hart et al., 1996) that specifically activates Rho, the low-molecular-weight Ras superfamily GTPase involved in cytoskeletal regulation (reviewed in Hall, 1994; Zohn et al., 1998). Independently, Fukuhara and co-workers (1999) detected a weak similarity to the RGS domain of RGS14 within PDZRhoGEF, a widely expressed protein closely related to human p115RhoGEF, its mouse orthologue (Lsc), and *Drosophila* DRhoGEF2. (Although it is clearly an RGS box within PDZRhoGEF, Fukuhara and colleagues refer to this region rather cryptically as an "LH" or "*L*sc-*h*omology" domain.)

Direct interaction of PDZRhoGEF with $G_{\alpha 12}$ and $G_{\alpha 13}$, but not $G_{\alpha i2}$, $G_{\alpha q}$, or $G_{\alpha s}$, was demonstrated by cellular co-immunoprecipitation studies employing transiently expressed PDZRhoGEF and GTPase-deficient, constitutively active Gα mutants (Fukuhara et al., 1999); deletion of the "LH-domain" abrogated $G_{\alpha 12/13}$ association. In addition to performing similar co-immunoprecipitation studies with p115RhoGEF (Hart et al., 1998b), Kozasa and colleagues directly tested full-length p115RhoGEF as a Gα GAP in *in vitro* single-turnover assays and found acceleration of $G_{\alpha 12}$ and $G_{\alpha 13}$ GTPase activities by factors of 6 and 80, respectively, with no apparent activity on $G_{\alpha i1}$, $G_{\alpha z}$, $G_{\alpha q}$, or $G_{\alpha s}$ (Kozasa et al., 1998). The N-terminal RGS box of p115RhoGEF is responsible for this GAP activity: a GST-fusion protein solely containing the RGS box possesses full $G_{\alpha 12/13}$ GAP activity and, conversely, Nterminal deletion of the RGS box eliminates the GAP activity of p115RhoGEF (Kozasa et al., 1998).

Not only was p115RhoGEF established as a *bona fide* Gα GAP in this manner, but, in an accompanying report, Kozasa and colleagues also established that activated $G_{\alpha 13}$ stimulates the ability of p115RhoGEF to exchange bound GDP for GTP on Rho *in vitro* (Hart et al., 1998b). Deletion of the RGS box also stimulates the GEF activity of p115RhoGEF, implying that $G_{\alpha 13}$ binding to the N-terminus may release intramolecular repression of exchange activity embodied in the C-terminal DH (*d*bl-*h*omology) domain of the hallmark DH/PH (*p*leckstrin-*h*omology) domain tandem (Figure 5). In a similar vein, Fukuhara et al. (1999) reported

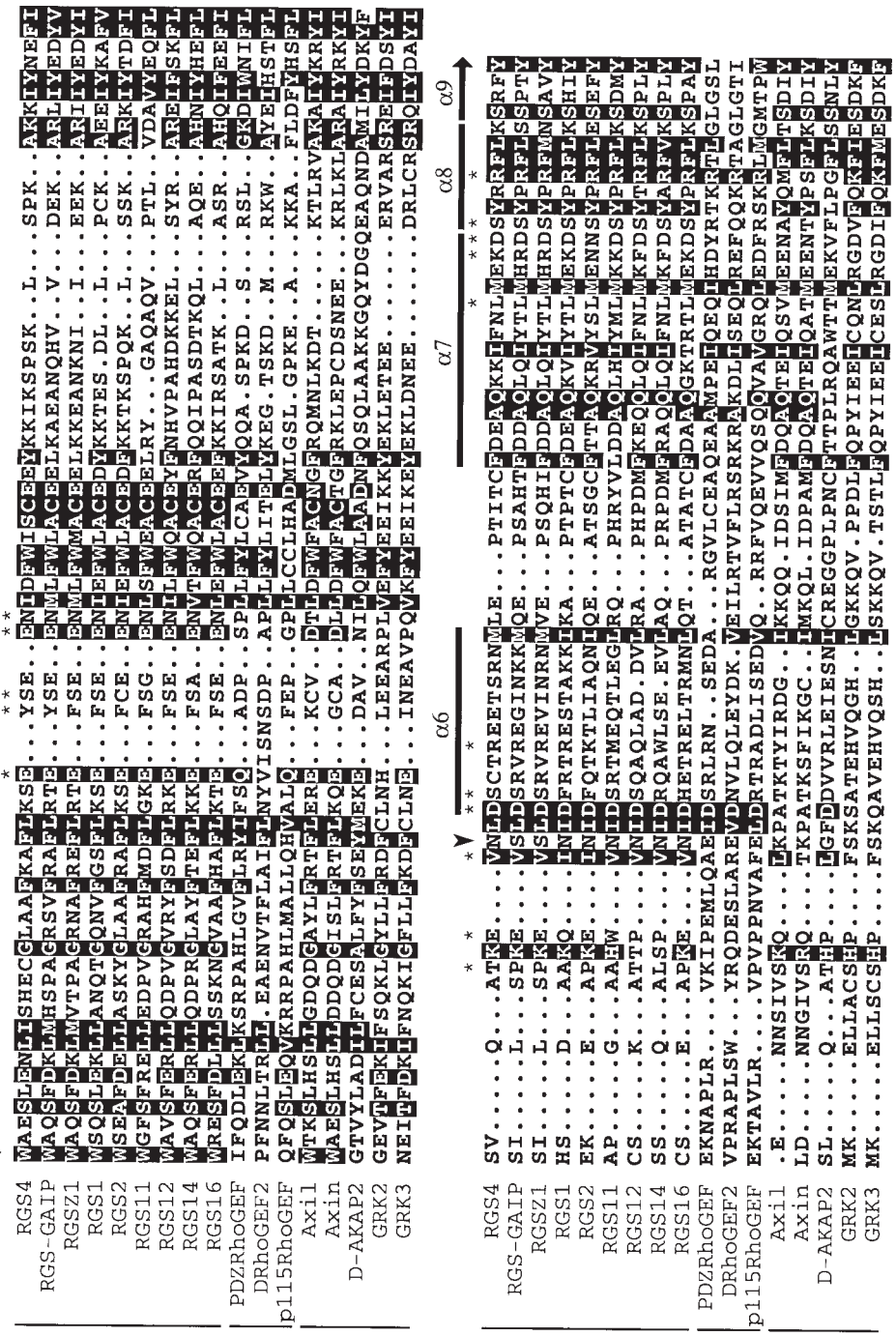

**FIGURE 4.** Multiple sequence alignment of RGS domains from (A) *bona fide* Gα GAPs, (B) $G_{\alpha 12/13}$ GAPs that also serve as Rho guanine exchange factors, and (C) "outlier RGS proteins". The alpha-helical secondary structure of RGS4, as observed in the crystal structure of RGS4 complexed with the transition state-mimetic $G_{\alpha i1}$-GDP-$AlF_4^-$ (Tesmer et al., 1997), is illustrated above the RGS4 primary sequence (α1 to α9); residues from RGS4 that contact $G_{\alpha i1}$ switch regions (interatomic distances <4.0 Å) are highlighted by asterisks (*). The critical asparagine-128 of RGS4, presumed either to specify binding to Gα subunits (Posner et al., 1999) or to interact directly with glutamine-204 of Gαi1 to cause GTPase acceleration (Coleman and Sprang, 1999), is denoted by the arrowhead. The primary sequences used in the alignment are as follows: aa 59-174 of human RGS4 (SwissProt P49798); aa 87-202 of human RGS-GAIP (P49795); aa 88-203 of human RGSZ1 (GenBank NP_003693); aa 69-183 of human RGS1 (Q08116); aa 80-195 of human RGS2 (P41220); aa 279-393 of human RGS11 (NP_003825); aa 712-828 of human RGS12 (NP_002917); aa 64-180 of rat RGS14 (O08773); aa 62-177 of human RGS16 (O15492); aa 310-430 of human PDZRhoGEF (GenBank AB002378); aa 924-1051 of Drosophila DRhoGEF2 (AF031930); aa 45-168 of p115RhoGEF (NP_004697); aa 78-196 of human Axil (NP_004646); aa 122-244 of human Axin (GenBank AAC51624); aa 86-211 of mouse D-AKAP2 (AAC61898); aa 51-171 of human GRK2 (P25098); aa 51-171 of human GRK3 (P35626). The sequence of Lsc, the mouse orthologue of human p115RhoGEF, is not illustrated as its RGS box is nearly identical to that of p115RhoGEF (six conservative substitutions; Kozasa et al., 1998). One-letter amino acid abbreviations are as follows: **A,** Ala; **C,** Cys; **D,** Asp; **E,** Glu; **F,** Phe; **G,** Gly; **H,** His; **I,** Ile; **K,** Lys; **L,** Leu; **M,** Met; **N,** Asn; **P,** Pro; **Q,** Gln; **R,** Arg; **S,** Ser; **T,** Thr; **V,** Val; **W,** Trp; **Y,** Tyr; **(.),** gap in alignment.

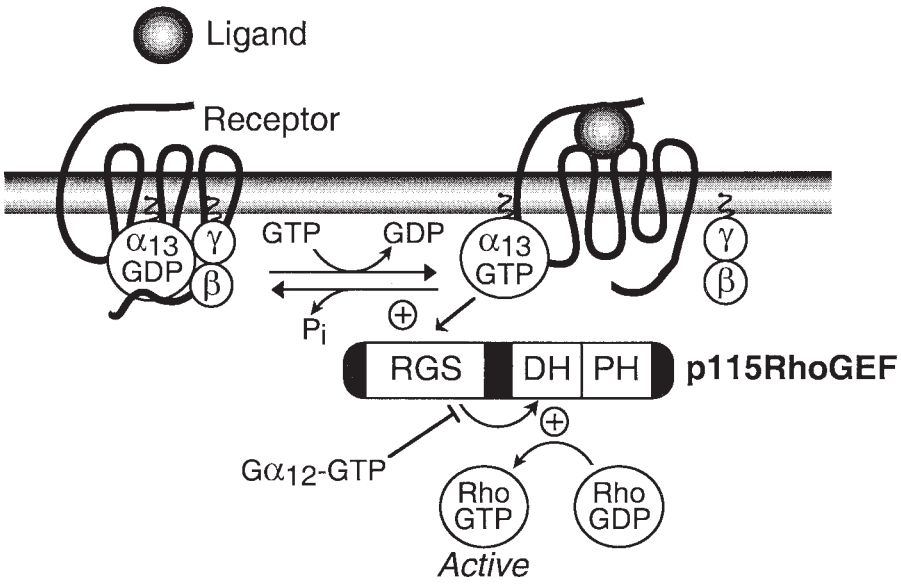


**FIGURE 5.** Current model of Gα-dependent Rho activation by p115RhoGEF. Activation of a heterotrimeric $G_{13}$-coupled receptor by ligand association leads to receptor-catalyzed exchange of bound GDP for GTP on $G_{\alpha 13}$. Subsequent binding of GTP-bound $G_{\alpha 13}$ to the RGS box of p115RhoGEF leads to both (a) activation of Rho exchange activity by the *d*bl-*h*omology (DH) domain and (b) 80-fold acceleration of the intrinsic GTPase activity of GTP-bound $G_{\alpha 13}$. Active, GTP-bound $G_{\alpha 12}$ is thought to inhibit $G_{\alpha 13}$-mediated Rho activation by forming an unproductive association with p115RhoGEF that fails to activate DH domain-mediated Rho exchange, coupled with weaker RGS box GAP activity on $G_{\alpha 12}$.

enhanced Rho-dependent transcriptional activation by PDZRhoGEF after deletion of its N-terminal RGS box. However, a simple mechanism of derepression may not totally account for the Gα-dependent activation of Rho exchange activity by these proteins; for example, binding of $G_{\alpha 12}$ to p115RhoGEF is insufficient to stimulate Rho exchange activity and actually blocks stimulation by $G_{\alpha 13}$ *in vitro* (Hart et al., 1998b). This finding suggests the existence of a secondary mode of $G_{\alpha 13}$/p115RhoGEF interaction that is not apparent during the binding of $G_{\alpha 12}$ and, consequently, raises a potential for dynamic interplay between activated $G_{\alpha 12}$ and $G_{\alpha 13}$ subunits in modulating Rho activation *in vivo* (Figure 5).

Hence, p115RhoGEF and PDZRhoGEF represent the first examples of RGS box-containing proteins that are at once both desensitizing agents (Gα-directed GAP activity) and signal generators (Gα-responsive Rho activation). As has been argued previously for phospholipase Cβ, the dual $G_{\alpha q}$-effector and $G_{\alpha q}$-GAP (Biddlecome et al., 1996), fast, local Gα inactivation by these RhoGEFs likely allows for GPCR-mediated Gα reactivation while the receptor/G protein/effector complex is still assembled, hence permitting rapid responsiveness to agonist and higher amplitude signal generation. In considering such complex formation between receptor, G protein, and effector, it is tempting to speculate that the N-terminal PDZ (*P*SD95/*D*lg/*Z*O1) domain of PDZRhoGEF might bind directly to the C-termini of $G_{12}$-coupled serpentine receptors (Figure 6), given the high percentage of known GPCR C-termini conforming to the general consensus for PDZ docking sites.

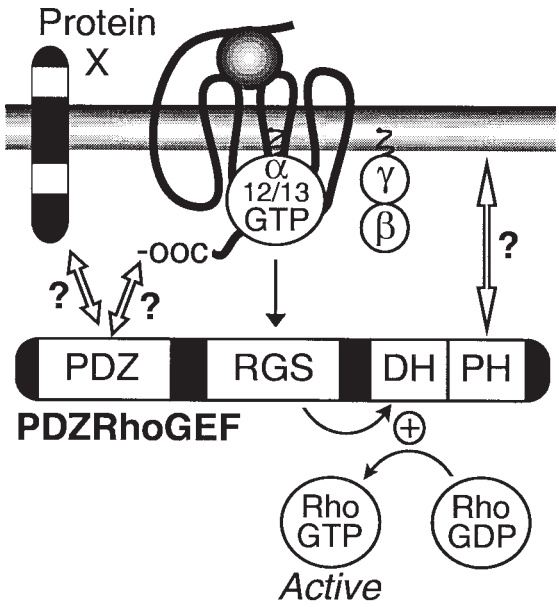


**FIGURE 6.** Speculative model of potential higher-order complex formation by PDZRhoGEF. Fukuhara and colleagues (1999) have demonstrated binding of GTPase-deficient, constitutively active $G_{\alpha 12}$ and $G_{\alpha 13}$ subunits to the RGS box of PDZRhoGEF. The N-terminal PDZ (*P*SD-95/*D*lg/*Z*O-1) domain of PDZRhoGEF represents a well-characterized protein-protein interaction module capable of binding to C-terminal (Songyang et al., 1997) or internal (Hillier et al., 1999) regions of proteins, here arbitrarily depicted by the C-terminus of the activating G12-coupled receptor and an anonymous transmembrane protein "X", respectively. The pleckstrin-*h*omology (PH) domain, universally present C-terminal to the *d*bl-*h*omology (DH) domain of Rho family GEFs (Whitehead et al., 1997), is thought not to play a direct role in Rho nucleotide exchange (Hart et al., 1994), but rather may mediate membrane association by binding to lipid and/or lipid-associated targets (Lemmon et al., 1996).

## B. Axin and Axil/Conductin: Scaffold Proteins for the Wnt Signaling Pathway

The study of embryonic axis development has led to identification of "outlier" RGS proteins (i.e., not *bona fide* Gα GAPs) that intersect with the Wnt/Frizzled signaling pathway (Figure 4). Costantini and colleagues originally described a random transgene insertional event in the mouse giving rise to a recessive lethal phenotype of embryonic axis duplication (Perry et al., 1995), similar to mouse *Fused* mutants (Glückson-Schönheimer, 1949). The gene disrupted by the insertional event encodes a 992 amino acid protein with an N-terminal RGS domain and a C-terminal DAX domain also found in *Drosophila* and vertebrate Dishevelled (Dsh) proteins (Zeng et al., 1997). To avoid confusion with an unrelated *fused* gene in *Drosophila*, the product of the mouse *Fused* gene was named Axin (for *ax*is *in*hibition (Vasicek et al., 1997; Zeng et al., 1997). Injection of wild-type *Axin* mRNA into *Xenopus* embryos blocked axis formation (Zheng et al., 1997). Moreover, wild-type *Axin* blocked the ectopic axis formation normally observed by injection of either *Wnt, Dsh,* or dominant-negative glycogen synthase kinase-3β (*GSK3*β)

mRNAs, thereby placing the inhibitory effect of Axin on Wnt signaling at the level of, or downstream of, GSK-3β.

The N-terminal RGS domain of Axin is critical for activity, as its deletion not only abolishes the axis-inhibiting function of Axin, but creates a dominant-negative Axin mutant able to induce ectopic axis formation (Zeng et al., 1997) One potential mechanism for Wnt signaling blockade by Axin is RGS domain-mediated GAP activity on Wnt-activated Gα subunit(s) (Figure 7, hypothesis ①). Involvement of heterotrimeric G proteins is possible in this pathway, as Frizzled proteins (the receptors for Wnt factors) are serpentine transmembrane proteins reminiscent of G protein-coupled receptors (Barnes et al., 1998), and at least one Frizzled family member is linked biochemically to phosphatidylinositol turnover via Gβγ subunits of pertussis-toxin-sensitive G protein(s) (Slusarski et al., 1997). However, recently several groups have described Axin as a scaffold protein (Hart et al., 1998a; Ikeda et al., 1998; Kishida et al., 1998; Sakanaka et al., 1998), binding to GSK3β, β-catenin, and APC (the *a*denomatous *p*olyposis *c*oli tumor suppressor protein) and thereby coordinating the effects of GSK3β and APC on β-catenin destruction, an action antagonistic to Wnt-dependent gene expression (Figure 7, hypothesis ②). A structurally related

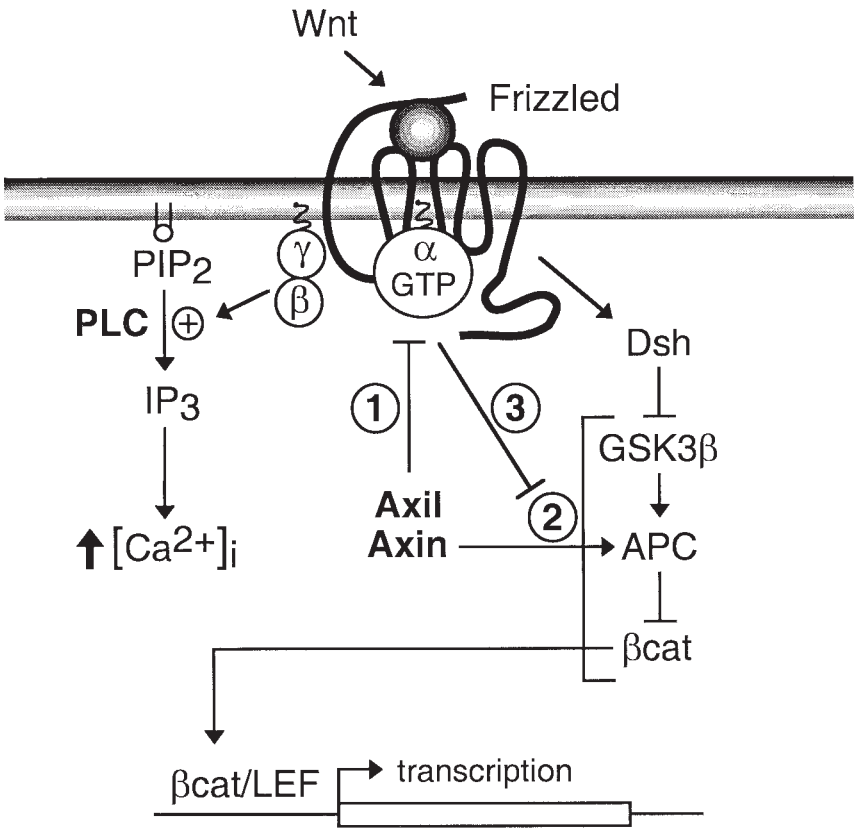


**FIGURE 7**. Presumed role of Axin and Axil/Conductin in coordination of Wnt signaling. See text for details regarding hypotheses (1), (2), and (3). **APC,** adenomatous *polyposis coli* tumor suppressor protein; **βcat,** β-catenin; **Dsh,** Dishevelled; **GSK3β,** glycogen synthase kinase-3β; **$IP_3$,** inositol 1,4,5-trisphophate; **LEF,** lymphocyte enhancer-binding factor; **$PIP_2$,** phosphatidylinositol 4,5-bisphosphate; **PLC,** phospholipase C.

protein, dubbed Conductin or Axil (for *Axin-l*ike), shares with Axin the ability to inhibit Wnt-induced axis formation by simultaneous binding of GSK3β, β-catenin, and APC.

APC was found to bind directly to the RGS domains of Axin and Axil (Behrens et al., 1998; Hart et al., 1998a; Kishida et al., 1998). While residues comprising the hydrophobic core of the RGS box are well conserved within Axin and Axil RGS domains (Figure 4C), many residues that directly contact Gα, as observed in the RGS4/$G_{\alpha i1}$ complex (Tesmer et al., 1997), are not conserved, including the critical asparagine-128 of RGS4 that, by one account (Coleman and Sprang, 1999), is presumed to elicit acceleration of GTPase activity by pushing the catalytic glutamine-204 of $G_{\alpha i1}$ out of an anticatalytic orientation (cf. Posner et al., 1999) This lack of conservation within the Axin and Axil RGS domains has perhaps shifted binding affinity away from Gα subunits and toward APC. We have been unable to observe detectable Gα GAP activity of GST-Axin fusion proteins in single-turnover GTPase assays (Krumins, A. M., Brothers, G. M., Gilman, A. G., and D. P. S., unpublished observations). Involvement of G proteins is still possible in this pathway if, despite the presumed lack of Gα GAP activity, Axil and Axin RGS domains retain binding affinity for Gα subunit(s): GTP-bound Gα, activated by Wnt-bound Frizzled receptors, could displace Axin/Axil binding to APC and thereby prevent β-catenin degradation (Figure 7, hypothesis ③).

## C. D-AKAP2: A Scaffold for Protein Kinase A Regulation?

Identification of new members of the *A-k*inase *a*nchoring *p*rotein (AKAP) family has resulted in the discovery of another example of an "outlier" RGS protein. Taylor and colleagues (Huang et al., 1997b) performed a yeast two-hybrid screen of mouse embryonic cDNA using a bait possessing the N-terminal 235 amino acids of $R^I\alpha$, a type I regulatory subunit of the cAMP-dependent protein kinase holoenzyme (PKA). Two novel proteins isolated in this screen were subsequently shown to interact with both type I and type II PKA regulatory subunits, and hence dubbed *d*ual-specificity *A-k*inase *a*nchoring *p*roteins-1 (D-AKAP1) and -2 (D-AKAP2) (Huang et al., 1997a; Huang et al., 1997b). While both D-AKAP1 and D-AKAP2 bind PKA regulatory subunits via a C-terminal "R-binding" domain, the latter protein also contains an Nterminal RGS domain, possessing, like Axin and Axil, the conserved hydrophobic residues thought to be important for the core RGS fold but with significant changes to the charged residues thought to be critical for Gα interaction (Figure 4).

The presence of both RGS and R-binding domains within D-AKAP2 suggests that this protein may serve as a scaffold coordinating G protein-mediated cAMP signaling pathway(s), as illustrated in Figure 8. Although its binding specificity remains to be determined, it is possible that the D-AKAP2 RGS domain binds and/or accelerates the GTPase activity of GTP-bound Gα subunits that either (1) directly activate adenylyl cyclase ($G_{\alpha s}$), (2) directly inhibit adenylyl cyclase ($G_{\alpha i}$), or (3) release Gβγ heterodimers that in turn modulate adenylyl cyclase activity and cAMP production (Figure 8). Local regulation of cAMP production hence could be coupled to local sensing of cAMP levels by D-AKAP2-associated PKA holoenzyme, with the regulatory (R) subunits binding cAMP and releasing active catalytic (C) subunits. On the other hand, given the example of Axin/APC association, it is equally possible that proteins other

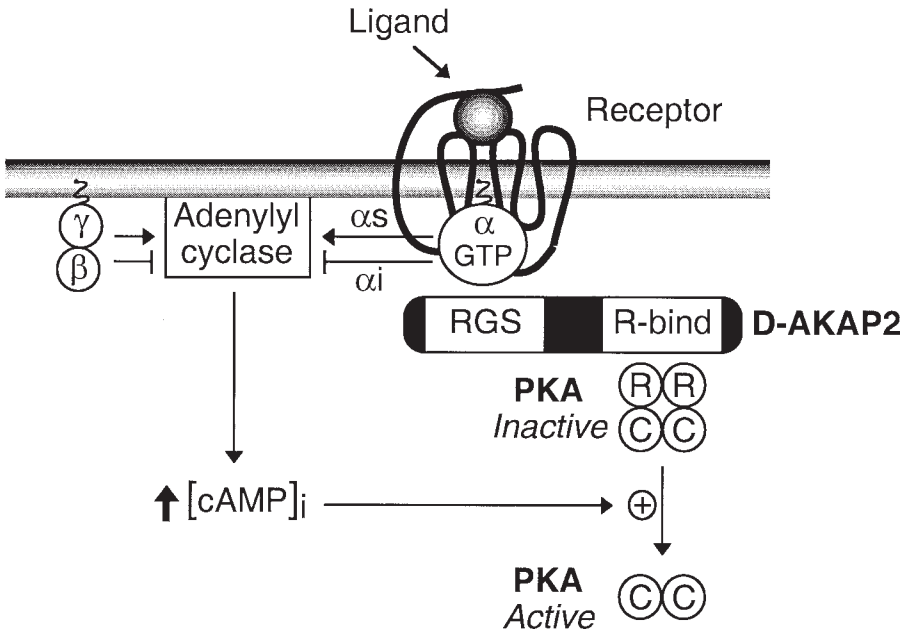


**FIGURE 8.** A hypothetical model of the role of D-AKAP2 as a scaffold protein coordinating G-protein receptor coupling to protein kinase A (PKA) activation, based on the domain substructure discerned by Taylor and colleagues (Huang et al., 1997a). See text for details.



than Gα subunits are bound by the divergent RGS domain of D-AKAP2; indeed, both Gα- and non-Gα-interacting partners for D-AKAP2 may be uncovered in the future.

## D. GRKs: Scaffolds for Multifocal Desensitization and Signal Generation?

Among the papers published in late 1995 and early 1996 that heralded the discovery of the RGS protein family (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996; Siderovski et al., 1996), ours was the only one to identify the RGS box signature within a preexisting class of signal desensitizers, the *G* protein-coupled *r*eceptor *k*inases (GRKs). The GRKs constitute a family of six serine/threonine kinases that phosphorylate GPCRs in the third intracellular loop and/or C-terminal regions, thereby allowing binding of arrestin proteins and, ultimately, functional uncoupling from G proteins and endocytosis of the phosphorylated receptor (reviewed in Krupnick and Benovic, 1998; Picher et al., 1998a). All GRKs share a common domain structure: a well-conserved ~180 amino acid N-terminal region historically assumed to play a role in receptor recognition (Inglese et al., 1993), a central ~270 amino acid catalytic domain most similar to the PKA and PKC families, and a variable C-terminal domain involved in membrane- and receptor-targetting via either posttranslational lipid conjugation (GRK1, GRK4, GRK6) or binding of membrane-bound lipid (GRK5) and Gβγ subunits (GRK2, GRK3).

Our recognition of the conserved amino-terminal region of GRKs as an RGS box (Siderovski et al., 1996) presents an opportunity to uncover hitherto unappreciated

modes of GRK regulation and action in both signal desensitization and signal generation, as speculatively illustrated for the β-adrenergic receptor kinase GRK2 (Figure 9). If the amino-terminal RGS box is capable of binding active (GTP-bound) Gα subunits, GRKs may desensitize GPCR signaling at the level of abrogating Gα/Gα-effector interaction(s); such an "effector blockade" role, irrespective of any Gα GAP activity, has been suggested previously for RGS4 and RGS-GAIP (Hepler et al., 1997). If the amino-terminal RGS box does indeed possess Gα GAP activity, GRKs may desensitize by accelerating Gα GTP hydrolysis prior to, or at the same time as, GRK-mediated receptor phosphorylation and functional uncoupling of receptor and heterotrimeric G-protein. This second scenario of Gα-directed desensitization (Gα GAP activity) is less likely than the first (Gα-effector blockade), given the lack of conservation of residues within GRK N-termini in the area corresponding to asparagine-128 of RGS4 (Figure 4C; Siderovski et al., 1996).

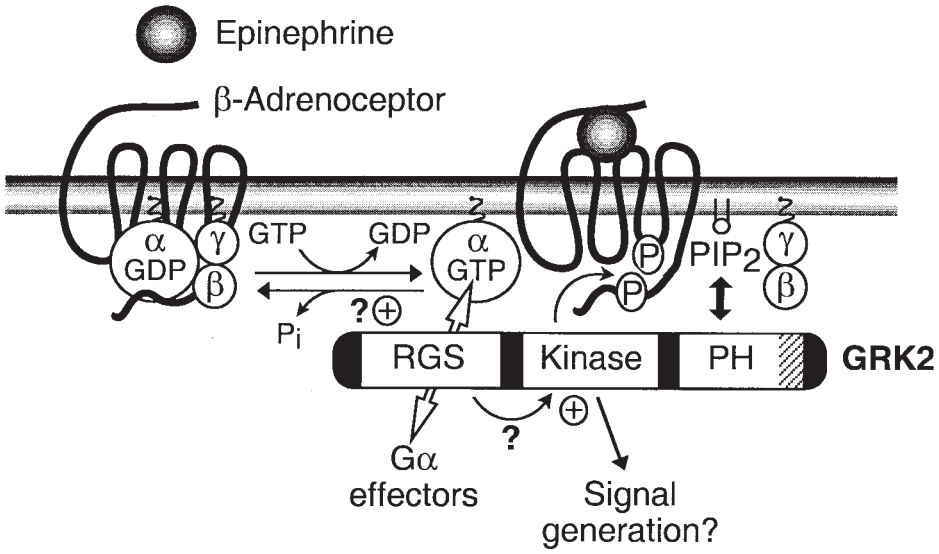


**FIGURE 9.** Documented and potential modes of desensitization and signal generation transacted by the RGS box-containing, β-adrenergic receptor kinase GRK2. Binding of membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) and free Gβγ dimer to pleckstrin homology (PH) and overlapping C-terminal (hatched) domains (Touhara et al., 1995) mediates membrane localization of GRK2. Recruitment of GRK2 to the activated receptor complex might also be facilitated by binding of active, GTP-bound Gα to the N-terminal RGS box; this binding event would also serve to desensitize signal generation by blocking effector binding to active Gα-GTP (open arrow). Potential Gα GAP activity in this N-terminal domain would result in signal desensitization by accelerating reversion to GDP-bound Gα. The central serine/threonine-kinase domain of GRKs phosphorylates sites (P) in the third intracellular loop and/or C-terminus of GPCRs, thereby allowing arrestin/β-arrestin binding and receptor endocytosis. This kinase activity could be activated by Gα-GTP binding to the N-terminal RGS box, in a manner similar to the activation of p115RhoGEF exchange activity by $G_{\alpha 13}$-GTP binding (Figure 5). Such enhanced kinase activity may serve not only to desensitize GPCR-mediated signaling, but also initiate a "second wave" of phosphorylation-dependent signal transduction (see text for details).

The central hallmark of GRK activity is preferential phosphorylation of agonist-occupied (i.e., activated) receptors rather than inactive or antagonist-occupied receptors. Thus, the presence of an RGS box with affinity for active Gα may also afford the means to both (1) recruit GRK protein to activated receptor complexes and (2) activate GRK serine/threonine kinase activity, independent of the roles previously documented for activated receptor itself (Palczewski et al., 1991; Brown et al., 1992) and free Gβγ subunits in the case of GRK2 and GRK3 (Pitcher et al., 1992). Indeed, in support of the latter possibility, there is one published report that the kinase activity of microsomal membrane-bound GRK2 is markedly enhanced after treatment with either $AlF_4^-$ or mastoparan/GTPγS, both known to stimulate heterotrimeric G-protein activation (Murga et al., 1996). The potential for stimulation of kinase activity by bound Gα-GTP creates a scenario of "GRK as Gα effector," akin to the roles of p115RhoGEF and PDZRhoGEF previously outlined above. This scenario is not far-fetched, as GRK-mediated receptor phosphorylation (and consequent arrestin binding) is now considered not only a receptor desensitization event, but also the start of a "second wave" of signal transduction independent of G protein involvement; in the specific case of the β2-adrenergic receptor, this second signal employs β-arrestin-mediated recruitment of c-Src and subsequent activation of the MAPK cascade (Luttrell et al., 1999). Moreover, at least one non-receptor substrate for active GRKs has been identified, namely, tubulin (Carman et al., 1998; Haga et al., 1998; Pitcher et al., 1998b); GRK-mediated phosphorylation of tubulin and other unidentified substrates thus could trigger downstream signaling cascades initially dependent on active Gα subunit association (Figure 9).

## E. The DEP/GGL/RGS Subfamily: *Coup de Grâce* Against a Central Tenet of Heterotrimeric G-Protein Assembly

Quite unexpectedly, our examination of the multidomain composition of another RGS box-containing protein has resulted in a radical rethinking of the very nature of heterotrimeric G-protein assembly. In our continuing efforts to characterize novel RGS family members (Snow et al., 1997; Snow et al., 1998a), we completed the cloning of *RGS11*, (Snow et al., 1998c) previously identified by Koelle and Horvitz (1996) only as a ~200 bp expressed-sequence tag (EST) from rat brain cDNA. The complete human *RGS11* open-reading frame was found to encode not only a carboxy-terminal RGS box but also an amino-terminal DEP (*D*ishevelled/*E*GL-10/*P*leckstrin) domain — a region of primary sequence conservation first identified by Ponting and Bork (1996) in a number of signal transduction-associated proteins, including the prototypic *C. elegans* RGS protein EGL-10. Studies of the Wnt/Frizzled signaling pathway have implicated the DEP domain of Dishevelled in controlling its membrane localization (Axelrod et al., 1998), but a particular binding partner for DEP domains has yet to be described.

The same DEP/RGS structure we identified for RGS11 was also reported for the retinal-specific, Gα-transducin GAP RGS9S/RGS9-1 (Cowan et al., 1998; He et al., 1998), the related, striatal-specific isoform RGS9L/RGS9-2 (Granneman et al., 1998; Thomas et al., 1998; Rahman et al., 1999), and RGS7 (Ponting and Bork, 1996; Saugstad et al., 1998). However, it was only with the amino acid sequence of RGS11 in hand that we were initially able to detect a region of similarity to Gγ subunits present between the DEP and RGS domains. We

dubbed this region the "*G* protein gamma subunit-*l*ike" domain or "GGL" domain (pronounced "giggle"). Subsequent multiple sequence alignment analyses convinced us that the GGL domain was also present in the analogous position within RGS6, RGS7, EGL-10, and both RGS9 isoforms (Snow et al., 1998c) (Figure 10).

The presence of a functional Gγ-like sequence within a much larger (>50 kDa) protein appeared at first to be a heretical notion. Conventional Gγ subunits are small (~10 kDa), C-terminally isoprenylated, alpha-helical polypeptides that not only make extensive contacts with the base of the Gβ seven-bladed β-propeller structure, but also form a parallel coiled-coil with the N-terminal alpha-helix of Gβ (reviewed in Clapham and Neer, 1997); in this manner, conventional Gγ subunits form functional heterodimers with Gβ subunits that are generally not dissociable except under denaturing conditions. Nonetheless, using an *in vitro* co-translation/immunoprecipitation experimental strategy (Snow et al., 1998c), we were able to demonstrate robust and selective binding by both RGS7 and RGS11 to two Gβ subunits: $G_{\beta 5}$ (Watson et al., 1994), and its N-terminally extended, retinal-specific isoform $G_{\beta 5L}$ (Watson et al., 1996a). Deletion and domain-swapping experiments confirmed the essential role of the GGL domain to Gβ subunit association (Snow et al., 1998c). Specific formation of RGS11/$G_{\beta 5}$ and RGS11/$G_{\beta 5L}$ heterodimers is also readily detectable after subunit co-expression in COS-7 monkey cells and Sf9 insect cells, with purified RGS11/$G_{\beta 5}$ heterodimers

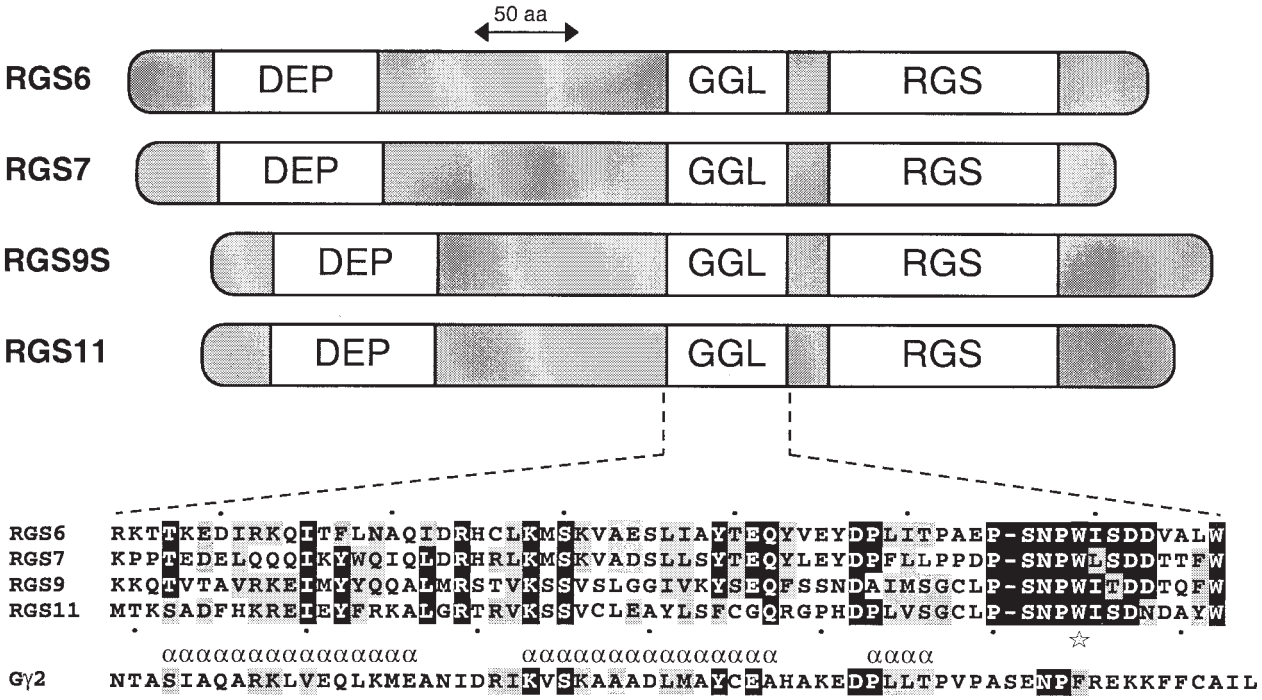


**FIGURE 10.** Domain structure of the DEP/GGL/RGS subfamily of mammalian RGS proteins (drawn to scale) and sequence comparison of GGL (*G* protein gamma subunit-*l*ike) domains to Gγ2. Regions within Gγ2 of alpha-helical secondary structure, as assigned by crystallographic structure determination (Wall et al., 1998), are denoted by the α symbol above the primary sequence. Position of Trp-274 residue within the RGS11 GGL domain is indicated by a grey star.

from the latter expression system exhibiting *bona fide*, *in vitro* GAP activity selective for $G_{\alpha o}$ in single-turnover GTPase assays (Snow et al., 1998c). In a follow-up study employing similar methods, we also reported the selective formation of RGS6/$G\beta_5$ heterodimers (Snow et al., 1999).

None of the other Gβ subunits ($G_{\beta 1}$, $G_{\beta 2}$, $G_{\beta 3}$, and $G_{\beta 4}$) are capable of binding to GGL domain-containing proteins; this absolute segregation of Gβ subunits into "GGL-binding" and "non-GGL-binding" subclasses parallels known Gβ sequence relatedness, as $G_{\beta 1}$ through $G_{\beta 4}$ are much more similar to each other than to the outliers $G_{\beta 5}$ and $G_{\beta 5L}$ (Watson et al., 1994; Watson et al., 1996a). Based on results from binding studies performed with chimeric GGL/PDZ (Snow et al., 1998c) and Gγ/RGS-box (Snow et al., 1999) fusion proteins, we believe that lack of interaction between $G_{\beta 1}$-$G_{\beta 4}$ subunits and DEP/GGL/RGS proteins is not the result of steric hindrance from N-terminal DEP or C-terminal RGS domain structures, but rather is intrinsic to the GGL domain. Through mutagenesis and molecular modeling studies, we have identified at least one potential area of GGL/$G_{\beta 5}$ contact that could explain their exquisite pairing fidelity — a C-terminal tryptophan present in all GGL domains (e.g., Trp-274 in RGS11; Figure 10) but not in conventional Gγ subunits (Snow et al., 1999). Future crystallography studies of GGL/$G_{\beta 5}$ heterodimer structure should allow us to pinpoint the molecular determinants that engender this strict binding fidelity of GGL domains for $G_{\beta 5}$ subunits.

Our observations of tissue-specific co-expression of mRNA for $G_{\beta 5}$ and *RGS11* (Snow et al., 1998c) or *RGS6* (Snow et al., 1999) suggest that RGS11/$G_{\beta 5}$ and RGS6/$G_{\beta 5}$ heterodimers do exist *in vivo*. More compelling evidence for *in vivo* heterodimer formation is present in independent reports from Cabrera et al. (1998) and Makino et al. (1999) that describe isolation of native RGS7/$G_{\beta 5}$ and RGS9/$G\beta_{5L}$ heterodimers from retinal photoreceptor cells. Consistent with the notion that formation of a GGL/$G_{\beta 5}$ complex precludes Gγ subunit association, Makino and colleagues (1999) chronicle their failure, by both immunoblotting and mass spectrometric analyses, to detect any Gγ protein within the RGS9/$G_{\beta 5L}$ complex. In addition, Levay et al. (1999) have demonstrated that *in vitro* formation of recombinant RGS7/$G_{\beta 5}$ heterodimers is unaffected by the presence of $G_{\gamma 2}$, a presumed native partner for $G\beta_5$ (Watson et al., 1994; Watson et al., 1996a; Zhang et al., 1996; Bayewitch et al., 1998a; Bayewitch et al., 1998b; Lindorfer et al., 1998). Our secondary structure predictions and mutagenesis studies suggest that the GGL domain interacts with $G_{\beta 5}$ in a generally similar fashion to conventional Gγ/Gβ pairings (Snow et al., 1999), thereby also implying that the GGL/$G_{\beta 5}$ complex, as a structural analogue of Gβγ subunits, excludes concomitant Gγ binding.

One central question has yet to be addressed: is the GGL/$G_{\beta 5}$ complex a *functional* analogue of Gβγ subunits? Conventional Gβγ subunits associate with GDP-bound Gα in the inactive state of Gαβγ heterotrimers (Figure 1); once freed of Gα association by receptor-catalyzed guanine nucleotide exchange, Gβγ subunits are able to modulate the activity of several signaling effector classes, including $K^+$ and $Ca^{2+}$ ion channels and particular isoforms of adenylyl cyclase, phospholipase-Cβ, and phosphatidylinositol 3-kinase. Hence, these functional roles are also conceivable for the GGL/$G_{\beta 5}$ complex; to illustrate these possibilities, a speculative model of coupled signal transduction and signal desensitization employing a $G_{\alpha o}$/RGS11/$G\beta_5$ "hetero-trimer" is presented in Figure 11.

The "simple" model of potential RGS11/$G_{\beta 5}$ heterodimer activities presented in Figure 11 pales in comparison to the complex

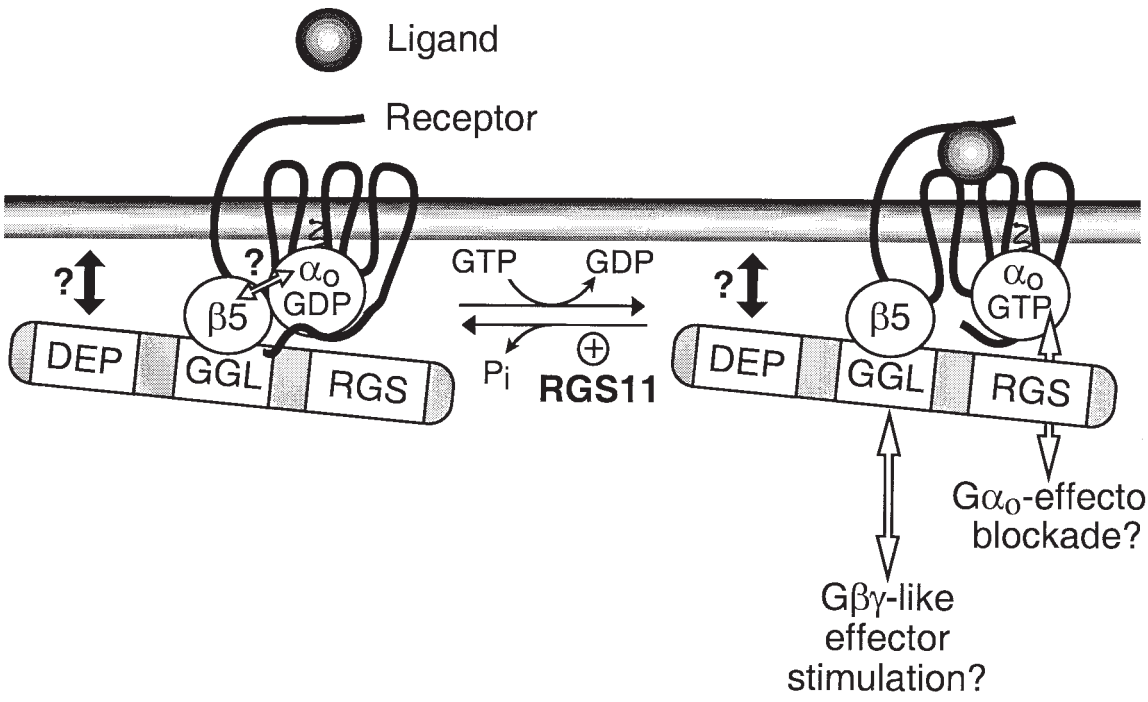


**FIGURE 11.** Possible modes of signaling and desensitization by a $G_{\alpha o}$/RGS11/$G_{\beta 5}$-coupled receptor. The DEP domain within the RGS11/$G_{\beta 5}$ heterodimer may serve to facilitate membrane recruitment in the absence of a conventional, lipid-modified Gγ subunit, as the DEP domain of Dishevelled has been reported previously to control its membrane localization (Axelrod et al., 1998). Akin to conventional Gβγ subunits, the GGL/$G_{\beta 5}$ moiety may associate with inactive, GDP-bound $G_{\alpha o}$; this association would presumably preclude any binding of $G_{\alpha o}$ with the RGS box, as both binding events involve the same regions on the Gα subunit (Tesmer et al., 1997; Ford et al., 1998; Li et al., 1998b). After receptor-catalyzed guanine nucleotide exchange, GTP-bound $G_{\alpha o}$ would release the GGL/$G_{\beta 5}$ moiety, which in turn could potentially stimulate either conventional Gβγ-effectors or novel effector proteins. Selective GAP activity of the RGS11/$G_{\beta 5}$ heterodimer on $G_{\alpha o}$ (Snow et al., 1998c) would revert the system back to ground state; the RGS box may also desensitize receptor signaling by acting as an effector antagonist, inhibiting $G_{\alpha o}$-GTP binding to downstream effector proteins.

machinations now considered possible for other RGS/$G_{\beta 5}$ heterodimers. In the case of RGS7, Kim et al. (1999) have recently described a unique feature to its GGL domain — the presence of two PEST sequences presumed responsible for targetting RGS7 to ubiquitin-dependent proteasome degradation. Kim and colleagues report that the binding of polycystin to the RGS7 GGL domain prolongs the half-life of RGS7 and alters its subcellular localization; it is currently unknown whether polycystin binding facilitates or hinders the interaction between RGS7 and $G_{\beta 5}$. The divergent nature of the RGS7 GGL domain vs. those of other DEP/GGL/RGS proteins may explain differences seen in $G_{\alpha o}$ association between RGS7/$G_{\beta 5}$ heterodimers and RGS11/$G_{\beta 5}$ heterodimers. While we have observed $G_{\alpha o}$directed GAP activity by both full-length and DEP-domain-deleted RGS11/$G_{\beta 5}$ heterodimers (Snow et al., 1998c), Levay and colleagues (1999) report that $G_{\beta 5}$ co-expression with RGS7 *inhibits* a $G_{\alpha o}$ association.

As RGS9 is now assumed to be the long sought-after membrane-associated GAP for

the phototransduction cascade (Arshavsky and Pugh, 1998; He et al., 1998), its GAP activity on Gα-transducin must be placed within the context of a larger complex also containing $G_{\beta 5L}$, the gamma subunit of cGMP phosphodiesterase (PDEγ), and retinal guanylyl cyclase (Figure 12). In this regard, the C-terminal region of PDEγ, as well as alpha-helical regions of Gα-transducin and RGS9, have been identified recently as critical contact points engendering synergistic activation of Gα-transducin GTPase activity by PDEγ and RGS9 (McEntaffer et al., 1999; Skiba et al., 1999). Further experimentation will undoubtedly result in the discovery of similar complex formations for RGS6/$G_{\beta 5}$ and RGS11/$G_{\beta 5}$ heterodimers.

## F. RGS12: A Scaffold for the Coordination of G Protein and Tyrosine Kinase Signaling?

Efforts to characterize novel RGS family members expressed in the brain led to our cloning of RGS12, the largest known protein bearing the hallmark RGS box (Snow et al., 1997). While clearly a *bona fide* GAP for $G_i$-class alpha subunits (Snow et al., 1998b), RGS12 also possesses at least three additional protein-protein interaction modules: a PDZ domain, a PTB domain, and a GoLoco motif. This multidomain structure suggests that RGS12 participates in an active, multifocal coordination of signal generation and signal desensitization.

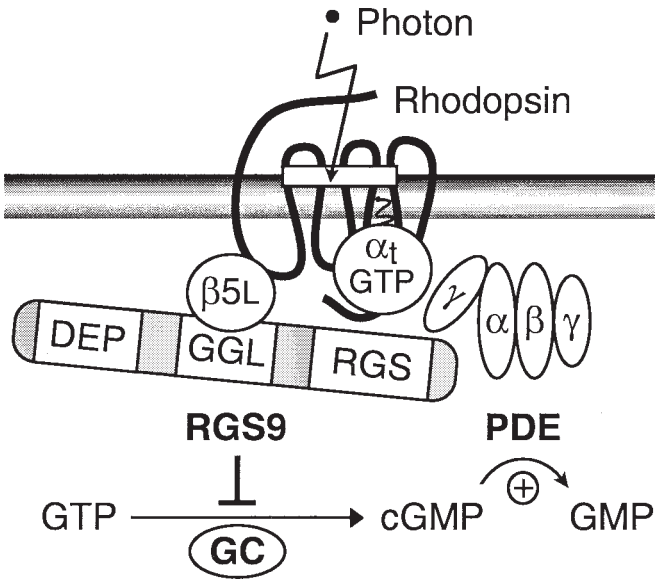


**FIGURE 12**. Multifaceted role of RGS9 in photoreceptor signaling. The covalently associated "ligand" of rhodopsin, 11-*cis* retinal (white bar), is isomerized to an all *trans* form by photon capture, leading to activation of rhodopsin's guanine nucleotide exchange activity on Gα-transducin ($\alpha_t$). The $G\alpha_t$-specific GAP activity of RGS9 is enhanced by binding of the gamma subunit (γ) of cGMP-phosphodiesterase (PDE), the effector of GTP-bound Gα-transducin (He et al., 1998). RGS9 has also been reported to bind retinal guanylyl cyclase (GC) and inhibit its ability to synthesize cyclic GMP from GTP (Seno et al., 1998).

Our original description of RGS12 protein sequences from rat and human reported an N-terminal region with significant similarity to the C-terminus of mouse Rhophilin (Watanabe et al., 1996; Nakamura et al., 1999). As the N-terminus of Rhophilin contains a class I Rho-binding motif shared with the Rho effector PKN (Fujisawa et al., 1998), we initially assumed that similarity between Rhophilin and RGS12 reflected a conserved but uncharacterized function in Rho-mediated signaling (Snow et al., 1998). However, after further analysis of *RGS12* splice variants, we reidentified this region as a PDZ domain (Figure 13), present only in the two longest RGS12 isoforms (Snow et al., 1998b). As previously mentioned in the context of PDZRhoGEF, PDZ domains are protein-protein interaction modules capable of binding internal regions of proteins (Hillier et al., 1999) or, more often, a three or four amino acid motif at the extreme carboxy-termini of target proteins (Songyang et al., 1997). PDZ domains are found singly, or in tandem repeats, in a wide variety of scaffold proteins that localize enzymatic activities and other protein-protein interaction modules to specific submembraneous regions (reviewed in Saras and Heldin, 1996; Fanning and Anderson, 1996; Ponting et al., 1997; Ranganathan and Ross, 1997; Craven and Bredt, 1998).

The first PDZ domain of NHERF, a protein that regulates the activity of the $Na^+/H^+$ exchanger type 3 (NHE3), is closely related in primary sequence to the RGS12 PDZ domain (Snow et al., 1998b) (Figure 13). As this first PDZ domain of NHERF mediates agonist-dependent binding to the carboxy-terminus of the β2-adrenergic receptor (Hall et al., 1998a; Hall et al., 1998b), we tested whether the RGS12 PDZ domain would also associate with GPCR C-termini *in vitro*. While no binding to β-adrenergic receptor C-termini was observed by either surface plasmon resonance or protein overlay assays, we did identify another GPCR C-terminal tail to which the RGS12 PDZ domain avidly bound — that of the interleukin-8 receptor B (CXCR2; Snow et al., 1998b). An association with the RGS12 PDZ domain was found to be exquisitely specific for CXCR2, as other GPCR C-termini sharing considerable sequence similarity failed to bind (e.g., CXCR1/interleukin-8 receptor A, metabotropic glutamate receptors 1α, 2, and 5). Extensive mutagenesis of the CXCR2 Cterminus revealed that the optimal C-terminal motif for binding to the RGS12 PDZ domain is (A/S)-T-x-(L/V). A version of this optimal motif is present at the C-terminus of an alternative splice variant of RGS12 itself (A-T-F-V) and was also found to bind the RGS12 PDZ domain *in vitro* (Snow et al., 1998b), albeit with lower avidity (Figure 14). The presence of this PDZ docking site within RGS12 itself has led us to speculate on both concatemerization and intramolecular self-assembly of RGS12 *in vivo* (Snow et al., 1998b). However, the true identity of physiologically relevant cellular docking site(s) for the RGS12 PDZ domain remains to be resolved.

Further sequence analysis of RGS12 has revealed the presence of a phosphotyrosine-binding (PTB) domain C-terminal to the PDZ domain (Figure 15). The PTB domain was first identified in Shc (Blaikie et al., 1994; Kavanaugh and Williams, 1994; van der Geer et al., 1995), a signaling adaptor protein that mediates phosphotyrosine-dependent interactions between growth factor receptor tyrosine kinases (e.g., EGF receptor) and their downstream effectors (e.g., Ras) (Pelicci et al., 1992; Margolis and Skolnik, 1994; Yokote et al., 1994). PTB domains bear structural relatedness to pleckstrin homology (PH) domains (Eck et al., 1996; Harrison, 1996; Lemmon et al., 1996) and were first assumed to be strictly targetted to phosphorylated tyrosine resi-

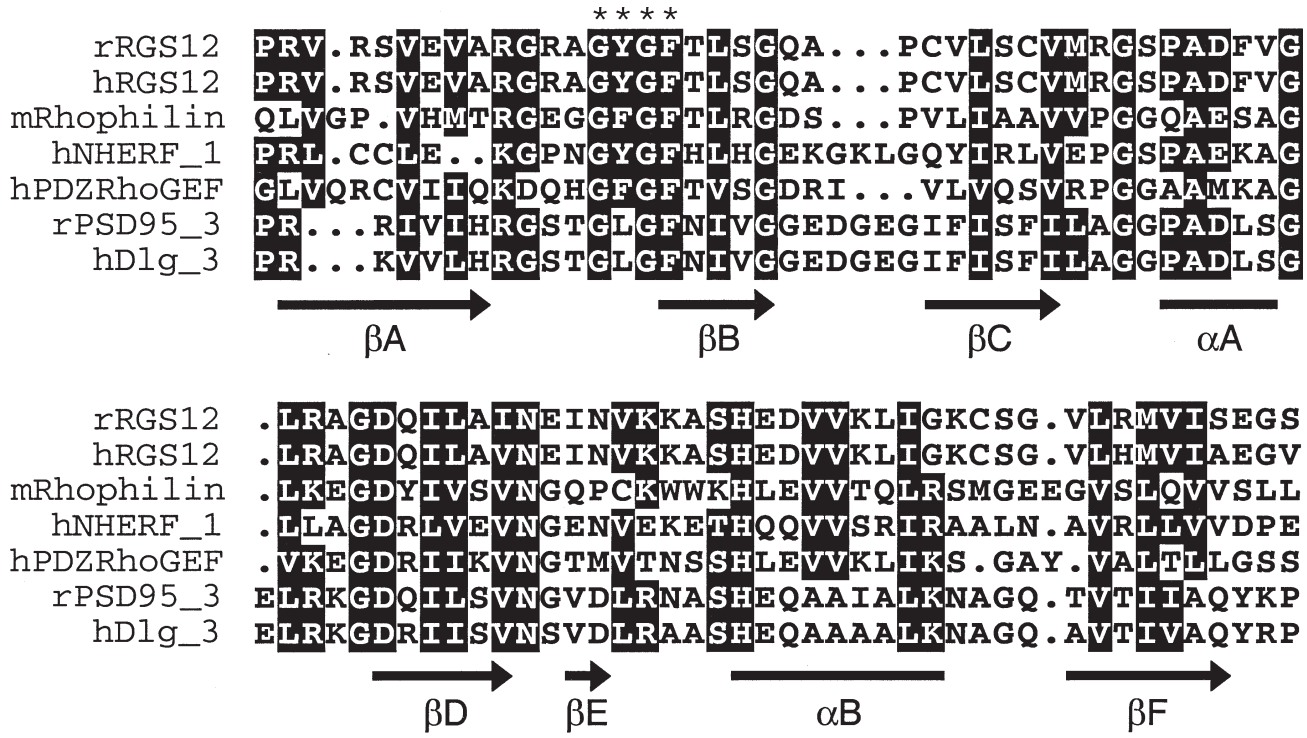


**FIGURE 13**. Alignment of the PDZ domains of rat and human RGS12 with other published PDZ domain sequences. Secondary structure elements present in the third PDZ domains of rat PSD-95 and human Dlg (β-strands as arrows, α-helices as rods) are shown below the Dlg sequence (Doyle et al., 1996; Morais Cabral et al., 1996). The conserved G-(L/F/Y)-G-F motif that forms the carboxylate-binding loop between β-strands βA and βB is highlighted with asterisks. The primary sequences used in the alignment are as follows: aa 17-98 of rat RGS12 (SwissProt O08774); aa 18-99 of human RGS12 (GenBank NP_002917); aa 496-578 of mouse Rhophilin (SwissProt U43194); aa 42-123 of human PDZRhoGEF (GenBank AB002378); aa 12-94 representing the first PDZ domain of human $Na^+/H^+$ exchanger regulatory factor (NHERF; GenBank NP_004243); aa 311-394 representing the third PDZ domain of rat presynaptic density protein-95 (PSD-95; EMBL X66474); aa 464-547 representing the third PDZ domain of the human homologue of *Drosophila* discs large protein (hDlg; GenBank NP_004078).

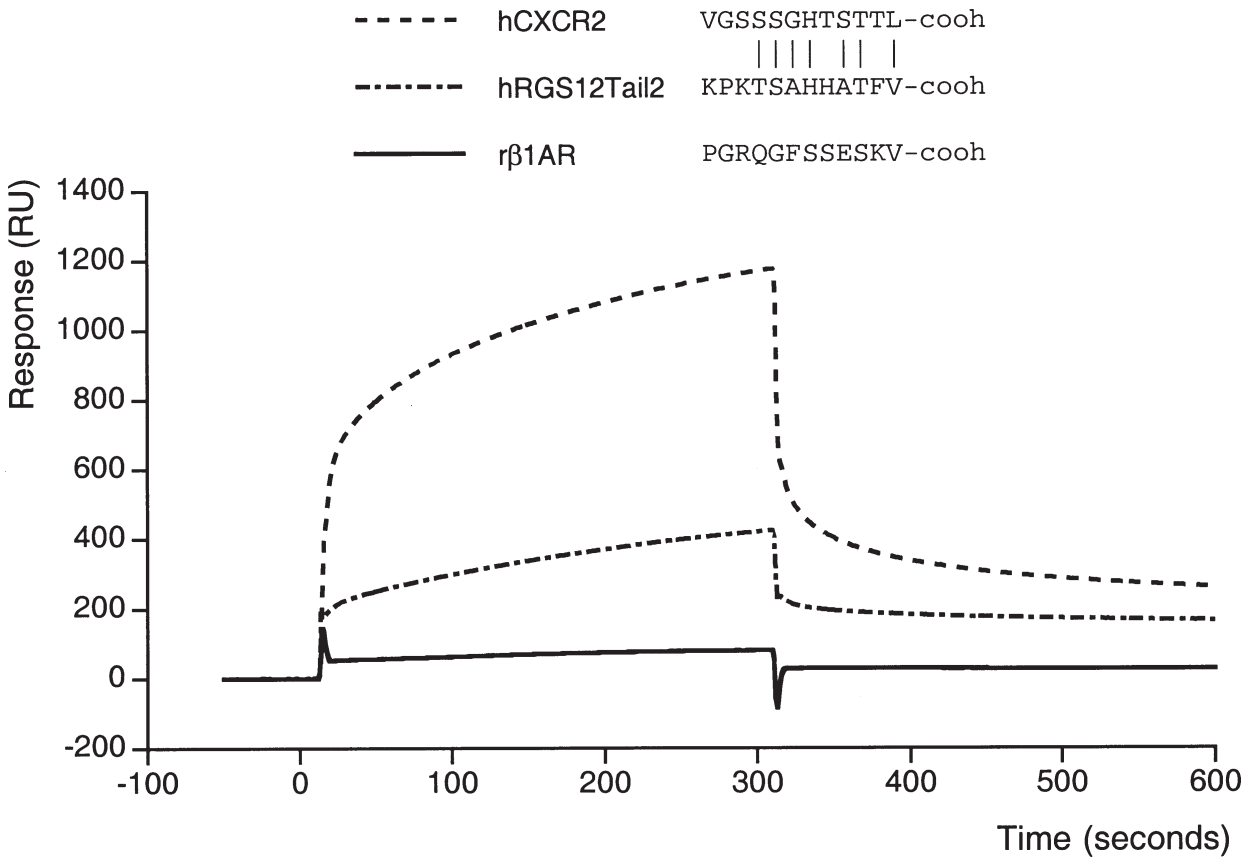


**FIGURE 14**. Simultaneous surface plasmon resonance measurements of human RGS12 PDZ domain binding to C-terminal peptides from the human interleukin-8 receptor B (hCXCR2), the alternative 3′ exon splice variant of human RGS12 (hRGS12Tail2), and the rat β1-adrenergic receptor (rβ1AR). Peptide sequences are listed to the right of the peptide name; amino acid similarities between hCXCR2 and hRGS12Tail2 peptides are denoted by vertical bars. See Snow et al. (1998b) for discussion of experimental procedures.

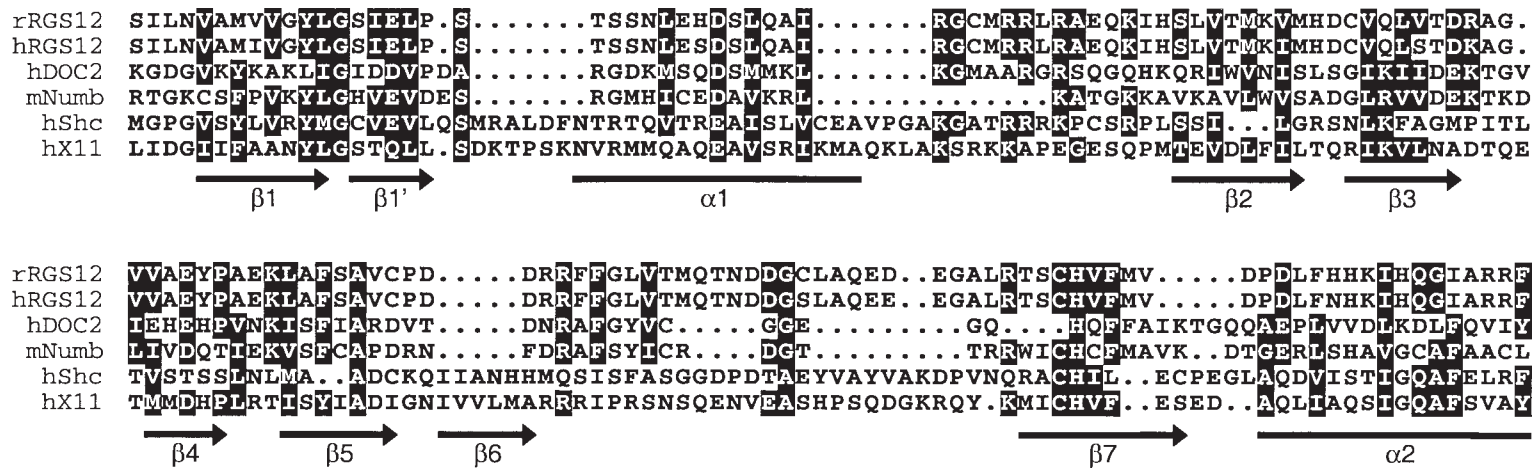


**FIGURE 15.** Alignment of the phosphotyrosine-binding (PTB) domains of rat and human RGS12 with other published PTB domain sequences. The secondary structure elements of the X11 PTB domain (β-strands as arrows, α-helices as rods) are shown below the X11 sequence (Zhang et al., 1997). The primary sequences used in the alignment are as follows: aa 223-358 of rat RGS12 (SwissProt O08774); aa 224-359 of human RGS12 (GenBank NP_002917); aa 44-170 of DOC2 (SwissProt P98082), the human orthologue of mouse disabled-2 (mDab2); aa 33-166 of the mouse homologue of Drosophila Numb (Verdi et al., 1996); aa 156-312 of human Shc (SwissProt P29353); aa 326-483 of human X11 (SwissProt Q02410).

dues; indeed, the prototypic PTB domains of Shc and the insulin receptor substrate (IRS-1) recognize phosphotyrosine sites on growth factor receptors in the context of a conserved sequence motif: Asn-Pro-X-Tyr(p) (van der Geer et al., 1995; Wolf et al., 1995; Zhou et al., 1995). However, as other proteins with PTB domains have been identified and their binding sites characterized, it is clear that a PTB domain can also have nonphosphorylated, and even nonproteinaceous, binding targets. For example, the PTB domains of X11 and mNumb specify binding to Asn-Pro-X-Tyr motifs within amyloid precursor protein (βAPP) and LNX, respectively, but neither of these interactions are dependent on tyrosine phosphorylation (Borg et al., 1996; Dho et al., 1998). *Drosophila* Numb and mouse Shc PTB domains have both been shown to interact with target polypeptide sequences divergent from the consensus Asn-Pro-X-Tyr motif (Charest et al., 1996; Li et al., 1998a). The PTB domains of Shc and mNumb also bind acidic phospholipids *in vitro*, and these interactions may help to constitutively localize Shc and mNumb isoforms to the plasma membrane *in vivo* (Ravichandran et al., 1997) (McGlade, C. J., personal communication).

This diversity of known PTB domain-binding interactions presents multiple possibilities for cellular target(s) of the RGS12 PTB domain, but a phosphotyrosine-containing binding site is most likely. The closest related PTB domain sequence to that of RGS12 is the PTB domain of mouse p96 (Xu et al., 1995), a mitogen-responsive phosphoprotein alternatively known as mouse disabled-2 or mDab2. mDab2 is the second of two mouse homologues of the Drosophila Disabled protein thus far identified. It has been speculated that mDab2 (Howell et al., 1997; Xu et al., 1998) serves as a negative regulator of growth factor signaling, as its human orthologue, DOC2, is expressed in normal ovarian epithelial cells but consistently absent in ovarian carcinoma cells. Similar to recent findings with *Drosophila* Disabled, mouse disabled-2 has been implicated in modulating receptor tyrosine kinase signaling to Ras by binding to Grb2, an SH2-SH3 adaptor protein (Xu et al., 1998). While not directly applicable to mDab2 binding specificity, the PTB domain of the highly related mDab1 was reported to bind phosphotyrosine proteins present in extracts of embryonic mouse heads (Howell et al., 1997). Using a similar experimental procedure, recently we have observed binding of the RGS12 PTB domain to phosphotyrosine proteins present in extracts of neurotransmitter-stimulated chick dorsal root ganglion neurons (D. P. S. and María A. Diversé-Pierluissi; submitted). RGS12 PTB domain binding to phosphotyrosine-containing target(s) is not only most likely based on this evidence, but it is the most compelling scenario to contemplate in light of recent work indicating extensive cross-talk between tyrosine kinase signaling and G protein-coupled signaling (Gutkind and Robbins, 1992; Daub et al., 1996; Dikic et al., 1996; Daub et al., 1997; Della Rocca et al., 1997; Diversé-Pierluissi et al., 1997; Herrlich et al., 1998; Igishi and Gutkind, 1998; Luttrell et al., 1999; Della Rocca et al., 1999).

Recent cloning of Loco, a *Drosophila* protein required for glial cell development, has led us to identify a novel Gα subunit interaction motif within RGS12 — the GoLoco motif (Siderovski et al., 1999). Trapped in a screen for genes whose glial cell-specific expression depends on the activity of the transcription factor Pointed (Granderath et al., 1999), *loco* was found to encode an RGS box-containing protein with greatest similarity to rat RGS12 and RGS14 (Snow et al., 1997). Sequence homology between Loco, RGS12, and RGS14 extends


**FIGURE 16.** Alignment of putative Gα binding sites, dubbed $G_{\alpha i/o}$-Loco or 'GoLoco' motifs, from RGS12 and other Gα-interacting proteins. The primary sequences used in the alignment are as follows: aa 1188-1206 of rat RGS12 (SwissProt O08774); aa 643-661 of Drosophila Loco (GenBank AAD24581); aa 498-516 of rat RGS14 (SwissProt O08773); aa 483-501 (W), aa 538-556 (X), aa 588-606 (Y), and aa 622-640 (Z) representing the four GoLoco motif repeats of human LGN (GenBank U54999); aa 27-45 of mouse Purkinje cell-protein-2 (Pcp2; SwissProt P12660). A multiple sequence alignment of GoLoco motifs is available from the European Bioinformatics Institute (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/; alignment number DS38614).

beyond the RGS box into three discrete regions labeled B, C, and D by Granderath and colleagues (1999). To test the hypothesis that the RGS box of Loco mediates interaction with Gα subunits, Granderath and colleagues performed a yeast two-hybrid screen with *Drosophila* $G_{\alpha i}$ and identified Loco as an interacting protein. Surprising, however, was the fact that the D region of Loco, rather than the RGS box, was repeatedly isolated in this screen. Subsequently, we have discovered that this D region contains an 19 amino acid sequence motif (Figure 16) also present, singly or in tandem arrays, in two other Gα-interacting proteins: LGN (Mochizuki et al., 1996) and Purkinje cell protein-2 (Pcp2; Luo and Denker, 1999). Recently, Pcp2 was shown to possess $G\alpha_o$-directed guanine nucleotide exchange activity, thus presenting the possibility that GoLoco motif-containing proteins such as RGS12 represent novel, receptor-*independent* guanine nucleotide exchange factors for Gα subunits (Siderovski et al., 1999). A summary of the documented and potential interactions for RGS12 is illustrated in Figure 17. The overwhelmingly rich, multidomain character of RGS12, with PDZ, PTB, RGS, GoLoco, putative coiled-coil (Snow et al., 1997), and PDZ-docking C-terminal domains, hints at a complex orchestration of signaling events that only further experimentation will unravel.

## V. CONCLUSION

Precedents exist to support the original view of RGS family members as simple desensitizers of G protein-coupled signaling. However, recent discovery of the RGS box within several multidomain proteins presages much larger roles for these molecules in the orchestration of signal transduction pathways — as effectors propagating signals forward and as scaffolds coordinating signaling component assembly to overcome diffusional limitations and facilitate rapid signal onset and termination. Confronted with this expanded realm of possible functions, one must consider the presence of an RGS box within a given protein to have implications beyond mere

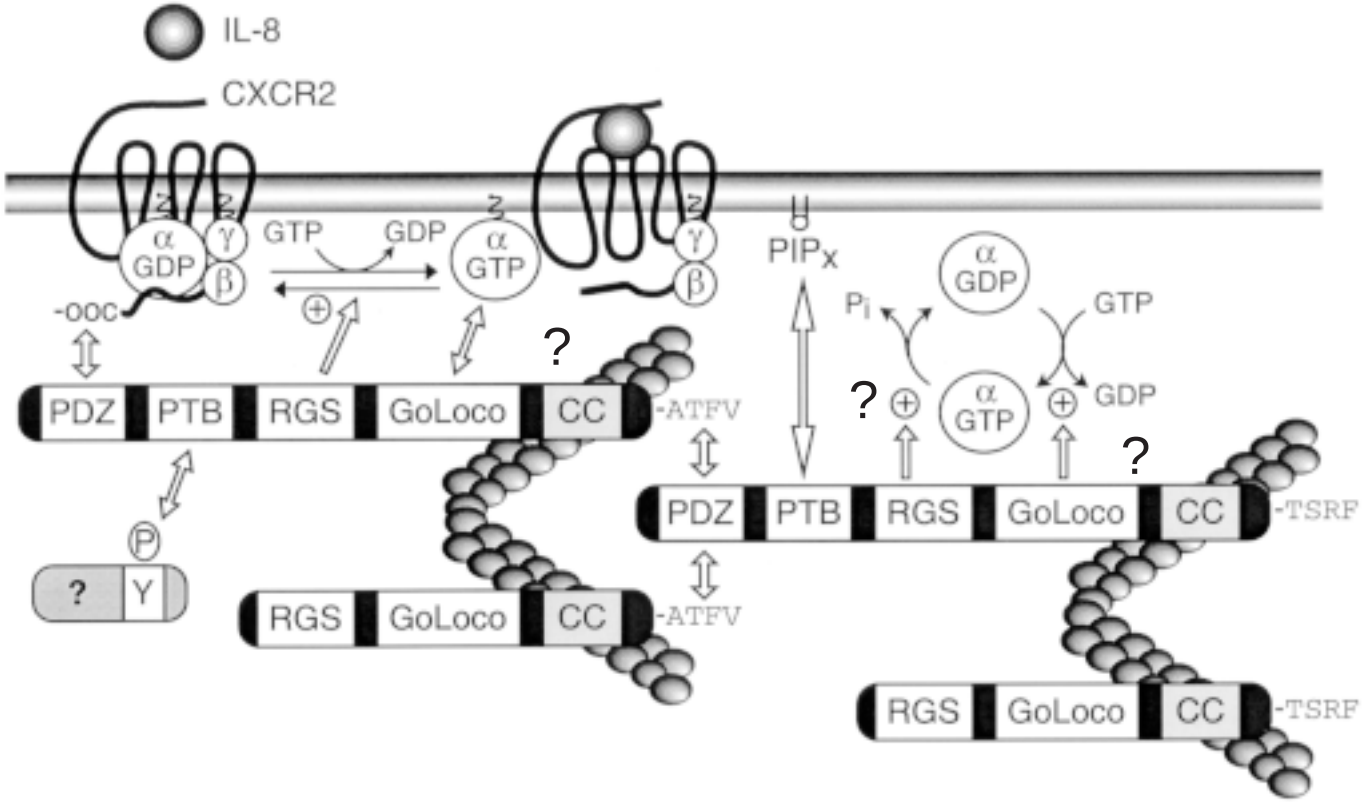


**FIGURE 17**. Documented and potential modes of protein-protein contact, signal generation, and signal desensitization transacted by the multidomain scaffold protein RGS12. Four RGS12 isoforms depicted above represent predicted translation products of the four alternatively spliced human *RGS12* mRNAs thus far identified (Snow et al., 1998b). The PDZ domain binds *in vitro* to the carboxy-terminal tail of the interleukin-8 receptor B (CXCR2), as well as to the alternatively spliced carboxy-terminus of RGS12 itself ("ATFV"; Snow et al., 1998b); "TSRF" represents the last four amino acids of RGS12 isoforms to which the N-terminal PDZ domain has little binding affinity *in vitro*. PTB domains have the potential to bind acidic phospholipids (denoted generically as "$PIP_x$") and/or protein targets in a phosphotyrosine-dependent or -independent manner (e.g., Howell et al., 1999). The RGS box of RGS12 is a GAP for Gi-class α subunits (Snow et al., 1998b), presumably acting to desensitize receptor-activated, GTP-bound Gα. Juxtaposition of an RGS box with a GoLoco motif (a putative Gα binding site potentially possessing guanine exchange factor activity) presents the puzzling prospect of a futile cycle of guanine nucleotide hydrolysis by receptor-independent Gα subunits. Contact between RGS12 and cytoskeletal components, illustrated by multimeric chains, is possible through a C-terminal heptad-repeat structure predicted to form a coiled-coil (CC; Snow et al., 1997).

Gα GAP activity — even beyond G protein-coupled signal transduction itself.

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